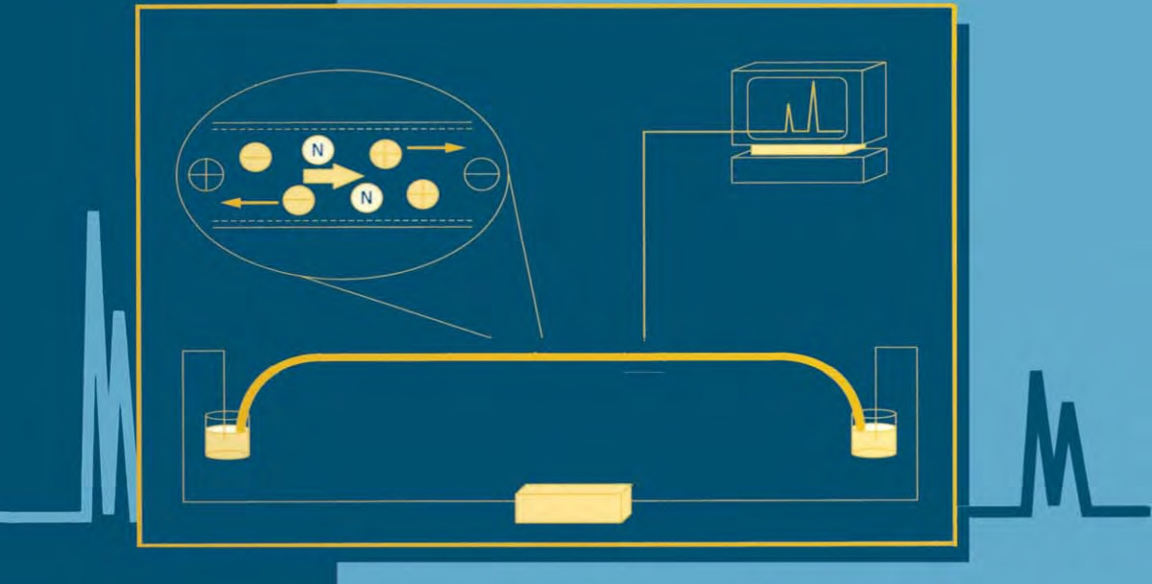
Progress in Pharmaceutical and Biomedical Analysis Volume 2

Pharmaceutical and Biomedical Applications of Capillary Electrophoresis



Edited by

Susan M. Lunte and Donna M. Radzik

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VOLUME 2

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INTRODUCTION

In the pharmaceutical industry, it is well documented that timely analytical support which provides quality information is critical to success in research and development. It is also true that acceptance of any new analytical technique in the industrial environment can be a slow and painstaking process. For example, it took almost twenty years for liquid chromatography (LC) to gain its now almost universal acceptance in bioscience laboratories. The relatively rapid growth of capillary electrophoresis (CE) over the past ten years may be related to its resemblance to LC. CE shares many features with LC, including the ability to separate and determine a broad range of molecules with a wide variety of sizes and chemical characteristics. In addition, many of the advances in automation and sample preparation that were developed for LC can easily be adapted for use with CE.

However, while CE may have similarities to LC, it is clearly superior for certain applications. Among the most easily recognized advantages of CE are the small sample sizes needed and the fast analysis times and high separation efficiencies that can be achieved. The importance of the small volumes of mobile phase required for CE separations is frequently overlooked, but will become more apparent as environmental considerations for separation techniques increasingly become an issue.

Our original goal for this book was to present an evenhanded, comprehensive overview of the technique rather than just a "snapshot" of current understanding and research. However, this became an almost impossible task because CE was evolving so rapidly. Therefore, we have tried to supply the basic information needed to understand the working aspects of CE as well as examples of applications of CE that would be of interest to pharmaceutical and biomedical scientists. It is our hope that this book will provide the necessary foundation for a better understanding of the current and future developments in CE. As always, for a particular analytical problem, the best way to learn about the state of the art is to refer to the recent literature.

This book, Volume 2 in the series *Progress in Pharmaceutical and Biomedical Analysis*, is divided into three major sections. The first part offers an overview of CE theory, beginning with a general informational chapter about the instrumentation and a brief discussion of the various separation modes. More detailed information on capillary modification and micellar electrokinetic chromatography is provided in the next two chapters. Part 2 deals with detection, and includes chapters on optical, mass spectrometric and electrochemical detection methods.

Part 3 of the book is dedicated to some of the more exciting applications of CE in pharmaceutical and biomedical analysis. The first chapter in this section concerns the issue of sample preparation for capillary electrophoresis, especially with regard to biological samples. The next three chapters consider some of the more conventional uses of CE—the isolation and determination of amino acids, peptides, proteins and nucleic acids. Next, an informational chapter is provided on the use of CE in the pharmaceutical industry, with special emphasis on regulatory considerations. In particular, the issues of validation and technology transfer are addressed. Finally, there is a discussion of applications of CE for single cell analysis and for use in conjunction with microsampling techniques such as microdialysis.

In all of the presentations in this book, we have attempted to take the "long view" of this technique. We feel that capillary electrophoresis is a method that will be remain a staple of the analytical laboratory. As members of the research and development community, we all have seen the introduction of "the next big technique." Many of these have actually led to changes in the way we acquire scientific information on a daily basis (liquid chromatography, mass spectrometry, laser-induced fluorescence, etc.). However, others that have been introduced with much fanfare have subsequently proved to offer little advantage over current methodologies. CE will avoid being placed in this latter category due to its versatility and ease of use and because it is complementary to LC.

As editors, there are many people we must thank for encouragement and contributions in putting this text together. First, we salute the authors, who provided their input into the development of this project and who were so extremely patient during the editing and publication process. The publishing staff at Pergamon was very supportive throughout; we would especially like to recognize David Claridge. Many friends and family members (several born during the writing process) provided support and inspiration. Finally, this book could not have been completed without the able assistance, coaching, motivating sarcasm and never-ending good will of Nancy Harmony, whom we can never thank enough.

Susan M. Lunte
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1996

Part One: Separations

CHAPTER 1

Principles of Capillary Electrophoresis

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1. Introduction

Scientists from many different disciplines tend to demand new analytically valid separation and detection techniques on a regular basis. This seems to be especially true of the biomedical and pharmaceutical industries. Among the issues are cost, speed, efficiency separation requirements, sample size, and even environmental factors. This need becomes more understandable if one considers currently available methods of separation. The available modes of chromatography (gas, liquid and thin-layer, for example) and electrophoresis (slab gel) can provide only certain advantages for long-term success. Is there a need for new modes of separation? The response may seem obvious; however, in terms of the cost of research and development, the answer may not be as easily understood. Field-flow fractionation and super-critical fluid chromatography are techniques that, although useful, have not created industries as gas and liquid chromatography have done. Whether an analytical separation technique becomes a "routine" technique or remains a research and development curiosity depends on many factors. For instance, what types of molecules are we as scientists attempting to separate on a routine basis? Gas chromatography (GC) satisfied the need for separations to evaluate the components of fossil fluids. GC methods could not be applied to nonvolatile analytes and, therefore, liquid chromatography (LC) was developed and has been used with much success for the past 25 years. In the pharmaceutical industry, measurement of the levels of small organic molecules (especially from biological matrices) is important, as are techniques to evaluate the impurity profiles of these molecules. LC addressed many of these issues. In many ways, the needs of the separation scientist have not changed significantly for several decades. Today, separation technologies are needed for large molecules, optical isomers, and the contents of a single cell. Our research and development activities require in-depth studies of material that is available only in nanoliter-to-picoliter (or lower) quantities, as well as the need to interface the separation system to a living animal.

Since there will always be the desire to conduct better and faster separations using less and less material, there will always be a need to examine alternative separation strategies. Capillary electrophoresis in its various forms is quite likely to be at the forefront of research and development as we move into a new century. In reviewing the history of this technique, it is interesting to note that it follows a long line of analytically useful strategies in which the basic theory was known long before successful application to "real world" problems was achieved. It will not be surprising, therefore, if the various forms of CE and its future adaptations become as numerous and indispensable as the menu of chromatographic techniques currently available.

1.1. A Brief History of Capillary Electrophoresis (CE)

Electrophoretic separations in narrow diameter tubes were reported in the literature as early as 1950 (Haglund and Tiselius, 1950). In the late 1960s, Hjertén (1967) described the separation of a large number of analytes using rotating gel-filled glass tubes of 3 mm i.d. In 1979, Mikkers and coworkers introduced the concept of the "wall effect" to minimize convention in tubes (Mikkers *et al.*, 1979). Teflon tubes of 200 μm i.d. were used for these electrophoretic separations. Efficiencies for a number of anions were reported, with the number of theoretical plates reported averaging 25,000. The major drawback of this method was poor detectability at low analyte concentrations due to the narrow pathlength of the detection window.

Modern capillary electrophoresis was described by Jorgenson and Lukacs (1981). Jorgenson's group used fluorescence to detect several derivatized amino acids and peptides following separation by CE. The electrophoretic separation employed a one meter glass capillary of 75 μm i.d. This paper introduced the basic principles of CE separation and described the effect of electroosmotic flow on resolution.

Since the publication of that paper, a multitude of reports on the theory and application of CE have appeared (*e.g.*, Grossman and Colburn, 1992; Guzman, 1993; Kuhn and Hoffstetter-Kuhn, 1993; Weinberger, 1993; Monnig and Kennedy, 1994; Baker, 1995). Initially, the focus was on the development of more sensitive detectors. While ultraviolet detection can often provide sufficient sensitivity, this technique is dependent on the pathlength of the detection window and, therefore, CE detectors are less sensitive than those used in LC. The development of laser-based spectroscopic methods of detection (Yeung, 1993) and microelectrode-based amperometric detectors (Ewing *et al.*, 1994) has significantly improved both the sensitivity and selectivity of CE. Mass spectrometric detection might be the most significant advance in detection technology since it combines the high separation efficiencies obtainable with CE with the ability to generate structural information while using very small sample volumes (Olivares *et al.*, 1987).

New modes of separation have been developed, including micellar electrokinetic chromatography (Terabe *et al.*, 1984), capillary gel electrophoresis (Cohen *et al.*, 1988) and isoelectric focusing (Kilar and Hjertén, 1989). Several investigators have also succeeded in modifying capillary walls to reduce interactions of free silanol groups with large molecules such as proteins and peptides (Hjertén, 1985).

Since the first commercial instrumentation became available in 1989, CE has been widely applied in the pharmaceutical and biochemical laboratories. This is understandable, since these workers require highly efficient separations for the limited amounts of sample they frequently have available.

1.2. Advantages of Capillary Electrophoresis

Most conventional electrophoresis is still performed using slab gels. This format is simple and inexpensive, and allows one to perform multiple analyses simultaneously by using several lanes on a single gel. While the presence of the gel is necessary to prevent band broadening due to Joule heating, it can also lead to undesirable secondary interactions with the analyte of interest. In most applications, the gel acts as a sieving medium; this tends to restrict the technique to separations based on molecular size. Small molecules are generally not well separated by slab gel methods. The field strengths that can be applied in this format are limited by Joule heating and, therefore, most separations will require several hours to complete.

Densimetric or radiometric detection is most often used, followed by staining. These methods can be time-consuming and are not generally amenable to automation.

In capillary electrophoresis, the walls of the fused silica capillary permit efficient heat dissipation. Therefore, field strengths much higher than those used for slab gel electrophoresis can be employed. This results in faster and more efficient separations. Since convection due to heat is not generally a problem in CE, it is not necessary to employ gels (although they can be used for their sieving properties). This substantially widens the range of separation applications from very small (pharmaceuticals, anions and cations) to very large (glycoproteins, oligonucleotides) molecules. Column efficiencies of one million theoretical plates have been reported (Guttman *et al.*, 1990). The time required for such separations has also been reduced significantly; some separations on glass chips occurring in less than 1 s have been reported (Jacobson *et al.*, 1994).

Capillary electrophoresis is applicable in almost every laboratory where LC is used. It is complementary to reversed-phase LC since most separations are based on charge or size. In fact, many investigators have begun to employ both LC and CE to verify peak purity or to look for highly charged degradation products. The simplicity of the CE system also lends itself to the development of new separation modes. A stationary phase is not required, and it is possible to improve the separation through the use of unique buffer additives that exhibit specific chemical or physical interactions with an analyte. In addition, because only very small amounts of run buffer are needed for CE separations, it is possible to use exotic solvents and reagents that could not normally be used as LC mobile phase modifiers (due to the large quantities generally required). Some elegant chiral separations have been achieved using CE with the addition of very expensive cyclodextrin derivatives to the run buffer. At the concentrations required to effect such separations, preparation of an analogous LC mobile phases would be far too costly.

CE is also useful for those analyses where one is limited by the amount of sample available. The total volume of the separation capillary is generally less than 2 μl and injection volumes are typically 1–30 nl. Therefore, one can analyze a single 1 μl sample several times without an appreciable loss in sample volume. Single cells, which possess a total volume of only a few nanoliters, can also be analyzed using this technique (Kennedy *et al.*, 1989; see also Chapter 12).

2. Description of Instrumentation

One of the reasons for the popularity of CE is that the necessary instrumentation is quite simple and easily assembled. A typical system is illustrated in Fig. 1A. While commercial CE systems are available, the components are such that even the most financially strapped laboratory can purchase them and build a system that will be useful for many investigations. The separation and detection modes are somewhat analogous to those used for liquid chromatography, which are familiar to many investigators. In addition, LC detectors and data systems can easily be modified or directly employed for CE analyses. In many laboratories, initial investigations using CE have been performed by appropriating available equipment from existing chromatography instrumentation and modifying it for use with CE.

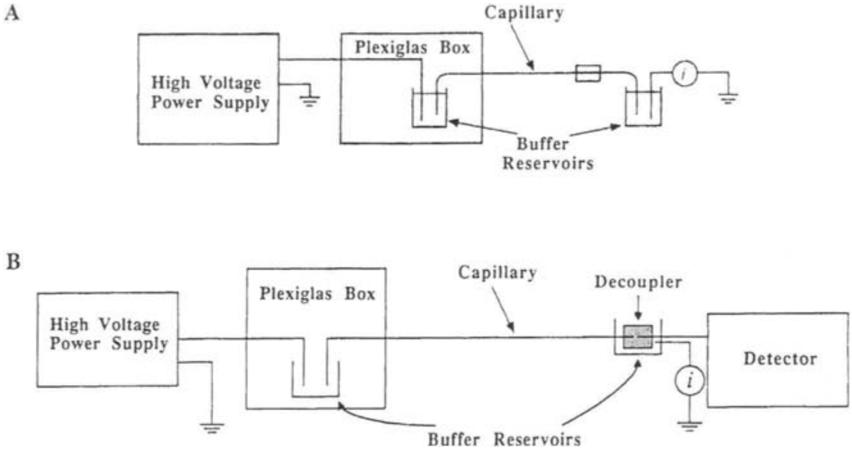

Fig. 1. Typical systems for capillary electrophoresis. A, on-column detection; B, off-column detection.

The CE separation takes place in a buffer-filled fused silica capillary with an i.d. usually 25–100 µm, although separations with capillaries as small as 1 µm i.d. have been reported. The length of the capillary used depends on the particular separation desired; however, lengths between 20 and 100 cm are most typical. To perform the CE experiment, both ends of the capillary are immersed in a buffer reservoir and a potential difference, typically 20–30 kV, is applied across the capillary using a high voltage power supply.

Detection can be accomplished either on-line (by measuring the analyte as it is moving through the capillary, Fig. 1A) or off-column (by measuring the analyte after it has eluted from the separation capillary, Fig. 1B). Spectrophotometric detection is the most commonly used method, and is generally accomplished on-column (Fig. 1A). In this case, a small portion of the protective polyimide coating on the capillary is removed to expose the fused silica surface. The light can then be focused on this point and absorbance or fluorescence measured. If mass spectrometric or electrochemical detection methods are employed, off-column detection is utilized (Fig. 1B). In this case, the detector is isolated from the electric field associated with the separation voltage by an electrical joint that will permit fluid flow, but will send the current to ground.

A very simple CE system can be constructed using a high voltage power supply, a plexiglas safety box and a commercial UV detector for LC that has been modified for the small optical characteristic of CE. In most cases, injection takes place at the anodic buffer reservoir (high voltage end), which is contained within the plexiglas box to minimize the possibility of electrical shock. In general, the cathodic buffer reservoir is connected to ground. Most commercial instruments also include temperature control, autosamplers, data acquisition systems and various injection modes.

3. Description of Separation Mechanisms

To maximize the separation mechanisms available using CE, it is necessary to understand the parameters that are most important in driving the separation. The two mechanisms with the greatest impact on separation are the electrophoretic mobility of the analyte and the electroosmotic flow generated in the fused silica capillary.

3.1. Electrophoretic Mobility

In an electrophoretic separation, two opposing forces act upon the analyte. Electrostatic force (F_e) pulls the ion toward the electrode of opposite charge. This force is proportional to the charge of the analyte (q) and the size of the electric field (E):

$$F_e = qE \tag{1}$$

Opposing this electrostatic force are the frictional forces (F_f). The magnitude of the frictional force is dependent upon the radius (R) of the ion, the viscosity of the run buffer (η) and the velocity of the ion (v).

$$F_f = 6\pi\eta Rv \tag{2}$$

If one sets the frictional force equal to the electrostatic force, then the velocity of the ion is equal to:

$$v = \frac{qE}{6\pi\eta R} \tag{3}$$

where q is the net charge of the analyte, E is the strength of the electric field, η is the viscosity, and R is the Stokes' radius. Therefore, the velocity of an ion in the electric field is directly proportional to the charge and inversely proportional to the radius (or size) of the molecule. It must also be noted that the frictional drag will increase as the viscosity of the medium increases; therefore, the value of v will decrease if everything else is held constant. Lastly, it can be seen from Eq. 3 that the velocity is dependent upon the field strength applied across the fused silica capillary. Thus, v will increase with increasing voltage if all else remains constant. Experimentally, the velocity is determined by measuring the length of time for the analyte to flow past the detector following the application of a potential difference. It is calculated by dividing the length of capillary from its end to the point of detection (L_d) by the migration time of the analyte (t_m). The resulting value has units of cm/s.

$$v = \frac{L_d}{t_m} \tag{4}$$

The electrophoretic mobility of an ion (μ_{ep}) is a constant for a particular ion in a given medium. It is defined as the steady-state velocity divided by the field strength ($\mu_{ep} = v/E$). The units for μ_{ep} are cm^2/Vs. Experimentally, it is calculated using the following equation, where V is the applied voltage:

$$\mu = \frac{L_d L_t}{V t_m} \tag{5}$$

Based on the above discussion, it could be inferred that in CE compounds are separated strictly on the basis of charge and size and that ions will always migrate to the electrode of opposite charge. However, another force, electroosmotic flow (EOF), also plays an important role in the separation.

3.2. Electroosmotic Flow

EOF is generated in fused silica capillaries because of the presence of free silanol groups on the capillary wall. The negatively charged wall attracts the buffer cations and creates an electrical double layer adjacent to the fused silica surface. These hydrated cations will migrate toward the cathode, moving the solvent with them. The resulting EOF is quite strong and is usually of a larger absolute magnitude than the electrophoretic mobility of the analytes. Thus, there is a net migration of all analytes, regardless of charge, toward the cathode. The basic separation mechanism in CE is illustrated in Fig. 2. Since negatively charged analytes have an electrophoretic mobility that is opposite in charge to the EOF, these analytes exit last. Neutral compounds migrate at the same speed as the EOF and are not resolved. Cations, which possess an electrophoretic mobility of the same charge as the electroosmotic flow, elute first.

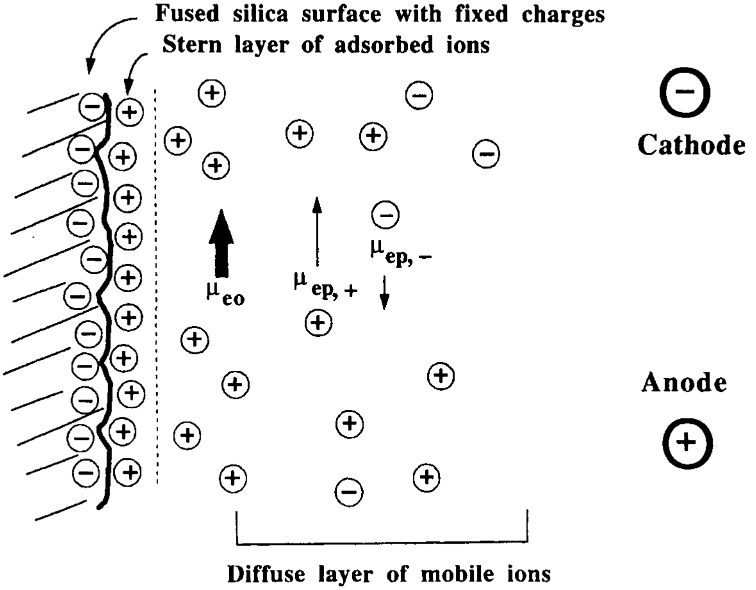

Fig. 2. Mechanism of separation in capillary zone electrophoresis.

Because of this electroosmotic flow, Eq. 5 is valid only when the electroosmotic flow has been reduced to zero (see below). This can be the case when using modified capillaries or low pH. Otherwise, the value calculated in Eq. 5 is the *apparent* electrophoretic mobility of the analyte (μ_{app}). To calculate the actual electrophoretic mobility, the electroosmotic flow must be subtracted from μ_{app}. The value for the EOF is determined by injecting a neutral marker (that is not influenced by the electric field) and measuring its apparent mobility (μ_{eof}). This is then subtracted from the μ_{app} calculated experimentally. The final value gives the actual electrophoretic mobility (μ_{act}) of the analyte.

$$\mu_{act} = \mu_{app} - \mu_{eof} \qquad (6)$$

The magnitude of the electroosmotic flow is dependent on the zeta potential of the electrical double layer at the capillary-liquid interface (ξ). It is also dependent on the dielectric constant of the background electrolyte (ε), the field strength (E) and the viscosity of the run buffer (η). The relationship of EOF to these parameters is given in Eq. 7:

$$v_{eo} = \frac{\varepsilon \zeta E}{\eta} \qquad (7)$$

EOF is beneficial in most cases because it permits fast, highly efficient separations while causing all analytes (regardless of charge) to flow past the detector. The magnitude of the EOF can be manipulated by changing the zeta potential, which is inversely related to the surface charge, the square root of electrolyte concentration, and the number of valence electrons.

Since the surface charge of a fused silica capillary is pH-dependent, EOF changes as a function of pH. The pI of the silanol group is approximately 1.5. Figure 3 shows a plot of the electroosmotic flow vs. pH for several different types of capillary material. It can be seen that at pH 2, the value of the EOF is approximately 10% of its value at pH 8. This will present a problem if a low pH is required for a separation, because it can lead to excessively long analysis times.

The electroosmotic flow can also be decreased by increasing the concentration of the electrolyte, which changes the zeta potential. As indicated in Eq. 7, increasing the viscosity of the solvent will also reduce the EOF. This can be accomplished through the use the run buffer additives such as carboxymethyl cellulose. Conversely, the η can be decreased (and, therefore, the v_{eof} increased) by raising the temperature of the separation capillary.

3.3. Separation Efficiency

The flow profile in CE is a "plug" rather than the parabolic shape characteristic of the laminar flow present in LC. This is due to the fact that the EOF is constant throughout the length of the capillary.

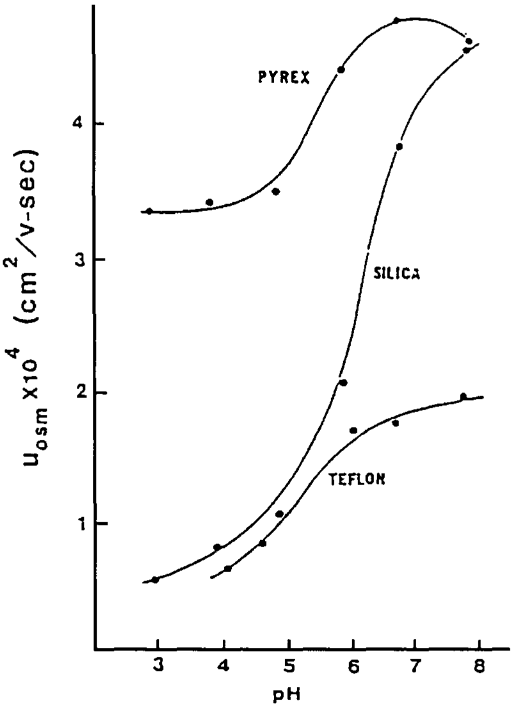

Fig. 3. Effect of pH on electroosmotic flow. (Reproduced with permission from Lukacs and Jorgenson, 1985.)

Therefore, in general, separation efficiencies attainable through CE are much higher than those obtained with LC. However, the actual efficiency obtained for each compound is dependent on several parameters. These include the electrophoretic mobility (μ_{ep}), the diffusion coefficient (D) of the analyte, and the applied voltage (V). Experimentally, the number of theoretical plates (N) is defined as:

$$N = \frac{\mu_{ep} V L_d}{2 D L_t} \qquad (8)$$

Based on this equation, it appears that the number of theoretical plates (N) should increase with increasing voltage. However, above a certain voltage, N usually decreases due to Joule heating in the capillary (see Fig. 4).

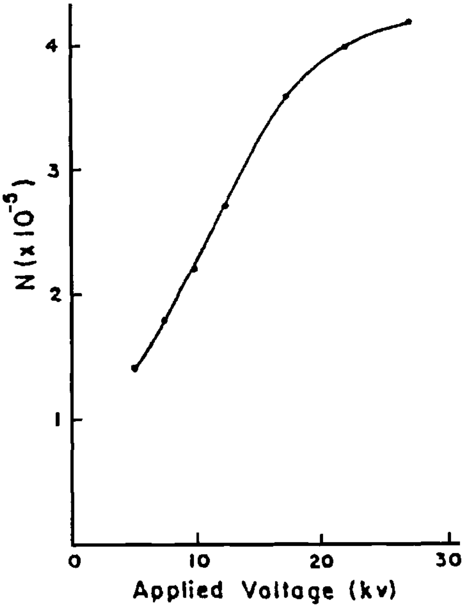

Fig. 4. Effect of applied voltage on separation efficiency. (Reproduced with permission from Jorgenson and Lukacs, 1981.)

It is also implied in Eq. 8 that compounds with smaller diffusion coefficients (*i.e.*, large molecules) will exhibit higher separation efficiencies. Once again, this is not always the case, since many of these same molecules (for example, basic proteins) undergo undesirable electrostatic or hydrophobic interactions with the silanol groups on the fused silica. On the other hand, small molecules that have large diffusion coefficients and are not generally separated by conventional slab gel electrophoresis exhibit high separation efficiencies in CE due to their large electrophoretic mobilities.

3.4. Joule Heating

There are several factors that can cause zone broadening and reduce the apparent separation efficiency in CE; the most important of these is Joule heating. The amount of heat (ΔH) generated in a capillary is proportional to the conductivity of the buffer (K) and the square of the applied voltage (V), and is inversely proportional to the square of the length of the capillary (L). These relationships are summarized in Eq. 9.

$$\Delta H = \frac{KV^2}{L_t^2} \tag{9}$$

As the field strength for the separation is increased, so also is the amount of Joule heating. It is apparent from Eq. 9 that the conductivity of the buffer should be kept as low as possible to minimize this heating. Because of thermal convection, a high voltage separation performed in a very short capillary is more likely to experience a reduction in efficiency than is the same separation performed in a longer capillary. Thus, one must balance the speed that is required for a separation with the possible loss of efficiency caused by Joule heating.

Efficient heat dissipation is essential in order to obtain high separation efficiencies with CE. In larger diameter capillaries, a thermal gradient will be produced whereby the temperature in the center of the capillary is higher than that at the walls. This problem can be lessened by using a capillary with a smaller i.d., where there is a larger surface-to-volume ratio. The current passing through a capillary is also directly proportional to the cross-sectional area of the capillary; therefore, the use of smaller diameter capillaries will minimize the amount of Joule heating due to current. Typical capillaries used for CE separations range from 5 to 100 μm i.d.

3.5. Additional Factors Contributing to Band Broadening

Conductivity differences between the analyte zone and the run buffer in the separation capillary may lead to band broadening in CE. This can occur if a very concentrated plug of analyte is introduced. In this case, the analyte zone will generate its own electric field, causing dispersion of the zone as it moves through the capillary.

Several physical factors can also influence band dispersion within a capillary. If the inlet and outlet ends of a capillary are not placed at the same level in the instrumentation, some siphoning through the capillary may occur. This will lead to broadening of the analyte zone, since some hydrodynamic flow will be introduced into the system. Larger pathlength flow cells, such as the Z cell, that are used for increased sensitivity with UV detection also decrease resolution by sampling the absorbance of a larger portion of the capillary. Lastly, off-column detection methods can lead to a decrease in efficiency if there is a large distance between the ground electrode and the detector. The analyte zone is driven over the decoupler by the electroosmotic flow. However, once over the decoupler, the magnitude of the flow is decreased gradually, which leads to zone dispersion.

3.6. Resolution

In order to increase resolution in CE, the difference in electrophoretic mobilities of two species must be maximized. Equation 10 shows the expression for calculating resolution in CE.

$$R_s = 0.177\Delta\mu\sqrt{\frac{V}{(\bar{\mu} + \mu_{eof})D_m}} \qquad (10)$$

where $\bar{\mu}$ is the average electrophoretic mobility of species 1 and 2 ($\bar{\mu} = (\mu_1 + \mu_2)/2$), N is the number of theoretical plates, $\Delta\mu = \mu_1 - \mu_2$ is the difference between the individual mobilities of species 1 and 2, D_m is the diffusion coefficient, and μ_{eof} is the electroosmotic mobility. The most common way to improve resolution is to maximize the difference in electrophoretic mobilities of the two analytes. This is most frequently accomplished by changing the ionization state of the analyte through adjustment of the pH of the buffer. Alternatively, one can alter the mode of separation, for example, by adding surfactants or using gels. Once this has been accomplished, the resolution can be further improved by increasing the voltage applied across the capillary.

4. Injection Methods

Injection in CE can be accomplished in several ways. Each of the methods has its own unique advantages and disadvantages.

4.1. Electrokinetic Injection

Electrokinetic injection is the simplest method of sample introduction. In this case, the anodic end of the capillary is removed from the run buffer and placed in the sample reservoir (while no voltage is applied). A voltage that is much lower than the run voltage is applied for a short period of time (5–60 s). During this period, analytes from the sample migrate slowly into the capillary. Since the field strength is low and the period of time for the injection is short, only a small amount of sample is introduced. This injection method is based strictly on the electrophoretic mobility of the analytes. Since the analytes will have different electrophoretic mobilities depending on their size and charge, there will be an unequal representation of positive, neutral and negative ions. Because this can lead to errors in quantitation, internal standards are almost always necessary. The major advantage of this technique is that it is very simple to implement and requires no additional equipment beyond that described above for performing the basic CE experiment. It is the method of choice for introduction of sample in capillary gel electrophoresis because the other forms of injection can disturb the gels.

In the electrokinetic injection mode, the electrophoretic mobility of the analyte must be known in order to calculate the actual amount injected. The quantity of material injected (A) is defined by the following equation:

$$A = \frac{(\mu_{ep} + \mu_{eof})\pi r^2 VCt}{L_t} \tag{11}$$

where μ_{ep} and μ_{eof} are the electrophoretic and electroosmotic mobilities of the analyte, respectively, r is the capillary radius, V is the applied voltage, t is the length of time that the field strength is applied, C is the concentration of each solute and L_t is the total length of the capillary.

4.2. Gravity Injection

Gravity injection is another very simple method; it is accomplished by raising the anodic buffer end a defined distance above the cathodic buffer reservoir. Analyte is siphoned into the capillary at a constant rate. The volume of materials injected per unit time (V_t) is equal to

$$V_t = \frac{\rho g \Delta h d^4 \pi}{128 \eta L} \tag{12}$$

where ρ is the density of the solution, g is the force of gravity, $\Delta h = h_f - h_i$ (h_i is the initial height and h_f is final height), d is the diameter of the capillary, L is the length of the capillary, and η is the viscosity. In contrast to electrokinetic injection, positive, negative and neutral compounds are applied to the capillary with equal efficiency.

4.3. Vacuum or Pressure Injection

This is probably the most reproducible method of injection, and injects positive, negative and neutral compounds with the same efficiency. A highly regulated pressure or vacuum is used to push or pull a plug of the analyte mixture into the fused silica capillary. An advantage of this technique is that the same system can be used to quickly fill or rinse the capillary. The total volume injected on capillary is described by Eq. 13.

$$N_t = \frac{\Delta P d^4 \pi}{128 \eta L} \tag{13}$$

where ΔP is the drop in pressure across the capillary.

4.4. Peak Stacking

With both electrokinetic and hydrodynamic injection methods, it is possible to use a technique termed "peak stacking" to improve the limits of detection for a particular analyte. In peak stacking, the sample is dissolved in a solvent of much lower conductivity than that of the run buffer. When the sample is injected and the high voltage applied, a larger portion of the electric field is applied across the sample zone due to its larger resistance. Therefore, sample ions move toward the buffer/sample interface at a faster rate than the electroosmotic flow. This movement toward the interface continues until the conductivity of the two zones become equivalent, after which normal electrophoresis takes place. As ions move toward the interfacial boundary, they stack up. Typical improvements in LOD are 10–100-fold. Peak stacking is most effective if the pH of the run buffer is below 5.

5. Modes of Separation

5.1. Capillary Zone Electrophoresis (CZE)

Capillary zone electrophoresis (CZE) or free zone electrophoresis is the simplest mode of CE separation. A voltage is applied across a fused silica capillary containing buffer and compounds are separated based on a combination of their electrophoretic mobility and the magnitude of the electroosmotic flow. In CZE, molecules are separated based on their size and charge. For most analytes, the charge is dependent on pH and, therefore, the separation can be manipulated by changes in pH. Amino acids and peptides are ideal candidates for separation by CZE.

Protein separations can be accomplished by CZE, but special consideration must be made for basic proteins, which tend to adsorb to the wall of the fused silica capillary (see Chapters 2 and 9). This interaction can be minimized by running the separation at a low pH, where the silanol groups are no longer ionized. However, this can lead to very long analysis times and denaturation of the analyte. Alternatively, the separation can be run at a pH greater than the pI of the protein. In this case, both the protein and the fused silica surface are negatively charged and thus repel one another. Problems with this approach include fast electroosmotic flows that can make resolution of proteins with similar pI values difficult or impossible. Again, protein denaturation can also occur at this pH. Currently, the most common approach to protein separations is modification of the wall of the fused silica capillary so that the silanol groups are no longer exposed. Several coated capillaries have been described in the literature and are examined in more detail in Chapter 2.

5.2. Micellar Electrokinetic Chromatography (MEKC)

CE is generally considered a separation technique for charged molecules. In order to separate neutral molecules by CE, a secondary mode of separation must also be employed. MEKC was introduced by Terabe and coworkers (Terabe *et al.*, 1984) and is described in much more detail by Foley and Ahuja in Chapter 3. In this form of CE, the run buffer contains a surfactant that acts in a manner similar to the stationary phase of a reversed-phase liquid chromatography column. The surfactant is introduced into the capillary at a concentration above the critical micelle concentration (CMC). These charged micelles act as a stationary phase. Compounds that are hydrophobic spend more time in the negatively charged micelle and therefore elute last. Hydrophilic compounds, on the other hand, do not partition into the micelle and are therefore separated based only on their electrophoretic mobility. The retention time t_r for an analyte by MEKC is described by:

$$t_r = \frac{t_o(1 + k')}{1 + \left(\frac{t_o}{t_{mc}}\right)k'} \qquad (14)$$

where t_0 is the migration time of an "unretained" analyte, t_{mc} is the migration time of the micelle and k' is the capacity factor of the analyte partitioning into the micelle. In order to achieve the best separation, one must maximize the difference between t_0 and t_{mc}, since that is the window of the separation. This method has been shown to work well with small hydrophobic drugs.

Sodium dodecyl sulfate (SDS) and cetyltrimethylammonium bromide (CTAB) are the most common MEKC reagents. If SDS is used, the order of elution will be the reverse of that with CZE due to electrostatic repulsion between the SDS and anions present in the sample. Neutral molecules can be separated by MEKC based on their relative hydrophobicity. Cations are retained longest on the capillary due to the electrostatic attraction between the SDS micelle and the positively charged ion. However, if a class of compounds is very hydrophobic, they may all spend the entire time in the micelle and elute at t_{mc}. Therefore, in order to separate very hydrophobic compounds, it is frequently necessary to add an organic modifier to the mobile phase (just as with LC). This decreases the amount of time that the analyte spends in the micelle and improves the selectivity.

A similar mechanism of separation can be employed for the separation of chiral compounds. In this case, cyclodextrins, bile salts and metal additives are used to complex (or include) chiral molecules and thereby change their relative migration times.

5.3. Capillary Gel Electrophoresis

Capillary gel electrophoresis (CGE) is becoming an increasingly popular form of CE and is being explored by both the biotechnology industry and the human genome project. In capillary gel electrophoresis, the fused silica capillary is filled with a sieving media. Gels can either be chemically crosslinked into rigid polymers or can take the form of physically crosslinked gels. Polyacrylamide gels have become very popular for the separation of oligonucleotides and proteins. These compounds cannot be resolved by CZE because the charge/mass ratio does not change significantly as the oligonucleotide grows. In CGE, the gel is used only for sieving purpose and not for its anticonvective properties. Generally, however, very high field strengths (greater than 500 V/cm) cannot be employed because of Joule heating and bubble formation. The use of CGE for the separation of oligonucleotides is discussed in greater detail in Chapter 10.

5.4. Isoelectric Focusing

Isoelectric focusing (IEF) is a popular electrophoretic technique because it is possible to separate proteins based on small differences in pI. It can also be used to determine the pI of an unknown protein. To carry out IEF in a capillary, the electroosmotic flow must be suppressed. This is accomplished by using modified capillaries or adding carboxymethylcellulose to the run buffer. To perform IEF, a sample is dissolved in a carrier ampholyte and pumped into the capillary. The two buffer reservoirs are then filled with an acid and a base and the electric field is applied. The analyte then moves to the pH-pI (*i.e.*, it is neutral) and stops. After all the analytes have been focused, the bands are mobilized by changing the run buffer and are detected at the electrode of opposite charge (see Chapters 2 and 9).

6. Summary

Capillary electrophoresis is a simple technique with many possible analysis modes. In this book, we will focus on methods of separation and detection that are relevant to the areas of pharmaceutical and biomedical analysis. The field of CE has grown considerably over the past 15 years, and it is anticipated that this growth will persist in the future. In this volume, we have attempted to emphasize fundamentals that will continue to be useful to workers as the field changes and advances.

7. References

Baker, D. R. (ed.) (1995). *Capillary Electrophoresis*, Wiley, New York.

Cohen, A. S., D. R. Najarian, A. Pauls, A. Guttman, J. A. Smith and B. L. Karger (1988). *Proc. Natl. Acad. Sci. USA, 85*, 9660–9663.

Ewing, A. G., J. M. Mesaros and P. F. Gavin (1994). *Anal. Chem., 66*, 527A–536A.

Grossman, P. D. and J. C. Colburn (1992). *Capillary Electrophoresis, Theory and Practice*, Academic Press, New York.

Guttman, A., A. S. Cohen, D. N. Heiger and B. L. Karger (1990). *Anal. Chem., 62*, 137141.

Guzman, N. (ed.) (1993). *Capillary Electrophoresis Technology*, Vol. 64, *Chromatographic Science,* Dekker, New York.

Haglund, H. and A. Tiselius (1950). *Acta Chem. Scand., 4,* 957–962.

Hjertén, S. (1967). *Chromatogr. Rev.*, *9*, 122–219.

Hjertén, S. and M. Zhu (1985) *.J. Chromatogr., 346*, 265–270.

Jacobson, S. C., R. Hergenröden, L. B. Koutny and J. M. Ramsey (1989). *Anal. Chem., 66*, 1114–1118.

Jorgenson, J. W. and K. D. Lukacs (1981). *Anal. Chem., 53*, 1298–1302.

Kennedy, R. T., M. D. Oates, B. R. Cooper, B. Nickerson and J. W. Jorgenson (1989). *Science, 246*, 57–63.

Kilar, F. and S. Hjertén (1989). *J. Chromatogr., 480*, 351–357.

Kuhn, R. and S. Hoffstetter-Kuhn (1993). *Capillary Electrophoresis, Principles and Practice*, Springer-Verlag, Berlin, New York.

Lukacs, K. D. and J. W. Jorgenson (1985). *J. High Resolut. Chromatogr., 8*, 411–417

Mikkers, F. E. P., F. M. Everaerts and T. P. E. M. Verheggen (1979). *J. Chromatogr., 169*, 11–20.

Monnig, C. A. and R. T. Kennedy (1994). *Anal. Chem., 66*, 280R–314R.

Olivares, J. A., N. T. Nguyen, C. R. Yonker and R. D. Smith (1987). *Anal. Chem., 59*, 1230–1232.

Terabe, S., K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando (1984). *Anal. Chem., 56*, 111–113.

Weinberger, R. (1993). *Practical Capillary Electrophoresis*, Academic Press, Boston.

Yeung, E. (1993). In: *Capillary Electrophoresis Technology* (N. A. Guzman, ed.), Vol. 64, *Chromatographic Science*, pp. 587–603. Dekker, New York.

CHAPTER 2

Column Technologies for Capillary Electrophoresis

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1. Introduction

Capillary electrophoresis (CE) is a rapid, high-resolution analytical technique for the separation of a wide variety of charged and uncharged species. Separations in electrophoresis are based on the differential migration rates of species through a solution or gel in the presence of an electric field. In contrast, separations in liquid chromatography (LC) are based on differential chemical interactions with a surface in all cases except size exclusion chromatography. The unique selectivity of CE and the need for additional high resolution separation modes have resulted in increased interest in this technique. Important applications of CE include the analysis of complex biological mixtures, for example, the separation and characterization of macromolecules.

The use of CE is no longer limited to "experts." Commercial instruments, buffer formulations, and column technologies are widely available. With the emphasis switching from theory and instrument development to "real life" applications, attention is being directed toward the capillary itself. It is disconcerting that when using a

$40,000 commercial system, success or failure of the analysis can depend on a $10 piece of fused silica tubing.

The fused silica tubing does not always provide the necessary separation performance; modification of the capillary is often required. The wide acceptance of the technique and practical applications can be attributed in large part to advances in column technology. It is now possible to move beyond the analysis of amino acids and peptides to that of macromolecules. This, in turn, leads to the prospect that CE can provide the necessary resolving power for separations that are currently not possible using present techniques such as chromatography and slab electrophoresis. Table 1 provides information on column modifications and applications employed for the various modes of capillary electrophoresis.

Table 1. Column Modifications and Applications Employed in Capillary Electrophoresis Methodologies

Methodology (*Column Modification*)	Application
Free-zone electrophoresis (*Column coating*)	
Neutral charged coating	Proteins and carbohydrates
Positive charged coating	Positively charged compounds basic peptides and proteins
Negative charged coating	Negatively charged compounds acidic peptides and proteins
Capillary gel electrophoresis (*Gel filled*)	Peptides and proteins large oligonucleotides single and double stranded DNA fragments
Capillary isoelectric focusing (*Ampholyte filled/coating*)	Peptides and proteins
Electroendoosmotic chromatography (*Particle filled*)	Organic compounds
Chiral capillary electrophoresis (*Chiral coating*)	Chiral compounds

This chapter focuses on advances in column technology for reducing solute adsorption, manipulating flow rate, and enhancing the efficiency and selectivity of CE. Special attention is given to the advantages and limitations of capillary electrophoresis for the separation of macromolecules. The tendency of some proteins to adsorb to the inner walls of fused silica capillaries and the problems associated with this are also discussed.

2. The Advantages of Electrophoresis in Capillaries

Conventional slab-gel electrophoresis is a powerful approach for the separation and analysis of charged biopolymers. This form of electrophoresis, however, is rather labor-intensive and does not provide data in a format that lends itself readily to quantitation. The major limitation in slab-gel electrophoresis is Joule heating, which results from the ionic current generated between the electrodes. Joule heating can cause density gradients and subsequent temperature gradients that increase convective zone broadening and affect electrophoretic mobilities. A supporting medium such as a gel is an effective means of eliminating convection, thereby minimizing these sources of band broadening. Stabilizers increase the surface area available for solute adsorption and complicate gel preparation. Conventional slab gel systems are not easily adapted to on-line sample application, detection, quantification and automated operation.

An advantage of using capillary columns is that their large surface area-to-volume ratio allows heat to dissipate more efficiently than in slab-gels (Hjertén, 1983). Stabilizing gels are not necessary in capillary electrophoresis. This permits the use of very high potential fields for fast, efficient separations.

2.1 The Optimum Capillary Dimensions

Tubes of small diameter are advantageous because they increase the rate of heat transfer per unit volume of buffer, reducing the magnitude of temperature differences within the capillary. Temperature differences between the fluid in the center and at the wall are roughly proportional to the square of the i.d. of the column (Wieme, 1975). This means that smaller tubes reduce temperature-induced zone spreading. A second advantage of smaller i.d. capillaries is that the drag of viscous fluid at the capillary wall (the "wall effect") diminishes bandspreading induced by convective flow. Lastly, analyte diffusion across smaller i.d. tubes leads to diffusional averaging of solutes and narrowing of zones.

Lukacs and Jorgenson (1985) found that a dramatic decrease in theoretical plates occurred when capillaries with diameters greater than approximately 80 μm were employed; this could be attributed to insufficient heat dissipation as described above. Capillary length is also important in separation optimization. At a constant voltage (30 kV), capillaries 100-cm long gave the maximum number of theoretical plates. Capillaries both longer and shorter had fewer theoretical plates. At a given applied voltage, shorter capillaries decrease the surface area available to dissipate heat, resulting in temperature gradients and zone broadening.

Selecting the optimum capillary diameter requires balancing detector sensitivity and heat dissipation. For optical detection methods, detection sensitivity is related to capillary diameter, *i.e.*, pathlength. Problems with small i.d. capillaries include small injection volumes (typically 10 nl), optical distortion, and scatter due to the rounded capillary tubing. Square and rectangular borosilicate glass capillaries were examined and found to minimize optical distortion and light scattering while still providing efficient heat dissipation (Tsuda *et al.*, 1990). The corners of the capillaries apparently do not degrade the separation. Rectangular (i. d. ranging from 16 μm × 195 μm to 50 μm × 1 mm) and square capillaries (50 μm × 50 μm) were shown to have separation efficiencies comparable to that of 50-μm i.d. cylindrical capillaries. These capillaries allow injection volumes to be increased without losing resolution.

The use of UV detection, however, has severe limitations for high-sensitivity analysis due to the short pathlength afforded by the capillary. Extending the optical pathlength offers a means of enhancing sensitivity without significant hardware modifications or chemical derivatization. Detection across the long cross-sectional axis of the rectangular capillary provided a substantial increase in sensitivity. At least two manufacturers have introduced modifications of the capillary for improved sensitivity. The Z-shaped (Moring *et al.*, 1993) and bubble (Heiger, 1992) cells have greatly increased detection sensitivity by increasing the pathlength with little loss in electrophoretic resolution and separation efficiency.

The protective polyimide coating on the outside of the capillary must be removed for on-column UV-visible detection. This results in a fragile, optically transparent detection window. The outside coating can be removed by somewhat labor-intensive methods ranging from burning with a flame to using hot sulfuric acid. When using these high temperature methods, care must be taken that coatings or gels inside the capillary are not destroyed. This type of damage may take the form of bubbles in gel-filled columns and increased adsorption of analytes due to the destruction of the coating in modified capillaries. An optically transparent capillary coating available from Polymicro Technologies (Phoenix, AZ) is an attractive alternative to polyimide-coated capillaries.

2.1.1. Capillary electrophoresis on a chip

Manz *et al.* (1992) eliminated the capillary column altogether in their work on electrophoresis by using a chip-like structure. Micromachining, a well-known photolithographic technique for creating structures in the micrometer range, provided the means to manufacture an entire microchannel system of very high precision. The photolithographically fabricated glass device consists of two glass plates, one containing etched channels and the other serving as a cover. The electrophoresis channel was 10 μm deep and 30 μm wide with rounded corners. Buffer reservoirs (pipette tips) contained platinum electrodes and were mounted directly into drilled holes at the ends of the channels. Figure 1 shows the separation of two fluorescent dyes using an applied voltage of 3000 V in a channel 13-cm long with laser fluorescence detection at 6.5 cm along the channel. Electrophoretic separations within such a glass structure are a further step in the miniaturization of CE to provide high efficiency, fast analysis time, and low reagent consumption.

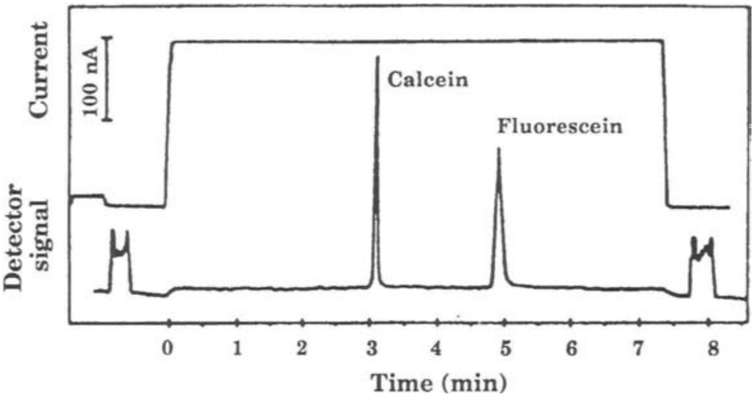

Fig. 1. Capillary electrophoretic separation of two fluorescent dyes. Sample: 20 mM calcein, 20 mM fluorescein. Background electrolyte: 50 mM borate, 50 mM Tris, pH 8.5, 3000 V on 13 cm. Detection at 6.5 cm, fluorescence, excitation 490 nm, collection 520 nm, injection through side channel, 500 V for 30 s. (Reproduced with permission from Manz *et al.*, 1992.)

2.2. Electroosmotic Flow

Electroosmosis is the flow of liquid that occurs when an electrical potential is applied to a liquid-filled porous medium (Pretorius *et al.*, 1974). In an uncoated capillary, this flow is the result of ionized silanol groups on the capillary wall being balanced by an excess of cations in the stagnant double layer adjacent to the capillary wall.

Under an applied potential, solvated cationic counterions in the diffuse layer migrate toward the cathode. The rate of electroosmotic flow is governed by the potential drop across the surface double layer, which in turn is a function of the charge density on the capillary wall (Pretorius *et al.*, 1974; Davies and Rieal, 1961). Because the flow originates at the diffuse region of the double layer, charge density at the capillary wall determines the magnitude and direction of flow. In uncoated capillaries, this flow is strong enough to cause all solutes, independent of charge, to migrate toward the cathodic buffer reservoir. Therefore, only a single detector is needed. As the capillary is shortened to decrease analysis time, the electrical resistance of the capillary is decreased and more Joule heat must be dissipated. At the same time, the surface area has been reduced and heat is dissipated more slowly. This results in serious thermal overloading, the effect of which ranges from zone broadening and bubble formation to electrical arcing in the capillary (Jorgenson and Lukacs, 1984).

The concentration of electrolyte is also a critical parameter in capillary electrophoresis (Wallingford and Ewing, 1989). High electrolyte concentrations result in an elevation of current, which in turn produces more heat. It would seem that very low ionic strength would be best. However, the electrolyte concentration must be significantly greater than the concentration of the individual analytes. If the analyte concentration is greater than that of the run buffer, there will be a discontinuity in conductivity when solutes separate into zones. This produces a change in the local potential gradient and results in uneven migration and zone asymmetry.

The shape of the flow profile across the capillary axis is unique in electroosmotically driven pumping. Because the flow is driven by the surface double layer, which is extremely small, the shape of the profile is "piston-like" over most of the capillary cross section and only drops to zero at the capillary wall (Rice and Whitehead, 1965). This flat profile of the electroosmotic flow results in increasing separation efficiency compared to that of the parabolic flow profile characteristic of hydraulic flow. The combination of a flat profile and lack of a stationary phase makes capillary electrophoresis an extremely high efficiency separation technique.

3. Limitations of Electrophoresis in Capillaries

Early research into the optimum material for capillaries focused on pyrex borosilicate glass, fused silica, and Teflon (Jorgenson and Lukacs, 1984). Recently, polymeric hollow fibers prepared from polypropylene and polybutylene terephthalate have been used as CE columns (Liu *et al.*, 1993). The hydrophobic nature of these capillaries, which leads to very strong interactions with proteins, makes modification of the capillary wall with polyacrylamide necessary for improved performance.

Fused silica is widely used due to its superior UV transparency, thermal conductivity, and thermal stability. However, a major problem in the analysis of proteins by capillary electrophoresis is adsorption of the analyte to the fused silica capillary. The exposed silica surface is rich in silanol groups which are weakly acidic, resulting in a negatively charged surface. In uncoated capillaries, exposed silanol groups are ionized above pH 4. These negative silanol groups cause problems in the analysis of proteins by adsorbing positively charged species onto the fused silica capillary.

3.1. Adsorption

High resolution capillary electrophoresis is of great utility in the separation of inorganic ions, amino acids, oligonucleotides, and peptides. Peptide separations yielding over a million theoretical plates have been observed (Jorgenson and Lukacs, 1983). This illustrates the high efficiency separations attainable with CE. It had been hoped that the separation efficiency for proteins might be equally high due to their small diffusion coefficients (Jorgenson and Lukacs, 1981). Unfortunately, protein separations have not achieved this high level of efficiency.

Proteins readily adsorb to silica due to charged and hydrophobic sites on the surface of both materials. Polypeptides generally contain acidic and basic side chains that give them substantial charge. Depending on the isoelectric point (pI) and the buffer pH, a protein can be either anionic or cationic. Kapaciewicz *et al.* (1983) showed that even at the pI a protein can have regions of localized positive or negative charge. This means that proteins having zero net charge can still adsorb to a charged surface. Green and Jorgenson (1989) have reviewed the mechanisms that have been proposed for this protein-capillary wall interaction. It is generally thought of as a combination of two mechanisms—electrostatic and hydrogen-bonding—which substantially reduces protein recovery, alters peak shape, reduces efficiency, diminishes resolution and changes electroosmotic flow. This results in irreproducible migration times and erroneous peak area determinations.

Although the irreversible adsorption of proteins to the capillary wall results in the loss of protein and a change in the wall characteristics, even reversible adsorption can cause serious problems beyond that of band broadening. There is the question of the structure of the protein when it desorbs from the silica surface. Adsorption of proteins on silica can cause denaturation (Regnier and Noel, 1976). This seriously limits the use of untreated fused silica capillaries for the separation of sensitive biological macromolecules. An additional problem of protein adsorption is its impact on electroosmotic flow. Table 2 summarizes the major effects of protein adsorption on separation performance in capillary electrophoresis.

Table 2. Adverse Effects of Protein Adsorption

Reduced protein recovery
Reduced peak symmetry
False peak area determination
Reduced elution time reproducibility
Reduced efficiency and resolution
Solute mixing

3.2. Adverse Effects of Protein Adsorption

3.2.1. Alteration of peak symmetry

The most noticeable consequence of protein adsorption at the capillary wall is peak tailing. A good example of the effect of protein adsorption on peak shape is illustrated in Fig. 2 with the positively charged protein cytochrome *c* (pI 10.2) using both A) uncoated and B) coated capillaries (Towns and Regnier, 1992). In evaluating the uncoated capillary, the first three injections of approximately 10 ng of protein were completely adsorbed to the wall. The fourth injection produced a peak that tailed badly and migrated much slower than expected. Improvement in terms of efficiency, resolution, recovery, and peak shape was observed with the coated capillary. The coating process resulted in a twofold reduction in electroosmotic flow and increased recovery from 0 to 91% (Towns and Regnier, 1991).

Tailing of positively charged proteins such as cytochrome *c* at neutral pH is attributed primarily to a reversible electrostatic interaction of the protein with negatively charged silanol groups at the capillary wall. The solute distribution coefficient for electrostatic adsorption, rates of adsorption and desorption, solute diffusion coefficient, and capillary diameter all play a role in determining the extent of band spreading. A noticeable feature of species such as proteins that are strongly, *i.e.,* irreversibly, adsorbed to the capillary wall is that the

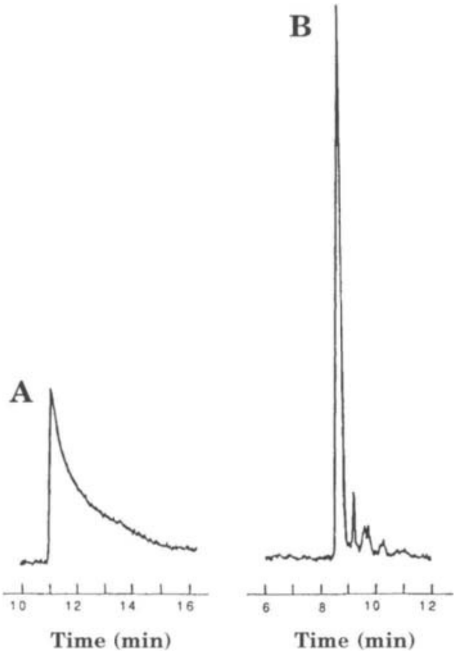

Fig. 2. Peak shape for cytochrome *c* on A) an uncoated capillary and B) a C-18/Brij coated capillary. Conditions: 0.01 M phosphate buffer, pH 7.0; 75 μm i.d. × 100 cm capillary (85 cm to detector); 28 kV, 30 μA. (Reproduced with permission from Towns and Regnier, 1992.)

baseline does not return to zero after the analyte passes the detector (Fig. 2A). This is due to irreversible adsorption of the protein onto the detector window, causing a constant absorption reading from the detector and an upward baseline shift.

The emergence of several components in the cytochrome *c* sample on the coated capillary is a good example of how peak tailing can completely obscure minor peaks in an electropherogram. The cytochrome *c* sample on an uncoated capillary shows excessive peak tailing, which completely masks the minor components. Using a coated capillary, the adsorption of protein to the capillary wall is reduced to the extent that the minor components are no longer obscured and are determined to make up 5% of the total sample.

3.2.2. Reduced protein recovery

In the analysis of proteins by CE, it is important to know the extent to which protein is adsorbed to the capillary column. Table 3 lists the percent recoveries of 11 proteins using a low ionic strengthbuffer (0.01 M phosphate) at pH 7.0 (Towns and Regnier, 1991). Recoveries were determined using two detectors 60 cm apart.

Table 3. Percent Recovery for Proteins of Varying pI Values

Protein	pI Value	% Recovery
Lysozyme	11.1	0
Cytochrome *c*	10.2	0
Ribonuclease A	9.3	0
Chymotrypsinogen	9.2	0
Myoglobin	7.3	63
Conalbumin	6.3	66
Carbonic anhydrase	5.2	72
β-Lactoglobulin B	5.2	74
β-Lactoglobulin A	5.1	76
Ovalbumin	4.7	81
Pepsin	3.2	90

Determined using two detectors 60 cm apart on a 75-mm i.d. capillary. Conditions: 0.01 M phosphate buffer, pH 7.0, detection at 214 nm, 300 V/cm, 30 mA

Positively charged proteins with pI values above 8, such as lysozyme (pI 11.1), cytochrome *c* (pI 10.2), ribonuclease A (pI 9.3), and chymotrypsinogen-A (pI 9.2), were readily adsorbed onto the silica wall and were not recovered. As the pI values of proteins decreased, recovery increased. Myoglobin, with a pI of 7.3, was the first protein recovered from an initial injection. As pI values decreased, recovery reached a maximum of 90% (pepsin, pI 3.2). The increase in recovery for proteins with low pI values is due to coulombic repulsion between the negatively charged protein and the negative wall of the capillary. With a pI value of 3.2, pepsin will be negatively charged at neutral pH and strongly repelled from the wall.

The same type of two-detector system was used to evaluate the effect of buffer pH on protein recovery for six selected proteins. The results showed a minimum in recovery near neutral pH with increased recovery at both high and low pH extremes (Fig. 3). The per-

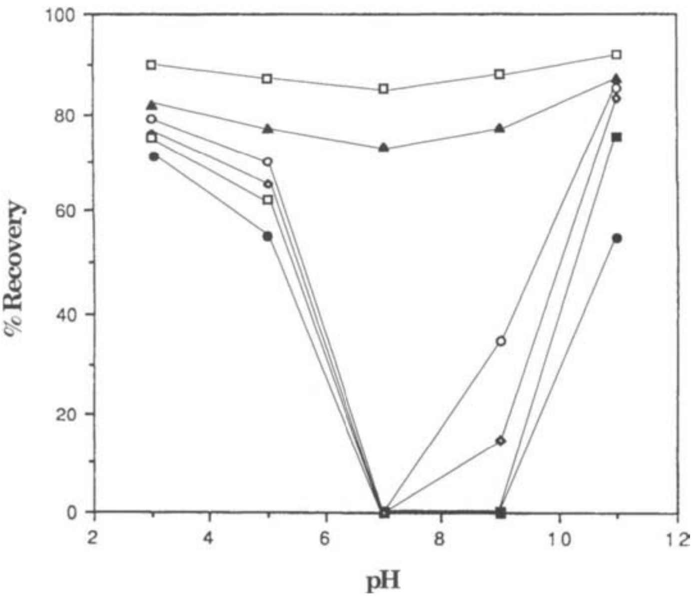

Fig. 3. Protein recovery vs. pH for six proteins ranging in pI value from 3.2 to 11.1 on an uncoated 75 µm i.d. × 100 cm capillary using two detectors 50 cm apart. Conditions: pH 3 and 5, 0.01 M acetate; pH 7, 0.01 M phosphate buffer; pH 9 and 11, 0.01 M diaminopropane. Detection at 214 nm; 300 V/cm, 30 µA. □, Ovalbumin; ▲, myoglobin; ○, chymotrypsinogen; ◇, ribonuclease A; ■, cytochrome *c*; ●, lysozyme. (Reproduced with permission from Towns and Regnier, 1992.)

cent recovery was also determined for the acidic protein ovalbumin (pI 4.7). The shallow minimum near neutral pH indicates that varying the pH of the buffer has less effect on protein recovery for acidic than basic proteins.

The results shown in Fig. 3 may be interpreted as being due to a combination of the charged properties of the fused silica capillary and individual proteins. It is well known that minimal ionization of surface silanols occurs at or below pH 3. At this low pH, the separation of proteins is facilitated by protonation of silanols on the capillary wall. Ionic suppression prevents adsorptio of cationic species, and the recovery of these proteins is relatively high. Ionization of surface silanols occurs broadly over a pH range starting at 3 and extending to greater than 9. Proteins, in contrast, are amphoteric; at pH values below their isoelectric point they are anionic, whereas above the pI they are cationic. Net charge increases on either side of the pI. At neutral pH, basic proteins have a net positive charge and are adsorbed to the negatively charged capillary wall, reducing recovery. As the pH is increased, the proteins with isoelectric points below the buffer pH have a net negative charge and are electrostatically repelled from the surface. Repulsion of the protein from the capillary wall results in an increase in recovery compared to that obtained at neutral pH.

Buffers of low or high pH are best for protein recovery. The use of these pH extremes, however, greatly limits conditions for the analysis of proteins. Maximum charge differences between proteins may result at intermediate pH values, and proteins may not be stable at low pH (McCormick, 1988). Denaturation of proteins may cause greater adsorption, dissociation of multimeric proteins, and/or loss of activity in post-separation, on-column functional assays of enzymes (Wiktorowicz and Colburn, 1990). Since pH is the best parameter for optimizing selectivity, limiting the pH greatly reduces flexibility in separations. The optimum resolution generally occurs in the pH range of 4 to 9. This, however, is the exact pH range that results in the largest adsorption of proteins to the capillary wall. Therefore, a gap exists between the usefulness of silica capillaries under non-adsorbing pH conditions and the pH range that would maintain proteins in their native state and induce the greatest charge difference.

3.2.3. *Alteration of electroosmotic flow*

The factors that control flow rate in CE are quite different from those in liquid chromatography, where the mobile phase is driven by a pump with a predetermined flow rate. Electroosmotic flow is determined by the zeta potential, which is a function of the density and sign of the charges on the capillary wall. The flow is fastest under conditions that maximize the zeta potential. Excluding adsorption effects, the major factors that alter zeta potential are pH, ionic strength, and buffer type.

Adsorption of proteins to the capillary surface greatly affects the electroosmotic flow. It is this dependence of the electroosmotic transport rate on the capillary wall characteristics (specifically, the zeta potential) that leads to irreproducibility of migration times when protein adsorption occurs. Variations occur within a single run as well as between successive runs. Separation performance, in terms of both sample quantitation and reproducibility of migration time, is affected.

The adsorption of proteins onto the capillary and, therefore, the zeta potential and electroosmotic flow, are not uniform across the length of the column. This results in a drift in electroosmotic flow during the run. It should also be noted that once a fused silica surface has been fouled with a protein, it cannot necessarily be cleaned with a base wash. Denatured conalbumin, for example, is difficult to solubilize with base. Both cationic and anionic proteins can foul a capillary and, therefore, zeta potential can vary between runs. Ideally, adsorption should be eliminated completely.

In addition to causing increased migration time and reduced reproducibility, this drift in migration time complicates the use of automatic peak identification software (Frenz *et al.*, 1989). Programs developed to identify peptide fragments from proteolytic digests of proteins based on ion mobility and ion migration cannot be employed. Electroosmotic flow must be reproducible to within 0.1% to avoid sizable errors in predicting migration times, especially at higher pH values (Sydor *et al.*, 1989).

3.2.4. Reduced efficiency and resolution

Martin and Guiochon (1984) found that even the slightest amount of adsorption will cause an appreciable decrease in efficiency, while moderate adsorption can lead to altered peak shape and poor reproducibility. Adsorption of solutes to the capillary wall in CE can be treated as a chromatographic phenomenon, with the inside surface of the capillary being termed the "stationary phase" and the buffer the "mobile phase." The amount of adsorption can be described quantitatively as k', the capacity factor, which is defined as:

$$k' = t_s/t_m \tag{1}$$

where t_s is the time the solute spends adsorbed to the capillary wall and t_m is the time the solute spends in the buffer. This k' is conceptually the same as the capacity factor in chromatography. Adsorption gives rise to band broadening in electrophoresis due to resistance to mass transfer in the "mobile" and "stationary" phases. Walbroehl (1988) predicted that capacity factors as small as 0.05 would be sufficient to cause a 20-fold reduction in plate numbers for proteins. Swedberg (1990) reported that with reversible protein-capillary wall interactions, a k' of less than 0.07 was needed for plate numbers of 300,000. It is of paramount importance that adsorption be significantly reduced in order to exploit the full benefits of the separation power of CE.

Researchers who have developed strategies to reduce protein adsorption have noted that observed plate numbers are still low compared to theoretical predictions (Swedberg, 1990; Lauer and

McManigill 1986; Cobb *et al.*, 1990; Towns and Regnier, 1991; Towns *et al.*, 1992). This has been attributed to extra band broadening, caused mainly by residual adsorption. An example of this is illustrated in Fig. 4 for a sample run of lysozyme and cytochrome *c* on a weakly deactivated coated capillary with buffers at two different ionic strengths.

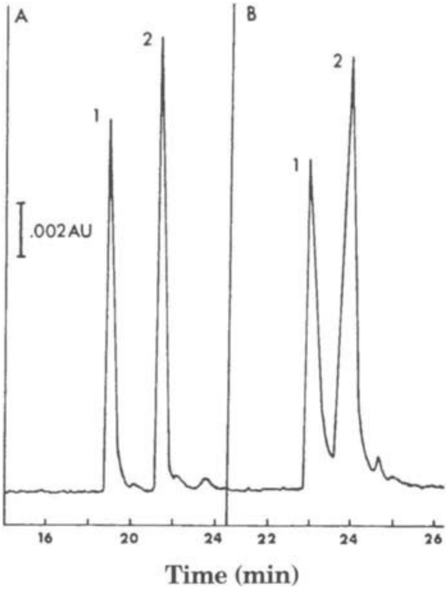

Fig. 4. Effect of adsorption on efficiency and resolution on a diol-based coated capillary using different ionic strength buffers. 1) lysozyme, 2) cytochrome *c*; pH 7.0 phosphate buffer run at A) 0.05 M and B) 0.01 M ; 75 µm i.d. × 100 cm capillary; Detection at 200 nm, 85 cm from injection; 280V/cm. (Unpublished data.)

A comparison of the results of the separation at two different salt concentrations demonstrates the way in which adsorption affects efficiency and, in turn, resolution. At high salt concentrations, cations in the run buffer compete more effectively with the basic proteins for anionic sites on the capillary wall, lowering protein adsorption and increasing efficiency (Lauer and McManigill, 1986). The separation with the higher ionic strength buffer (0.05 M) shows improved efficiency and resolution compared to that with a lower ionic strength buffer (0.01 M). The higher salt concentration showed a twofold increase in resolution and a fivefold increase in efficiency. The efficiency declines at moderate ionic strength, which causes a lack of resolution for basic proteins with the complete absorption of lysozyme at neutral pH (Swedberg, 1990).

Although it has been shown that flow rate varies inversely with buffer concentration (Altria and Simpson, 1986), this was not the case in the example above. A longer migration time obtained using a lower salt concentration buffer is primarily due to adsorption of these positively charged proteins to the capillary wall, which slows the electroosmotic flow. The increase in ionic strength can improve separation performance for uncoated or poorly coated capillaries, but cannot adequately mimic the merits of a coating alone in reducing protein adsorption. Ionic strength becomes less a factor as the deactivation of the capillary wall is improved through coating chemistry (Towns *et al.*, 1992). The attractive feature of a low ionic strength buffer is that it minimizes convective flow inside the capillary due to excessive heat generation.

Theory suggests that molecular diffusion is the sole cause of peak broadening. However, one must conclude that other factors contribute to band broadening in CE of proteins. These factors include both electrostatic interactions (Towns and Regnier, 1990), as noted above, and hydrophobic-like interactions (Cobb *et al.*, 1990).

3.2.5. Effects on quantitation by peak area

Changes in electroosmotic flow can drastically change the quantitation of peak areas, as seen for two separate runs of the neutral marker mesityl oxide (Fig. 5). Both runs were performed on an uncoated capillary, with the second run after a single injection of lysozyme. The capillary was shortened for the second run to give comparable migration times. Adsorption of lysozyme to the capillary wall decreased electroosmotic flow threefold. An increase in the area of the mesityl oxide peak was seen (Fig. 5B). This increase is in part the result of a reduction in sample velocity as it moves past the detector. Therefore, as a result of the adsorption of proteins, the analyte velocity and, thus, area may be affected.

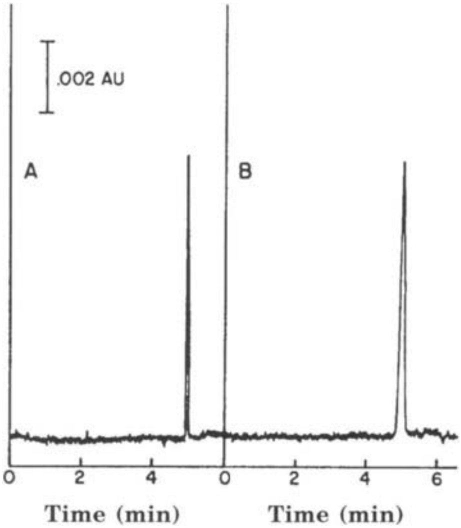

Fig. 5. Peak shape for mesityl oxide A) before and B) after a single injection of lysozyme on an uncoated capillary. Conditions: 0.01 M phosphate buffer, pH 7.0; 75 μm i.d. capillary 60 cm long (45 cm separation length) for A; 42 cm long (28 cm separation length) for B; 300 V/cm, 30 μA. (Unpublished data.)

3.2.6. Band broadening due to mixing of solutes

The increased band broadening caused by lysozyme adsorption may not be solely due to the reduction in velocity of the sample zone as it passes by the detector. Differences in the magnitude and sign of the charges along the capillary due to the adsorption of positively charged lysozyme may also play a role. This was confirmed by the use of a positively charged polymer adsorbed onto the first few centimeters of an uncoated capillary. This gives a positively charged section of capillary wall at the beginning of the capillary with the remaining section of uncoated capillary being negatively charged (Towns and Regnier, 1992). Adsorption of positively charged species on the negatively charged, uncoated capillary wall reduced electroosmotic flow and separation efficiency as measured by the transport velocity and peak shape of the neutral marker. In fact, fouling only 2% of the total capillary length with a positively charged polymer reduced separation efficiency by 50%.

The large increase in band spreading for capillaries fouled with positively charged species may be the result of the formation of complex flow patterns in the capillary. Differences in both the sign and magnitude of charge at various points in the capillary are thought to cause electroosmotic flow (EOF) to be in opposite directions in different portions of the capillary. It was proposed that these differences in the direction and velocity of the flow act in concert to cause dramatically increased band spreading (Towns and Regnier, 1992).

4. Manipulation of Electroosmotic Flow

Electroosmotic flow can have both a positive and a negative influence upon the CE separations. Some positive aspects of EOF are i) a plug flow profile that minimizes band spreading, ii) transport of both anionic and cationic species past a single detector, and iii) transport of mobile phases through packed capillary chromatography columns. Negative aspects of EOF are i) substances often elute from electrophoresis columns before they can be fully resolved, ii) mixing is initiated in columns that have a non-uniform charge distribution, and iii) flow rate and mixing are very sensitive to surface fouling, as discussed earlier. Factors that influence EOF are buffer type and concentration, pH, amine additives, and capillary coatings.

4.1. Manipulation of Electroosmotic Flow through Buffer Composition

Electroosmotic flow is a major mechanism of analyte transport when electrophoretic separations are carried out in open tubular columns. Transport by EOF is greater than by electrophoretic mobility in all but rare cases. EOF as a function of pH in untreated capillaries varies tenfold over the pH range 3–11 (Fig. 6), and is greatest at basic pH where surface silanol groups are totally ionized. The largest increase is seen from pH 4–8 (Towns and Regnier, 1991). The large difference in EOF over this pH range for the uncoated capillary demonstrates the dramatic effect of pH on EOF. Under acidic conditions, silanol ionization is repressed sufficiently that EOF essentially drops to zero. As the pH is increased, the silanol groups become deprotonated and EOF is increased. This increase in EOF can greatly affect solute residence time in the capillary and, therefore, separation efficiency and resolution. This dramatic EOF sensitivity to pH in uncoated capillaries is in sharp contrast to that found with coated capillaries. The EOF is relatively constant with pH using coated capillaries because the silanol groups are chemically bonded to the coating and are no longer able to be ionized.

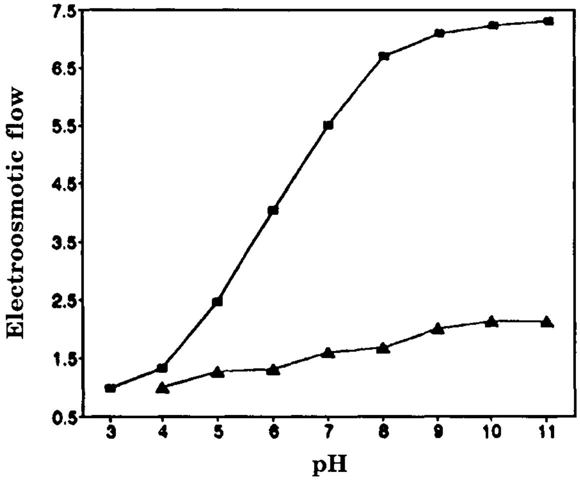

Fig. 6. Dependence of electroosmotic flow (m^2/V s) × 10^8 on pH for EOF vs. pH (■) uncoated and (▲) Brij 35/alkylsilane-coated capillary (75 μm × 50 cm). Reproduced with permission from Towns and Regnier, 1991.)

Ionic strength of buffers as well as the concentration of added surfactants (Altria and Simpson, 1986; Tsuda, 1987; Foret *et al.*, 1989; Huang *et al.* 1989), amine additives (Lauer and McManigill, 1986; Rohlicek and Deyl, 1989; Neilsen *et al.*, 1989), or organic solvents (Altria and Simpson, 1986; Fujiwara and Honda, 1987) can also affect EOF. A cationic polymer that reverses EOF has been adsorbed to the capillary (Wiktorowicz and Colburn, 1990). Addition of NaCl to decrease the thickness of the double-layer is yet another method of reducing EOF (Fujiwara and Honda, 1986), albeit at the expense of increased specific conductivity and Joule heating. Taken together, these studies suggest that the magnitude of EOF is predictable and controllable in fused silica capillaries.

In the specific case of protein separations in fused silica capillaries, it has already been noted that there is a serious problem with the analysis of cationic proteins. One method of dealing with this problem is with extremes in pH. Early work with the separation of proteins in fused silica capillaries was accomplished with buffers at pH 8-12 (Lauer and McManigill, 1986). Under these conditions, many proteins are anionic and are repelled from the capillary wall. The negative consequences of this approach are that many proteins are denatured at basic pH, and electroosmotic pumping is at a maximum. Very acidic pH buffers have the opposite effect. Silanol ionization is almost totally repressed, while all polypeptides are positively charged. This has proven to be a very effective method for peptide separations.

4.2. Manipulation of Electroosmotic Flow through Column Coatings

Manipulation of EOF through coating chemistry has taken the form of either derivatization of the silanol group on the surface of the capillary or masking of silanol groups through an adsorbed, non-covalent coating on top of the silanol surface. Table 4 lists the favorable attributes of a coating to control surface zeta potential and, thus, electroosmotic flow. It has been noted above that EOF was reduced by decreasing charge density at the capillary walls either through protonization of surface silanols, high ionic strength, or adsorption of amines. Another approach is to deactivate or mask silanol groups through the application of either semi-permanent or permanent organic coatings. Ideally, such coatings would control both EOF and protein adsorption at any pH.

Table 4. Desirable Properties of a Coating in the Control of Electroosmotic Flow

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- Ease of Application
- Uniformity
  - Column-to-column reproducibility within a single synthesis
  - Synthesis-to-synthesis reproducibility
- Long-term durability under various conditions
  - pH
  - Temperature
  - Ionic strength
  - Exposure to proteins
  - Exposure to detergents
  - Electric fields
- Consistency over wide pH range
  - Alter selectivity without adversely affecting analysis time
- Non-Toxic

Organic coatings are frequently coupled to silica surfaces by organosilanes. During silylation, organosilanes are bonded to the inorganic surface through siloxane bond formation between silanol groups at the surface and those on the silane. This process serves the dual role of attaching an organic moiety to the inorganic surface and sequestering surface silanols. Unfortunately, the reduction in surface silanols is seldom more than 50%, even in the most ideal cases. Because electrical potential at a surface decreases more slowly than charge density, it is difficult to eliminate EOF by sequestering surface silanols alone (Davies and Rieal, 1961). Contributions of the coatings themselves on EOF will be discussed below.

Control of protein adsorption through silane-coupled coatings is quite different. Adsorption of polymers at surfaces decreases dramatically with reduction in surface charge density. This is due to the fact that polymers are adsorbed at surfaces in a cooperative process involving multiply charged groups on both the adsorbent and adsorbate. Reduction in charge density on the sorbent produces an exponential decrease in the force of interaction (Kapaciewicz *et al.*, 1983). When the forces controlling EOF and protein adsorption at surfaces are considered together, the decrease in electrostatic adsorption of proteins to surfaces is expected to be more rapid than the reduction of electroosmotic flow by silylation of the surface silanols.

Coatings particularly if they are polymeric, appear to extend the plane of shear beyond the double layer at the capillary wall, resulting in a reduction of zeta potential. Herren *et al.* (1987) showed a direct relationship between reduction in EOF and increasing molecular weight of adsorbed polyethylene glycol (PEG). Presumably, the larger molecular weight PEG shifts the plane of shear farther from the surface. The problem with adsorbed PEG coatings is that they are not equally effective in controlling the adsorption of cationic proteins. Adsorption of surfactants to octylsilane-derivatized capillaries is a more effective approach. EOF could be varied by using surfactants that differ in head group size and alkyl chain length (Towns and Regnier, 1991) much as in the case of PEG. Surfactants with the largest head groups were the most effective in reducing EOF. Reductions in EOF resulted in greater resolution and longer migration times. Capillaries of comparable efficiency and adsorption characteristics can give very different resolution based on differences in EOF. Dynamic coatings allow the surfactant to be switched to optimize separations.

Covalently bonded coatings appear to reduce EOF in the same manner as does the dynamic coating described above. Covalently immobilized polyacrylamide, epoxy layers, and adsorbed methylcellulose all appear to diminish EOF by reducing the number of surface silanols and extending the plane of shear away from the surface. Large polymers, such as methylcellulose, may be sufficiently large that EOF is effectively reduced to zero.

In the study of positively charged coatings on EOF, Herren *et al.* (1987) noted that the glass surfaces studied were expected to possess approximately three silanol groups per 100 Å^2, not all of which were ionized. Silylation of the quartz capillaries with positively charged aminopropyl groups using a pH 5.8 running buffer was found to reverse the glass surface charge and the direction of the EOF. Herren *et al.* (1987) determined that 1.4 aminopropyl groups per 100 $Å^2$ would be expected to reverse the surface charge and the direction of EOF. Although this was not tested, they concluded that surface coatings that were less complete would be expected only to reduce EOF, not to reverse it. This process creates an amphoteric surface with a titration curve that is a function of both the density and ionization characteristics of the individual charged groups. At basic pH, silanol groups would predominate, and the surface would be electronegative. The opposite would be true at acid pH, while at intermediate pH the surface would be isoionic. This produces a material of unusual EOF characteristics: toward the cathode at basic pH, zero at intermediate pH, and toward the anode at acidic pH.

This phenomenon was observed in the analysis of adsorbed polyethyleneimine, where polyamine molecular mass had a large influence on EOF over a wide pH range (Towns and Regnier, 1990). A strong correlation between the magnitude and direction of EOF and polymer size exists. Low-molecular mass polymer on the surface resulted in EOF moving from the negative to the positive electrode at low pH, but a sharp reversal of direction in EOF occurred near pH 8. This reversal was due to the low coverage of positively charged coating that was unable to fully mask the negatively charged silanol groups as the pH was increased. Above pH 8, surface silanol groups are more abundant than amine groups, causing the reversal in flow. As the polymer molecular mass is increased, the EOF is increased due to either an increase in the positive charge on the larger polymer or a more efficient masking of the negative groups on the silica. As the pH is increased, the EOF stays relatively constant over the pH range, indicating a good masking of the silanol groups for this polymer. An estimation of coating thickness for three different polymers indicated that the larger the molecular mass of polymer, the thicker the coating. In the case of a 20,000 molecular weight polymer, where the EOF stays relatively constant over the entire pH range, a coating thickness estimated to be 30 Å results in a significant masking of the negative capillary wall charges.

4.3. Stabilizing Electroosmotic Flow Over a Wide pH Range

The rate of EOF depends on the magnitude of the zeta potential across the solution-solid interface, which in turn is dependent on the charge density at the capillary wall. An increase in silanol ionization at higher pH results in an increase in electroosmotic pumping. This makes it possible to run coated capillaries between pH 4 and 11 without significant change in capillary-dependent separation variables. The best pH can be employed to give optimum selectivity without adversely affecting the analysis time.

The net velocity of a charged solute in a fused silica capillary is the sum of the rates of both convective and electrophoretic transport. Ideally, the convective component of transport should remain constant while the electrophoretic component is being varied with pH to optimize selectivity in a separation. This is possible only in capillaries with no charge or constant charge at the surface. To this end, modification of capillaries by either masking or deactivating surface silanol groups has been performed by physically coating capillary walls. The effects shown in Fig. 6 are a good example of the reduction and leveling of the EOF over a wide pH range using a coated capillary (Towns and Regnier, 1992a).

The pH independence of EOF was also observed with a negatively charged coating (sulfonic acid groups) (Kohr and Engelhardt, 1991). As with the coating described above, the flow is reduced to approximately that of an uncoated capillary at pH 3. This low flow rate may result in strongly cationic species not passing by the detector due to their movement in the opposite direction of EOF. It has been observed, however, that positively charged proteins such as lysozyme (pI 11.1) are easily eluted at neutral pH using the coating described in Fig. 6 (Towns and Regnier, 1991).

It has been shown that surface charges on a coating or on the masked silanol groups do not change significantly over the pH range 3–12, *i.e.,* fluctuation in EOF is minimal over this range. This uncoupling of EOF and pH allows the manipulation of pH to enhance selectivity. In the separation of amphoteric compounds such as peptides and proteins net charge and electrophoretic mobility can vary significantly with pH. The masking or elimination of silanol groups on the capillary surface due to coatings has a leveling effect on the dependence of pH on EOF.

The most notable advantage of coated capillaries lies in their ability to retain a relatively constant surface charge over a wide pH range. This makes it possible to vary pH conditions to optimize selectivity without adversely affecting the analysis time. In contrast, uncoated fused silica capillaries are very different. The EOF is extremely dependent on pH, with silanol ionization beginning at pH 3 and continuing to increase through pH 8.

4.4. Mechanical Means to Control Electroosmotic Flow

Other means of directly controlling EOF in capillary electrophoresis have included the use of an additional electric field applied from outside the capillary (Lee *et al.*, 1990, 1991). This technique couples the externally applied potential with the potential across the buffer solution inside the capillary. This electric potential gradient across the capillary wall controls the polarity and magnitude of the zeta potential on the interior surface of the capillary wall, which in turn controls the direction and flow rate of electroosmosis. The effectiveness of the direct control of the EOF has been demonstrated at various operating conditions, including different solution pH values, electrolyte concentrations, and capillary dimensions (Lee *et al.*, 1991).

Another approach to the control of EOF is through the coupling of capillaries with different magnitudes of EOF (El Rassi and Nashabeh, 1992). A multiple capillary device permitted the change in electroosmotic flow during analysis. This approach has the advantage of carrying out rapid biopolymer analyses at relatively low field strengths. The pulling of solution through the first low flow surface-modified capillary by use of a second high flow uncoated capillary did not appreciably sacrifice the high separation efficiency. Care must be taken to not foul the second capillary with solute that will affect its EOF. This type of device has been used in the separation of biopolymers, including proteins and oligosaccharides.

5. Strategies to Reduce Protein Adsorption

The adsorption of proteins to the capillary greatly reduces separation performance, and acceptable protein separations are typically much more difficult to achieve than those of peptides and small molecules. Steps must be taken to alleviate the problems discussed above. It is of paramount importance that adsorption is eliminated in order to exploit the full benefit of CE separations. Several research groups and instrument manufacturers have directed their efforts toward solving the problem of protein adsorption. Table 5 lists the major approaches that have been adopted to minimize protein adsorption arising from silanol groups on the capillary wall, the advantages and limitations of each, and appropriate references. These strategies have taken the form of either physically coating the inside of the capillary with a deactivating agent or manipulating the sample and/or running buffer to reduce adsorption.

5.1. Run Buffer Composition

The composition of the run buffer may be used to enhance separation performance. This usually takes the form of changing the charge either on the protein or on the capillary wall or adding a com-

Table 5. Strategies Employed to Reduce Protein Adsorption to the Capillary Wall

Method	Advantages	Limitations
Coatings	* Increased pH range * Low conductivity * No limitations on running buffer * Reduced EOF (increased resolution)	* Instability of coatings * Solute/coating interactions * Long preparation time * Reduced EOF (increased analysis time)
Low pH	* Minimal capillary preparation	* High conductivity (heat) * Limited pH range * Increased analysis time * Reduced charge differences * Denaturation of proteins
High pH	* Fast analysis time * Minimal capillary preparation	* Limited pH range * Instability of silica * Denaturation of proteins
High Salt	* Increased elution time reproducibility	* High conductivity (heat) * Increased analysis time
Zwitterionic Salts	* Low conductivity * Minimal column prep. * Full pH range	* Increased analysis time * Need for added salt * Unexpected elution order
Amine additives	* Minimal column prep.	* Incomplete competition of wall sites with proteins * Increased analysis time * Increased conductivity (heat)

ponent to compete for negatively charged sites on the capillary wall. The charge of the solute is important in that anionic components are repelled electrostatically from the negatively charged capillary walls, thereby minimizing adsorption effects. Cationic species can interact with the negatively charged capillary walls, resulting in reduced recovery and broadening of zones. The following approaches have been employed to alleviate this problem area.

5.1.1. Effect of pH

One approach to reducing protein adsorption is the use of high pH buffers to induce a negative charge on the protein, which is then repelled from the capillary wall (Lauer and McManigill, 1986; Walbroehl and Jorgenson, 1989; Zhu *et al.*, 1989; Cobb *et al.*, 1990; Zhu *et al.*, 1990). Lauer and McManigill (1986) observed that at pH values above the pI of proteins, coulombic repulsion between the negatively charged protein and the negatively charged wall results in increased separation performance. High pH may also have a negative impact on separation, the possible denaturation of proteins, and the strong dependence of wall conditions to EOF, impeding reproducibility.

Low pH buffers have also been used, usually in conjunction with column coatings, to protonate the silanol groups, resulting in minimal charge on the capillary surface (McCormick, 1988; Bruin *et al.*, 1989; Green and Jorgenson, 1989; Grossman *et al.*, 1989a,b; Cobb *et al.*, 1990; Swedberg, 1990; Vinther *et al.*, 1990). This approach also limits the pH range that may be employed to optimize selectivity; in addition, there is the danger of structural alterations to the molecule at low pH. McCormick (1988) showed how the separation efficiency and resolution of six cytochrome *c* variants were affected at low pH. At pH 1.5, the difference in the mobilities of the six variants was minimal, and the proteins comigrated. As the pH is raised, the mobility differences become more pronounced, and a more complete separation is achieved. A loss of resolution above pH 5 was observed and was attributed to adsorption of the positively charged proteins to the negatively charged capillary wall. Using a hydrophilic coated capillary that sufficiently masks the wall charges on the capillary at neutral pH, the analysis of the cytochrome variants has been extended up to pH 8 (Towns, 1991). By varying the pH from 5 to 8, the mobilities of the individual variants could be manipulated to give optimum separation. The variant migration order switched as the pH was varied. This was attributed to changes in individual charged amino acid residues, which alter the electrophoretic mobility of the protein. The manipulation of pH to induce a charge difference in the variants was found to be quite effective over this pH range.

5.1.2. Addition of reagents

Another approach to the elimination of protein adsorption is the use of additives such as zwitterionic salts (Bushey and Jorgenson, 1989), salt at high concentrations (Bushey and Jorgenson, 1989; Green and Jorgenson, 1989; Walbroehl 1989; Swedberg, 1990; Chen *et al.*, 1992), or amine additives such as morpholine (Nielsen *et al.*,

1989) or putrescine (Lauer and McManigill, 1986) to compete with proteins for charged sites on the capillary wall. The use of high salt concentration or competing ions in the buffer medium has resulted in highly efficient separations. Problems with thermal effects (Joule heat generation) at high buffer concentrations, however, necessitate the need for narrower capillaries and low voltages. Narrow capillaries strain detection limits, while low voltages increase analysis time. The addition of low molecular-mass amines to the buffer to counteract the negatively charged silica capillary surface gives a considerably improved separation, but some residual tailing persists (Nielsen *et al.*, 1989). The effects of different polymethylenediamines, such as cadaverine or putrescine, were almost identical in preventing protein adherence (Rohlicek and Deyl, 1989). Charge reversal reagents reduce adsorption of cationic proteins. Another approach is to add surfactants to buffers. Fluorocarbon surfactant FC-134 (3M) forms a bilayer of hydrophobic chains along the wall (Emmer *et al.*, 1991). The addition of ethylene glycol to the protein sample buffer and modified buffer pH and concentration to reduce adsorption have also been employed (Gordon *et al.*, 1991). For the separation of membrane proteins, Josic *et al.* (1990) found that reproducible results in terms of peak height and migration time were obtained only after a high concentration of urea was added to denature the proteins. The use of cellulose derivatives such as hydroxypropylmethylcellulose has also greatly increased in resolution and efficiency by preventing protein absorption through shielding (Beckman Instruments, 1994).

The use of additives to compete with absorption sites on the wall has recently found favor as a method for reducing protein adsorption. This may be the best of the three approaches discussed because the use of pH extremes greatly limits the ability to manipulate the state of solutes to enhance selectivity. The addition of high salt concentrations significantly increases Joule heating. The strategies that have been widely implemented are those discussed above. The success of a particular reagent in reducing adsorption is dependent upon the type of solute. Success depends largely on how well a particular reagent can compete with the solute for sites on the capillary wall. Large molecules such as proteins may easily displace small reagents from the capillary wall. As a result, buffer modification has often fallen short of reducing adsorption of solutes onto the capillary wall, leading to the use of coated capillaries, as discussed in the next section.

5.2. *Modification of the Capillary Surface*

An attractive alternative for controlling protein-wall interactions, as well as EOF, is permanent modification of the capillary wall. In

CE, the primary goal of surface modification of fused-silica capillaries is to minimize protein-wall interactions without completely inhibiting the EOF. Using column technology borrowed from liquid chromatography and capillary gas chromatography, surface modification through the reactive surface silanol groups has provided a means of permanently modifying the capillary surface to reduce solute adsorption.

The current column technology is focused on deactivating the capillary wall by means of a silanization reagent and then coating with a functional group. The proven and most popular strategy is to modify the capillary wall by attaching hydrophilic polymers to the Si-OH sites on the silica surface, either directly or through suitable spacers. This coating process is not overly complicated and is fairly reproducible for each section of capillary both within a lot and from lot-to-lot. In the coating process, the fewer the steps the better. A downside is that, as seen with silica-based columns, capillaries quickly deteriorate above pH 8. Changing from coated capillaries to additives has proved less than successful. The reagents added to the running buffer do not completely reduce analyte absorption, and elaborate cleaning steps are often needed to return the capillary to its original condition.

Deactivation of the silica surface by chemical bonding has taken several forms. Table 6 lists coating chemistries that have been employed to deactivate the silica surface, the features of each, and the appropriate references. These coatings can be broadly categorized as neutral and charged coatings.

5.2.1. Neutral coatings

To mask the silanol groups, many coatings have been used in the hope of leaving a perfectly neutral coating. The advantage of hydrophilic neutral coatings is that they are non-interactive in terms of either charge or hydrophobicity. The bonding of neutral coatings to reduce wall interactions began in the early 1980s when Jorgenson and Lukacs (1983) bonded (3-glycidoxypropyl) trimethoxy silane to the capillary wall. Organo-silane-based surfaces are the most popular; examples include surface linkage with methylcellulose and non-crosslinked polyacrylamide (Hjertén, 1985; Hjertén and Johanson, 1991; Kohr and Engelhardt, 1991), epoxy-diol (Jorgenson and Lukacs, 1981, 1984; Bruin *et al.*, 1989a), polyethylene glycol (Herren *et al.*, 1987; Bruin *et al.*, 1989b), crosslinked epoxides (Towns *et al.*, 1992), and poly(vinylpyrrolidinone) (McCormick, 1988). An alternative to attaching the coating through the silane is to deposit a monomer onto the silanol surface and polymerize it on-column. Examples of this type of

Table 6. Coatings Used in the Deactivation of Fused Silica

Coating	Features	Reference
Polyacrylamide (non-crosslinked)	*Useful at low pH *Limited stability	Hjertén, 1985
Diol	*Useful at low pH *Low efficiency, limited stability	Jorgenson and Lukacs, 1981
Maltose	*Useful up to pH 7 *Limited stability, low efficiency	Bruin *et al.*, 1989b
Glyceroglycidoxy propyl	*Separations at low pH *Limited stability	McCormick, 1988
Poly(vinylpyrrolidinone)	*Useful up to pH 5 *Adsorption of some proteins	McCormick, 1988
Aryl pentafluoro	*Run at moderate ionic strength *Separation at neutral pH *Useful over a wide pH range *Day-to-day RSD of 7%	Swedberg. 1990
Polyethylene glycol	*Separation at low pH *Low efficiency at neutral pH	Bruin *et al.*, 1989a
Polyethyleneimine	*Positively charged coating *Leveling of EOF over pH range	Towns and Regnier, 1990
Non-ionic surfactant/ alkylsilane	*High protein recovery *Leveling of EOF over pH range *Coating can be regenerated *Good reproducibility, stability	Towns and Regnier, 1991

cont'd.

Table 6 cont'd.

Coating	Features	Reference
Vinyl bound polyacrylamide	*Increased stability of Si-C bond *Separations at low and high pH *Good reproducibility	Cobb *et al.*, 1990
Aminopropyl	*Positively charged at low pH *Stable to several weeks	Moseley *et al.*, 1991
Immobilized lactalbumin	*Charge dependent on pH *Low EOF *Separation at neutral pH	Maa *et al.*, 1991
Poly(methyl-glutamate)	*Run at neutral pH *Reasonable efficiency at neutral pH	Bentrop *et al.*, 1991
Polyacrylamide (through an alkene)	*Highly cross-linked, stable coating *Run at high and low pH *Good efficiency and reproducibility	Huang *et al*, 1992
Crosslinked epoxides	*Leveling of EOF over pH range *Stable to 150 hr *Multiple step synthesis	Towns *et al.*, 1992
Polyoxyethylene silane	*One-step synthesis *Leveling of EOF over pH range *Stable to two weeks	Towns, 1991
Polyethers (fuzzy and interlocked)	*One to three step synthesis *Stable to >80 hr *Coating can be restored	Nashabeh and El Rassi, 1991

coating are poly(methylglutamate) (Bentrop *et al.*, 1991) and poly(methacrylate) (Towns, 1991). Coatings produced by both procedures have improved protein separations; varied success has been obtained in terms of long-term stability, lowering the effect of EOF over a large pH range, and usefulness at neutral pH values. A measure of the completeness of deactivation is the extent to which the EOF is reduced, an indication that the silanol groups have been masked, neutralized, or chemically altered. This results in a reduction or elimination of charge and the reduction in EOF can be attributed to a change in the zeta potential on the wall and/or the moving of the plane of shear out beyond the double layer.

An example of a neutral surface modification for the separation of proteins is shown in Fig. 7 for two types of hydroxylated polyether chains covalently attached to the capillary inner surface (Nashabeh and El Rassi, 1991). The "fuzzy"-type coating (Fig. 7A) consists of two layers–a crosslinked glyceropropyl-polysiloxane sublayer covalently

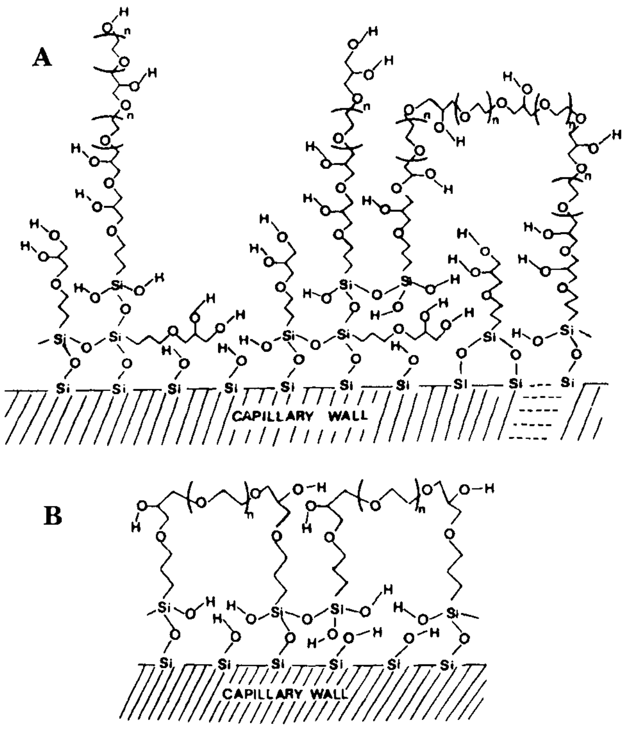

Fig. 7. Schematic illustration of the idealized structures of fuzzy (A) and interlocked (B) polyether coatings of fused-silica capillaries. (Reproduced with permission from (Nashabeh and El Rassi, 1991.)

attached to the capillary inner surface and a hydrophilic polyether top layer. The "interlocked"-type (Fig. 7B) consists of polyether polysiloxane chains whose monomeric units at both ends are covalently attached to the surface with the potential of possible interconnection.

These two coating schemes are good examples of capillaries with different properties giving quite different separation performances. The "fuzzy" coating exhibited lower electroosmotic flow than did the "interlocked" coating. This was proposed to indicate that the concentration of unreacted surface silanols is relatively low for the "fuzzy" coated capillaries. This greater surface coverage results in less variability in EOF when going from low to neutral pH and greater efficiency for the "fuzzy" coating. The "interlocked" coating is slightly more reproducible than the "fuzzy" coating, simply because the surface modification is accomplished in a single step while the "fuzzy" coating takes three steps. The effect of synthetic steps on the reproducibility of coated columns is discussed in greater detail below.

Due to stability concerns about organosilane-based coatings, alternative bonding chemistries have been employed to produce stable, uncharged hydrophilic surfaces. Cobb *et al.* (1990) used a Grignard reaction with a chlorinated surface, through which a vinyl moiety is attached to the silica. A subsequent reaction of the vinyl moiety with acrylamide is a variation of Hjertén's technique (1985), except that the resulting monomolecular layer of linear polyacrylamide is attached via stable Si-C bonds rather than Si-O-Si-C linkages.This treatment exhibits long-term stability over a pH range from 2 to 10.5. The theoretical plate count and peak symmetry were significantly improved with this coating treatment. This is illustrated in Fig. 8, which compares the separation of model proteins at pH 9.5 on a coated and untreated capillary. This treatment appears to be highly reproducible on a day-to-day and capillary-to-capillary basis (Table 7).

An example of the improved separation at neutral pH achieved by using coated capillaries is shown in Fig. 9, which compares the separation of five basic proteins on A) an uncoated and B) a coated capillary at neutral pH using a low ionic strength buffer (Towns *et al.*, 1992). The buffer pH and ionic strength were held constant for both capillaries. The electropherogram in Fig. 9A shows all the detrimental effects of protein adsorption discussed earlier—loss of peak symmetry, loss of recovery, reduced efficiency and resolution, and reduction in EOF. Figure 9B illustrates the dramatic increase in separation performance achieved with the proper deactivation of fused silica. The added advantage of coatings such as the Brij 35/alkylsilane, where the wall is deactivated to the extent that the EOF does not change with pH, is that there is no limitation on the buffer system in terms of pH or ionic strength. This allows the operator the freedom to alter these parameters in order to optimize the separation.

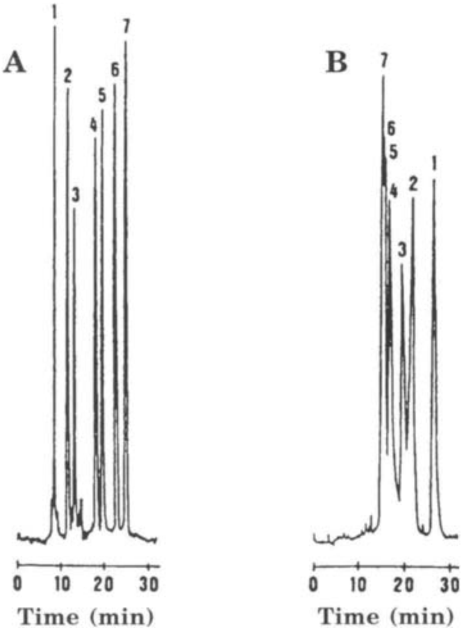

Fig. 8. Capillary electrophoretic separations of model proteins at pH 9.50, using (A) vinyl bound polyacrylamide coating and (B) uncoated fused silica capillaries. Peak identification: (1) insulin chain A (porcine); serum albumin (bovine); (3) ovalbumin (chicken); (4) insulin (porcine); (5) lactalbumin (bovine); (6) β-casein (bovine); (7) insulin chain B (porcine). (Reproduced with permission from Cobb *et al.*, 1990.)

5.2.2. Charged coatings

To help overcome adsorption, positively charged capillary coatings have been employed for the separation of positively charged solutes. In this approach, positively charged solutes are electrostatically repelled from the positively charged wall and have limited wall interaction. This is similar to the approach of Lauer and McMannigill (1985), who used high pH values to induce a negative charge on the proteins and repel them from the negatively charged wall.

Table 7. Reproducibility of Coated Capillaries

Coating (pH tested)	Run to Run %	Day to Day %	Column to Column %	Solute Tested	Reference
Uncoated (pH 8.0)	----	1.5	1.8	Riboflavin	VanOrman, 1990
Aryl pentafluoro (pH 7.0)	1.4	7.6	----	Ribonuclease	Swedberg, 1990
Polyethylene imine (pH 7.0)	0.9	2.4	4.7	Mesityl oxide	Towns and Regnier, 1990
Vinyl bound polyacrylamide (pH 2.7)	0.2	----	< 5	Ribonuclease	Cobb *et al.*, 1990
Brij 35/ODS (pH 7.0)	0.8	1.3	2.4	Mesityl oxide	Towns and Regnier, 1991
Interlocked PEG-200 (pH 6.5)	0.9	1.6	1.5	Lysozyme	Nashabeh and El Rassi, 1991
"Fuzzy" PEG-600 (pH 6.5)	1.6	1.4	4.2	Lysozyme	Nashabeh and El Rassi, 1991

Two examples of such coatings are polyethyleneimine (Towns and Regnier, 1990) and aminopropyltrimethoxysilane (APS) (Moseley *et al.*, 1991). The synthetic route used to prepare the polyethyleneimine-coated capillary was derived from techniques used in liquid chromatography to prepare silica-based anion-exchange packing materials. High-molecular mass polymers produced thick coatings that sufficiently masked the silanol groups on the wall, giving a positively charged surface over the entire pH range. This resulted in durable hydrophilic modified capillaries that resolved proteins quickly and efficiently with good recovery. Using a neutral buffer, the coulombic repulsion of proteins from the positively charged capillary surface overcomes solute wall adsorption. The coating reversed the EOF, requiring that the polarity of the power supply be switched from that used for uncoated fused-silica capillaries.

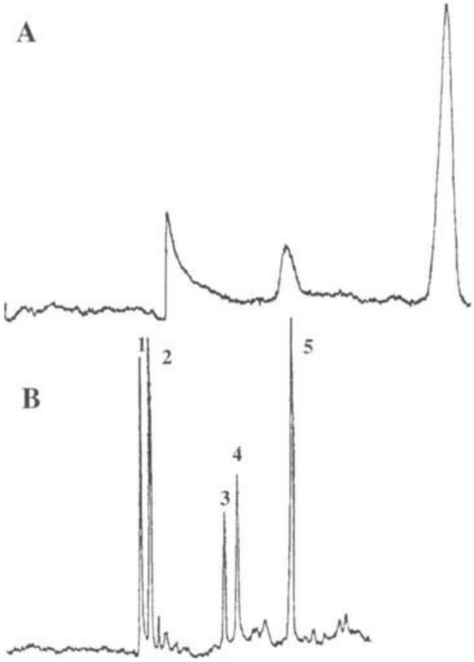

Fig. 9. Separation of five basic proteins on A) uncoated and B) Brij 35/alkylsilane coated capillary (1) lysozyme, (2) cytochrome *c*, (3) ribonuclease A, (4) chymotrypsinogen, and (5) myoglobin using a 0.01 M phosphate buffer (pH 7.0) on a 75 µm × 50 cm (A, top) uncoated capillary and (B, bottom) Brij/alkylsilane capillary at 300 V/cm. (Adapted with permission from Towns *et al.*, 1992.)

A good example of the dramatic difference in separation performance for positively coated vs. uncoated capillaries is the CE analysis of a solution of two basic peptides, bradykinin and lys-bradykinin (Fig. 10) (Moseley *et al.*, 1991). The analysis of this mixture with pH 7.5 buffer using bare fused-silica (Fig. 10A) results in severe zone broadening caused by adsorption of the positively charged peptides onto the wall. Optimum conditions on this uncoated capillary (deprotonation of amino acid residues to induce a negative charge for electrostatic repulsion) would necessitate the severe conditions of a buffer pH of ~13. Analysis of the mixture of basic peptides using the APS column (Fig. 10B) with pH 3.4 buffer resulted in greatly improved peak shape and separation efficiency. The low pH used in the APS column was

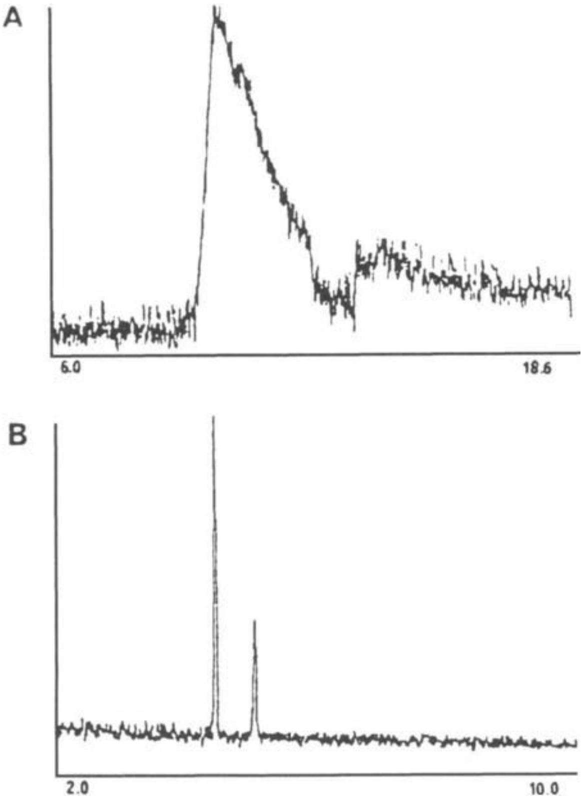

Fig. 10. CZE separation of two basic neuropeptides, detected by UV absorption at 200 nm. (A) separation with bare fused silica CE column; (B) separation with APS-treated CE column. (Reproduced with permission from Moseley *et al.*, 1991.)

necessary to induce a net positive charge (pK_a of *n*-propylamine is 3.4) that will electrostatically repel the positively charged basic peptides. The results show the power of working under conditions where the solute and capillary wall have similar charge.

As was expected, a surface coating having strong acidic groups is also independent of buffer pH (Towns and Regnier, 1990). To explore this, Kohr and Engelhardt (1991) coated the capillary with a strong cation exchanger (sulfonic acid groups). This modification of the capillary was accomplished by first bonding trichlorovinylsilane with the surface silanols, then depositing 2-acryloylamino-2-methylpropanesulfonic acid and polymerizing at 120°C for 3 h. The EOF was nearly independent of pH; only at pH values above 7 could a slight increase in mobility be observed. This was presumably due to the dissociation of surface silanols. Two different coating procedures showed almost identical EOF vs. pH curves. Thus, this type of coating allows the manipulation of pH without adversely altering EOF. Although no separations of proteins were shown, it would be expected that a capillary with such a coating would resemble an uncoated capillary with respect to readily adsorbing positively charged proteins.

5.2.3. *Reproducibility of coatings*

The existence of EOF does not, by itself, lead to poor reproducibility of migration times. It is the dependence of the EOF rate on the zeta potentials that results in these variations. As proteins adsorb to the uncoated capillary wall, the zeta potential of the wall changes and causes the EOF to vary, both within a single run and between successive runs. Thus, by reducing both EOF and adsorption sites on the capillary wall, the use of coating techniques has a fundamental advantage over other methods. In any analytical technique, reproducibility is a major concern. In the case of capillary electrophoresis, reproducibility of migration time is a measure of the completeness of the coating process, the effectiveness of suppressing adsorption, and the stability of the coating.

The dependence of migration time on EOF requires that these coatings be stable between runs and over a number of days for good reproducibility. Kohr and Engelhardt (1991) compared the reproducibility of EOF with uncoated and polyacrylamide-coated capillaries at pH 5. They found that migration time could only be reproduced with a RSD of ±15%. If a specific conditioning method, including flushing with sodium hydroxide and organic eluents, is used, flow fluctuations can be reduced to ±8% RSD. The day-to-day and column-to-column reproducibility of uncoated capillaries was examined by VanOrman *et al.* (1990) using a pretreatment scheme of rinsing the capillary with four capillary volumes of 1 M KOH followed by six volumes of running buffer. A day-to-day RSD of ±1.5% and a capillary-to-capillary RSD of ±1.8% for a neutral marker were obtained using this capillary treatment procedure. Although these results give a good idea of the variability of fused silica capillaries, they do not represent actual results obtained for protein analysis in capillary electrophoresis. Proteins readily adsorb to the capillary surface, quickly reducing EOF and therefore migration time. This results in double-digit run-to-run migration time RSDs for basic proteins on uncoated capillaries.

Reduction or elimination of the EOF by operating at low pH should yield more reproducible separations that depend on flow for transport through the capillary. Separations that depend solely on the electrophoretic mobility of the proteins for transport through the capillary are not subject to these flow variations. To minimize EOF variations due to solute adsorption, coatings have been employed. These, however, have their own reproducibility problems. Table 7 lists run-to-run, day-to-day, and column-to-column migration time RSDs for several coatings. The general trends observed are 1) the more stable the coating, the better the reproducibility, and 2) the fewer synthesis steps, the better the column-to-column reproducibility.

The number of steps used in the synthesis is important to consider in the reproducibility of a coating. A more complicated synthesis usually results in more complete coverage of the capillary surface for improved stability and efficiency. However, each step in a synthesis increases the probability that column-to-column reproducibility will be poor. A good example of a rather exhaustive synthetic route is the coating scheme using a gaseous acid-catalyzed crosslinked epoxide (Towns *et al.*, 1992a). Figure 11 shows that each step resulted in a further masking of the surface charges, producing increased durability, peak symmetry, recovery, efficiency, and resolution. This coating scheme required five steps to fully deactivate the coating for long-term stability and improved efficiency. The column-to-column reproducibility for this rather complex synthesis decreases dramatically with each step. To further complicate matters, the gaseous catalyst makes complete synthesis difficult over the entire capillary length. The overexposed front section and the underreacted back section must be removed before the next synthesis step. This significantly reduces the useful capillary length for the next synthesis step. The problems of reproducibility with the multiple-step synthesis have been reduced by using a two-step synthesis with a basic catalysis in solution (Towns *et al.*, 1992).

To evaluate coatings, a test mixture of cytochrome *c* and lysozyme has been very useful for measuring column-to-column reproducibility. These two basic proteins provide a worst-case scenario for column performance as they readily adsorb to unmasked charged sites on the capillary wall. The proteins are good indicators of the completeness of the coating process with respect to column-to-column and lot-to-lot variations. Sections of capillary that are not properly coated will quickly adsorb protein, resulting in a reduction of EOF and an increase in migration times. Multiple runs can show steady increases in migration times, leveling off once the protein(s) has adsorbed to most of the exposed sites on the capillary wall. Washing steps are often ineffective in returning the capillary to its original performance. In addition, these positively charged proteins provide valuable information on separation performance.

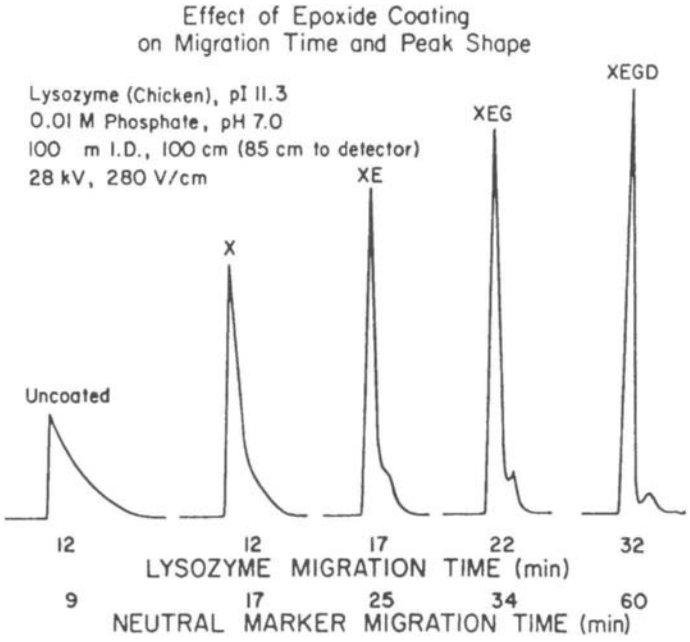

Fig. 11. Peak shape for lysozyme and migrations times for lysozyme and a neutral marker (mesityl oxide) for each step of an acid-catalyzed epoxy coating process. Conditions: 0.01 M phosphate, pH 7.0, detection at 200 nm, 100 cm × 75 mm capillary (85 cm to detector), 28 kV, 30 mA. (Unpublished data.)

6. Capillary Gel Electrophoresis (CGE)

A great deal of interest has been generated by gel-filled capillaries for the separation of large biological molecules that are not readily separated in free solution. The electrophoretic mobilities of related biomolecules are sometimes so close in free solution capillary electrophoresis (FSCE) that resolution is not possible. It is therefore desirable to adapt slab gel electrophoresis to a capillary. This method permits rapid separation with on-line detection at the nanogram level. The problems inherent in staining of slabs—reproducibility, quantitation, and speed—are eliminated.

6.1. *Sieving in the Capillary*

Gel-filled capillaries are produced by filling fused silica capillaries with a degassed monomer (*e.g.*, acrylamide and/or bisacrylamide) in buffer and polymerizing the mixture within the capillary. Pore sizes may be controlled in polyacrylamide gels by varying the percentage of acrylamide monomer and crosslinking agent (Guttman *et al.*, 1990). The polymer is covalently bound to the silica surface using a bifunctional reagent (Cohen and Karger, 1987; Karger *et al.*, 1989; Drossman *et al.*, 1990). The fibrous nature of the gel causes a sieving effect in which small molecules migrate faster than larger species.

The size of the pores is controlled by the monomer concentration (%T) and the degree of polymer crosslinking (%C). A 5% T, 5% C gel indicates that the total monomer concentration in the filling solution is 5%, and 5% of these monomers are cross-linked. Gels with higher %T and %C values have smaller pores that are more effective for resolving smaller molecules. The difference in resolving power, depending on the % T and % C, is illustrated in Fig. 12, which shows the single base resolution of oligonucleotides [pd (A)] ranging from 25–30 and 40–60 bases for both (3% T, 3% C) and (5%T, 5% C) (J & W Scientific Catalog, 1992). Both columns (50 cm separation length) exhibit over 2,000,000 theoretical plates. Poorer resolution is seen for low molecular weight compounds with the larger pore 3% T, 3% C gel column than for the smaller pore gel column (5% T, 5% C). The opposite would be observed in the separation of larger molecules.

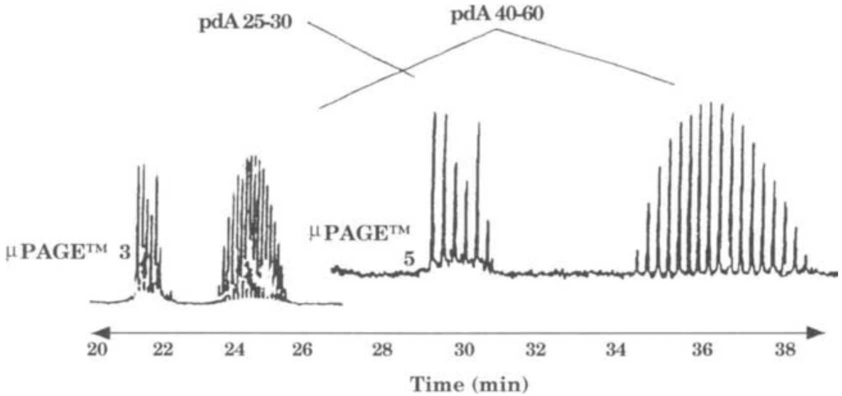

Fig. 12. Single base resolution of oligonucleotides [pd (A)] ranging from 25–30 and 40–60 bases for both µPAGE-3 (3%T, 3%C) and µPAGE-5 (5%T, 5%C) gel-filled columns. (Reproduced with permission from J & W Scientific catalog, 1992.)

Larger pores are optimal for separating large single- and double-stranded DNA fragments, and allow timely analysis of large biomolecules. Batch-to-batch changes in the gel that produce variation in analyte mobility may be compensated for by the use of an internal standard. In a case in which the RSD in mobility was 4.8%, the RSD in relative mobility (mobility of analyte/mobility of standard) was only 0.7% (Guttman *et al.*, 1990).

6.2. Gel Capillary Electrophoresis of Proteins

Although FSCE is the most widely used method for the separation of proteins, other applications such as isoelectric focusing (IEF) and capillary gel electrophoresis (CGE) are easily incorporated into the system. Significant growth has been seen in the use of gels for the separation of proteins in CE. Capillary gel electrophoresis allows for separation due to size along as well as charge. As with slab gels, capillary gels may be fabricated with optimum pore size for the molecular weight range of interest.

Cohen and Karger (1987) demonstrated the first sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) separation of proteins in capillary electrophoresis. Acrylamide was polymerized and attached to the surface of a capillary derivatized with (methacryloxypropyl) trimethoxy silane. The mobility of SDS-denatured proteins (molecular weight 14,000–34,500) was linear with molecular weight, and the effect of crosslinking was found to be similar to normal SDS-PAGE. The use of SDS causes each species to have the same charge density and shape, so that the separation is based solely on size or molecular mass.

Non-denaturing polyacrylamide gels can also be employed (Cohen *et al.*, 1987). In the case of non-denaturing gels for peptides and proteins, both size and charge will contribute to the separation. The gel concentration can easily be varied to enhance the resolution by varying pore size. A notable application is the capillary PAGE separation of human growth hormone from a synthesis byproduct. This byproduct (known as "two-chain") has a proteolytic enzymatic cleavage (clip) between Thr 142 and Tyr 143, and has essentially the same molecular weight but a different effective size. For this separation, Coomassie Blue-stained lab gels yielded a limit of detection of 5% compared to an estimated 0.1% detected impurity in CGE. Other advantages include increased plate numbers and the possibility of focusing of the samples upon injection into the gel column. Additives can also be incorporated into the gel matrix to increase resolution.

One problem with PAGE is Joule heating due to the high ionic strength of the buffers used, which results in thermal breakdown.

This emphasizes the need for efficient heat dissipation. To circumvent this problem, thermoelectric (Peltier) devices have been used to control the air temperature surrounding the capillary (Nelson *et al.*, 1989). Cohen and Karger have had success using a cooling system to remove the Joule heat generated in the capillary medium for the separation of four proteins using a gel-filled capillary (Fig. 13). The effect of air temperature variation on CE current as well as the importance of heat dissipation in capillaries under high power conditions were investigated. Thermal breakdown was found to occur at the high power levels needed for some protein separations. The cooling efficiency (related to air temperature) was shown to affect the peak shape of horse heart myoglobin.

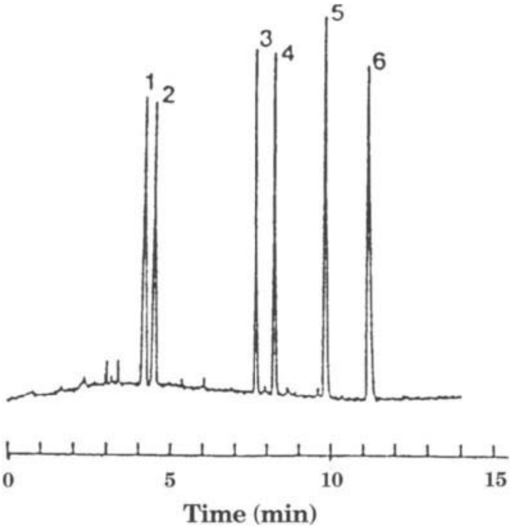

Fig. 13. Capillary SDS-polyacrylamide gel electrophoresis of proteins. Conditions: 300 V/cm; 12 μA; detection at 220 nm, separation length 15 cm; Gel: T = 7.5%, C = 5%; 0.1M Tris borate buffer, pH 7.3, 0.1% SDS, 8 M urea. Peak identification: (1) tracking dye; (2) lysozyme; (3) β-lactoglobulin; (4) trypsinogen; (5) pepsin; (6) egg albumin; (7) bovine albumin. (Reproduced with permission from Karger *et al.*, 1989).

Widhalm *et al.* (1991) separated four test proteins according to their masses using a medium of linear, non-cross-linked polyacrylamide gel. The proteins were applied as SDS complexes. The separation was performed at pH 5.5 and the migration times of the proteins were found to correlate with the log of their molecular mass.

6.3. Gel CE of Oligonucleotides and DNA Fragments

The rapid separation of oligonucleotides and DNA fragments has been accomplished with polyacrylamide gel-filled capillaries. The use of capillary electrophoresis/PAGE for the separation of large DNA restriction fragments was reported by Cohen *et al.* (1988). Kasper *et al.* (1988) used micellar electrokinetic chromatography and capillary PAGE to separate nucleotides and large DNA molecules. PAGE was found to be superior for separation of large, linear DNAs, since these have identical mass/charge ratios. Baba *et al.* (1991) prepared polyacrylamide gel-filled capillaries capable of very efficient separation of polynucleotides. A mixture of 250 polyadenylic acids was baseline resolved within 60 minutes using these columns. Paulus *et al.* (1990) separated oligonucleotide samples using polyacrylamide gel-filled capillaries. A plot of migration time vs. base number was linear with identical slopes for the three oligonucleotide samples examined. This provided for calibration of the capillary for the determination of molecular mass. They used this method of calibration to evaluate capillaries for the analysis of unknown samples for their molecular weight. Motsch *et al.* (1991) reported on the use of agarose gel-filled capillaries for the separation of medium-sized oligonucleotides (DNA restriction fragments) and some small, unsaturated and sulfated disaccharides. For further discussion of this topic, see Chapter 10.

6.4. Buffer Additives as Sieving Media

Buffer additives have also been used as sieving media for the separation of DNA fragments. The preparation of this type of capillary is very simple, and the gel is easily removed from the capillary and refilled. This greatly increases the versatility of the capillary system (Widhalm *et al.*, 1991). Strege and Lagu (1991) used a polyacrylamide coating and a buffer containing methylcellulose as a sieving medium to separate all fragments in the 1-kBP DNA ladder, as well as the very large fragments in a DNA-*Hind*III digest. MacCrehan *et al.* (1992) separated DNA fragment standards in the range of 50 to 10,000 base pairs using methylcellulose as a size-selective agent. All 23 fragments in a 1-kb DNA ladder were resolved within 25 min. Wehr (1993) used non-crosslinked dextran gels in the separation of proteins. These gels are inherently less rigid than cross-linked polyacrylamide gels and exhibit poorer resolution. Capillary wall deactivation, using a siloxane-bound polyacrylamide, greatly improved resolution and reproducibility, but the instability of the coating resulted in lifetimes of only about 30 runs. Guttman and Cooke (1991) incorporated an affinity ligand within the polyacrylamide gel to manipulate the selectivity of the separations. Selectivity was optimized by varying the electric field and the concentration of ethidium bromide, a soluble intercalating agent.

6.5. *Synthesis of Gels to Avoid Bubbles*

A major problem with gel columns is the development of vacuum bubbles inside the capillary tube during polymerization of the monomer solution. This is due to the fact that the polymerization occurs in a fixed volume, which is not a problem with slab gels. This results in a loss of separation current and renders the capillary unusable. Several reports have addressed the development of new strategies for the polymerization and stabilization of gels in fused-silica capillaries, emphasizing the minimization of bubble formation during polymerization and use. Dolnik *et al* (1991) described a procedure for preparing polyacrylamide gel-filled capillaries using isotachophoretic polymerization. In this method, the polymerization initiator is introduced by electromigration, allowing gradual polymerization of the acrylamide in a sequential manner along the length of the capillary and reducing formation of bubbles in the gels. Tsuji (1990) used ethylene glycol in both the gel formulation and the running electrolyte to prevent the formation of bubbles. Lux *et al.* (1990b) used gamma radiation to initiate polymerization of the polyacrylamide gel. Wang *et al.* (1991) produced polyacrylamide gels in fused-silica capillaries by adding riboflavin to the acrylamide/bisacrylamide solution before filling the capillary. After filling the capillary with this solution, UV radiation was used to initiate the polymerization.

In summary, disadvantages of gel-filled capillaries include production of bubbles in the column, lack of column-to-column reproducibility, difficulty in detection at lower than 210 nm due to interference by the gel, and hydrolysis of acrylamide limiting the number of runs for a given capillary. Gels, however, offer an added mode of selectivity with high resolution that makes this technique a significant advance in the separation of native proteins and other biomolecules by capillary electrophoresis.

7. Capillary Isoelectric Focusing

Isoelectric focusing is based on the separation of species by their isoelectric point (pI). It is performed in a medium with a pH gradient, and is particularly useful for protein separations. Charged proteins migrate to the pH at which they possess a net-neutral charge based upon the pK_a of the functional groups (pI). At this point, the neutral solutes will no longer migrate. The resolution of this method is limited only by the quality of the pH gradient.

When IEF is performed in capillaries (CIEF), the main issue is EOF. It was postulated that electroosmotic flow must be eliminated in order to obtain stable focused zones in CIEF (Hjertén *et al.*, 1987). Typically, the capillary is first coated to eliminate zone distortion caused by electroosmotic flow and to reduce adsorption of proteins to the capillary wall. Hjertén (1985) developed methylcellulose- and linear polyacrylamide-coated capillaries for CIEF. These coatings nearly eliminate EOF, allowing stable focused zones. It is not necessary to completely eliminate electroosmotic flow, only to slow the flow rate enough to allow for focusing of the zones before they pass by the detector.

The method is divided into two major steps: focusing and detection. Focusing occurs in a coated capillary column and is followed by the simultaneous application of precise vacuum and/or voltage to draw the focused bands past the detector at a steady, even rate. The capillary is filled with a carrier ampholyte (pH 3 to 10) to which the protein sample is added. Because the whole tube is filled with protein, larger amounts of sample can be employed than in free zone electrophoresis. Under an applied field, the proteins are focused along with the carrier ampholytes. The focusing process can be monitored by monitoring the current, which will reach a minimum value when focusing is complete.

Elution of the protein zones for on-column detection can be performed either by hydrodynamic flow or electrophoretically. In the electrophoretic elution mode, one of the reservoirs is switched to a salt buffer. When the field is reapplied, there will be an excess of either H^+ or OH^- entering the capillary. This excess will cause the pH gradient to become charged, and the solution will migrate toward the cathode if excess H^+ is present (cathodic mobilization) or toward the anode if excess OH^- is present (anodic mobilization) (Hjertén *et al.*, 1987). This will then permit detection of protein bands as they pass the detector. Resolution in IEF is dependent on the differences in pI of species in the mixture. The best resolution results with species possessing smaller diffusion coefficients and a high mobility slope at the isoelectric point. A shallow rate of change of pH with tube distance and a high electric field which speeds focusing also improve resolution.

Protein precipitation has been a common problem in conventional gel isoelectric focusing, and capillary IEF is no exception. Proteins are confined in zones at extraordinary high concentrations in a state of zero net charge, conditions which favor reduced solubility and aggregation. Stabilizing counterions are stripped from the protein as it is focused, further encouraging precipitation. This can yield poor reproducibility of peak size and migration time. A neutral detergent such as Triton X, which does not absorb at 280 nm, is often added to the focusing medium to suppress precipitation of the proteins (Hjertén, 1985).

Capillary IEF requires the use of coated capillaries because the magnitude of electroosmotic flow in uncoated capillaries prevents attainment of stable focused zones. As in free zone electrophoresis, coating chemistry in terms of column-to-column reproducibility and stability will be a key issue in the success and future development of IEF (Bolger *et al.*, 1991). Linear polyacrylamide coating is known to suffer from poor stability at high pH. Since it is not necessary to eliminate EOF but only to slow it down enough to allow bands to focus before migrating past the detector, methods that can control EOF will be of major interest for this technique. This approach will circumvent the need to mobilize the zones and move them past a detector, and will permit the focusing and elution in one step. The limitation of positioning the detector other than at the absolute end of the capillary is that species focused between the detector and the capillary end go undetected. The presence of salts in the sample changes the distribution of the pH gradient, and increases the time required for focusing and mobilization. Additives can act as dynamic coatings of the silica wall to cover many of the possible adsorption sites. EOF may be controlled, but not totally eliminated, through the careful choice of additive concentration. Maintaining some flow mobilizes the focused zones past the detector during the focusing step. To perform salt mobilization, the field must be turned off, the buffer changed, then the field turned back on.

An alternative to performing CIEF in coated capillaries is using methylcellulose in the sample/ampholyte mixture (Fig. 14). Proteins are separated efficiently, with good resolution and high peak capacity. The separation of nine proteins by isoelectric focusing using 0.5% methylcellulose in a 3% ampholyte mixture with a pH range of 2–11 is shown (Mazzeo and Krull, 1991). The proteins were focused for 20 min at 6 kV, followed by a 40-min mobilization at 10 kV. Thormann *et al.* (1990) added hydroxypropylmethylcellulose to the catholyte solution to separate the four proteins. CIEF has also been used to separate the isoforms of transferrin (Kilar and Hjertén, 1989), human

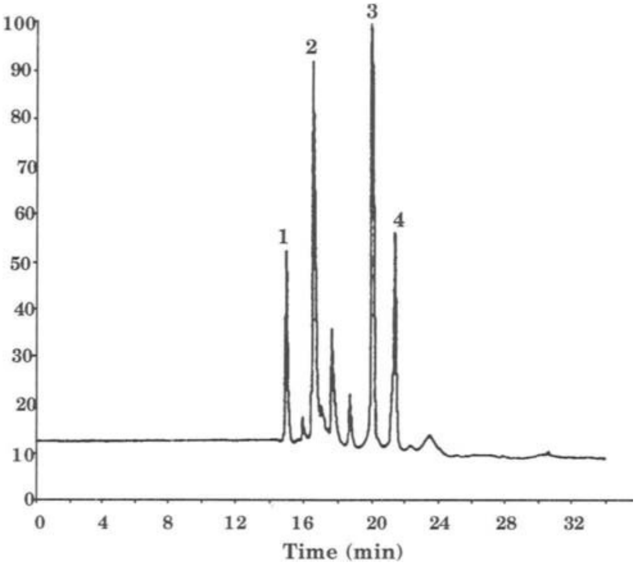

Fig. 14. CIEF of cytochrome *c*, chymotrypsinogen A and myoglobin in an uncoated capillary with TEMED. Capillary: 75 μm i.d. uncoated, 60-cm total length, 40 cm from the anode to detection. IEF: analyte 10 mM H_3PO_4, catholyte 20 mM NaOH, 30-kV voltage. Detection: UV, 280 nm. Sample: 1 mg/ ml each protein, 5% Pharmalyte 3–10, 0.1% methylcellulose, 1% TEMED. Peaks: (1) cytochrome *c*, pI 9.6; (2) chymotrypsinogen A, pI 9; (3) myoglobin, pI 7.2; (4) myoglobin, pI 6.8. (Reproduced with permission from Mazzeo and Krull, 1991.)

growth hormone variants (Frenz *et al.*, 1989), and the glycoforms of human recombinant tissue plasminogen activator (Yim, 1991).

8. Liquid Chromatography Packing Materials

In electroendoosmotic chromatography, a packed column is used, but the eluent is driven by electroosmotic flow rather than by pressure. Partitioning of solutes between the mobile and stationary phases occurs by normal distribution equilibria. The liquid, as in an open tube, is in contact with the tube walls and the particle surfaces, so electroosmotic flow occurs as a result of the fixed charges on the various surfaces. One interesting aspect of EOF is that, unlike other forms of flow, it generates a flat flow profile. In an open tube the flow is strictly plug flow with little variation of flow velocity across the section of the column. The flow in a packed bed is less perfect due to the nature of the channels (Knox and McCormick, 1989). However, it approximates plug flow and is substantially more uniform than in a pressure-driven system. This results in higher efficiencies for EOF than for pressure-driven flow. Since the tube walls form only a small proportion of the total superficial area of the particles, their conditionis not so critical. This is because the electroosmotic flow velocity, to a first approximation, is independent of the channel diameter between particles, while the pressure-driven flow rate is proportional to the square of the channel diameter. Although it is clear that uniformity of packing is less critical than in pressure-driven chromatography using packed beds, it is not yet known how uniform the packing structure should be.

Jorgenson and Lukacs (1981) studied electroosmotic flow for chromatography using a 170 μm i.d. × 68 cm capillary packed with 10 μm reversed-phase packing. The column ends were sealed by sintering 5-mm plugs of 30 μm ODS pellicular packing at each end of the capillary. With acetonitrile as the mobile phase, the separation of 9-methylanthracene and perylene resulted in efficiencies of 31,000 and 23,000 theoretical plates, respectively. They concluded that the plate heights were quite good considering the crude method and low pressure used to pack the column . They observed that if the column was indeed poorly packed, the good results were obtained due to the flow being uniform and independent of packing irregularities.

Knox and Grant (1987) looked into the factors that determine the optimum conditions for electrically driven LC separations. They studied the current and power requirements, the particle and tube diameters, and the electrolyte concentration on zeta potential. Their work involved the separation of polynuclear aromatic hydrocarbons using a drawn, packed capillary 50 μm i.d. × 60 cm with 5 μm Hypersil ODS-bonded *in situ* using methanol/water as the eluent. They also found no evidence of reduction in EOF in columns packed with particles as small as 1.5 μm in diameter (Knox and Grant, 1991). They found that higher chromatographic efficiencies can be obtained using EOF flow when the same column is used for both types of chromatography. Plate numbers of 100,000 were achieved in relatively short times (30 min) using *in situ* derivatized drawn packed capillaries containing 3 and 5 μm ODS-silica gels.

Yamamoto *et al.* (1992) described the possibilities of applying fast electroosmotic flow in packed capillary reversed-phase chromatography. No loss of column efficiency up to a linear EOF of 3 mm/s was observed. An example of the chromatograms obtained by electrokinetic reversed-phase chromatography is shown in Fig. 15 for the separation of isradipin, its byproducts, and thiourea. The dependence of theoretical plate number on linear velocity, stability and reproducibility of chromatographic performance was studied. A possible problem with this technique is the stability of the ODS silica packing at high pH. A high pH is necessary to obtain high EOF. It was found that capillaries 50 μm i.d. × 28.5 cm packed with Hypersil ODS (3 μm) were reproducible with run-to-run RSD of 1.6%–2.2%. Stable results were obtained for longer than 1 month.

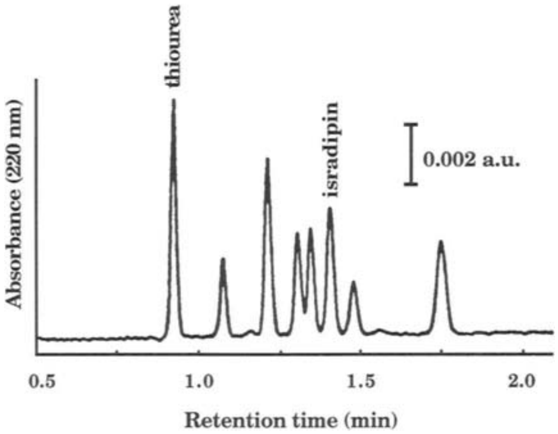

Fig. 15. Electrokinetic reversed-phase chromatography with packed capillaries in the separation of a mixture of Isradipin, its byproducts, and thiourea. Applied voltage, 30 kV, 1.8 μA. (Reproduced with permission from Yamamoto *et al.*, 1992.)

Limitations of EOF in chromatography include thermal effects that cause the same losses in efficiency seen in open tubes. Possible new technological limitations may be imposed by the establishment of the required electrical field. Martin and Guiochon (1984) noted that the piston-like character of the flow is less of an advantage when coupled with the limitation of peak broadening, which increases significantly with longer retention times. Their experience was that using EOF in a chromatographic system was difficult and inconvenient. This was largely due to the fact that the EOF lacked the direct and simple control over mobile phase flow that is customary with conventional pumping systems.

9. Column Technologies for Chiral Capillary Electrophoresis

Coated capillaries have been quite useful in enhancing chiral selectivity. This enhancement is mainly due to the elimination of EOF and the prevention of solute adsorption. Static coating with polyacrylamide (Fanali, 1991) and dynamically coating with polymers such as cellulose derivatives or polyvinyl alcohol have been used to improve peak shape and chiral resolution.

The elimination of EOF is important to maximize interaction between analyte and the chiral agent. For basic compounds, the chiral separation often utilizes a low-pH run buffer. Amino acids and many pharmaceuticals contain amine functional groups that will be protonated at low pH. At low pH, electroosmotic flow is minimal and the interactions of the solute with the chiral recognition agent are increased. As an example, cyclodextrins are often used in chiral CE where the solute is included into the cyclodextrin cavity, and its mobility is greatly reduced. If the two enantiomers have differing association constants, one enantiomer will migrate more slowly than the other and the two chiral species will be resolved.

For acidic compounds, however, the EOF is much stronger as the pH is often increased above the pK_a of the solute to enhance selectivity. Ionization of both the analyte and chiral agent can be controlled by buffer pH to increase enantioselectivity by optimizing the solute/agent interactions. However, as pH increases so does EOF, which may result in insufficient interaction of the solute with the chiral agent. The use of neutral coating on capillaries permits the reduction of EOF at elevated pH, providing the interactions necessary to enhance chiral selectivity and resolution.

Chiral selector-coated capillaries have been used for electrically driven chromatographic separations of chiral compounds (Mayer and Schurig, 1992). A 0.2-mm layer of Chirasil-Dex (permethylated ß-CD) was immobilized onto the capillary surface using polysiloxane for the separation of 1,1'-binaphthyl-2,2'-diyl-hydrogen phosphate and 1-phenylethanol. The separation was achieved in less than 30 minutes using a pH 7 phosphate buffer.

Gel columns have also been used with complexing agents in the analysis of chiral species (Guttman *et al.*, 1988). A column is made by entrapping the neutral inclusion compound β-cyclodextrin during the polymerization of polyacrylamide. Incorporation of this agent into the polymeric fiber gel matrix results in the complexing agent being immobile when uncomplexed and migrating only slowly when complexed with ionic species. Coated capillaries were used in conjunction with the gels resulting in a decrease in EOF and a reduction in analyte-wall interactions. Figure 16 shows the separation of D,L-dansylated amino acids using a β-cyclodextrin gel column. High field

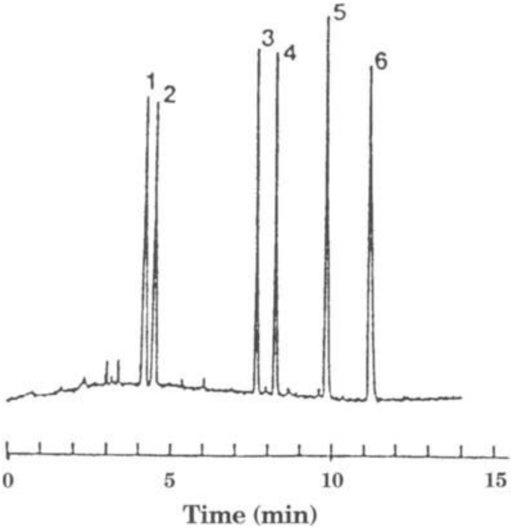

Fig. 16. Separation of D,L-dansylated amino acids using β-cyclodextrin gel column. Conditions: 0.1 M Tris, 0.25 M boric acid buffer (pH 8.3), 7 M urea. Gel: T = 5%, C = 3.3%. Capillary: 150 mm × 0.075 mm i.d.; E = 1000 V/cm. Peak identification: (1) Dns-L-Glu; (2) Dns-D-Glu; (3) Dns-L-Ser; (4) Dns-D-Ser; (5) Dns-L-Leu; (6) Dns-D-Leu. (Reproduced with permission from Guttman *et al.,* 1988).

strengths of 1000 V/cm were used with an effective separation length of 15 cm to achieve over 100,000 theoretical plates. Other gel columns include the co-polymerization of allyl carbamoylated-β-CD with acrylamide (Cruzado and Vigh, 1992) and crosslinking bovine serum albumin with gluteraldehyde (Birnbaum and Nilsson, 1992)

It should be noted that treatment of the wall surfaces and manufacturing gels is inherently more difficult and more complex than the use of carrier electrolytes incorporating specific chiral recognition agents added to the buffers in FSCE.

10. Commercial Columns

As discussed throughout this chapter, column technology plays a pivotal role in CE applications. The advancement of capillary electrophoresis as a standard analytical tool will depend largely on column technology and, in turn, on the commercial availability of columns.

There is a growing list of commercially available columns, reagents for the deactivation of capillary walls, sieving media and reagents. This allows analysts to move away from the manufacture of columns and instead to concentrate on applications.

Prior to the availability of commercial columns, laboratory-generated capillary surface modifications were frequently employed to reduce analyte adsorption. However, the capillaries produced were often unstable and gave irreproducible migration times. Commercial capillaries, priced at approximately $100/meter, are a welcome alternative to laboratory-built capillaries and possess useful lifespans of several weeks or hundreds of runs. The cost of a capillary, however, is more often measured by the time and energy required to coat a capillary. Therefore, in this case, commercial capillaries are quite valuable considering the separation efficiency and reproducibility they provide. However, as is the case with HPLC columns, the lot-to-lot reproducibility of coating capillaries can be variable.

10.1. Commercially Available Coated Columns

One of the first commercially available coated columns was manufactured by Bio-Rad Laboratories (Richmond, CA). These were produced in cartridges using a covalently bonded linear polyacrylamide after the method of Hjertén (1985). They were coated with a polymeric material that eliminates EOF to improve reproducibility and allow higher pH separation of proteins and isoelectric focusing. Each cartridge has prealigned optics to be used in their capillary electrophoresis instrument.

Beckman manufactures a neutral coated capillary designed for protein separations in the pH range 3–8 as well as CIEF applications. This coating is similar to the polyacrylamide coatings of Hjertén (1985). A kit includes buffers for use in the normal polarity (pH 3–6) or reversed polarity (pH 8) mode. A second type of coating available from Beckman utilizes a polyamine-modified surface. The resulting coating has a strong cationic charge that reverses the EOF. Basic analytes are repelled from the wall surface, thus minimizing the adsorption of positively charged analytes.

Supelco (Bellefonte, PA) offers C1, C8 and C18 bonded-phase CE columns, as well as a hydrophilic column. Column coatings are designed to provide a reduced but consistent EOF for improved protein separations. These surface modifications also reduce the dramatic effect of pH on EOF seen with untreated fused silica. Also available is an untreated fused silica capillary with internal diameters of 25 and 100 μm, which has an external protective coating transparent to UV to 200 nm.

Isco (Lincoln, NE) has available three different coated columns. The Isco CE-100 capillary column is for the separation of proteins and peptides. This capillary is filled with 0.5% Brij-35, 10% methanol in water. It is pH compatible from 4 to 10, shows no change under prolonged storage, and can be regenerated/cleaned by running several column volumes of a solution of Brij 35, 0.5% in water through the capillary. Reproducibility of this column was found to give run-to-run RSD of less than 1%, day-to-day RSD of 1.2%, and column-to-column RSD of 2.2%. The CE-200 capillary column is coated with a glycerol group attached through an octyl linker and is also recommended for protein and peptide separations. The CE-300 capillary column is coated with a sulfonic acid group attached through an octyl linker and is recommended for nucleotide separations. The capillaries measure 75-μm i.d. and 80 cm long with the optical window at 50 cm.

J & W Scientific (Folsom, CA) μSil products are bonded fused silica open tubular columns in 1 m × 50 μm and 1 m × 100 μm sizes. Their capillary gas chromatography columns are popular for the elimination the CE wall effects. These coatings have applications in isoelectric focusing of proteins and separation of large DNA fragments. Their DB-17 Microbore GC column and DB-WAX have been used for isoelectric focusing and the analysis of basic proteins.

LC Packings (San Francisco, CA) markets a packed-capillary C18 column with 75 μm i.d. for electrochromatography that achieves high capacity, high selectivity, and efficiencies of 600,000 theoretical plates.

Millipore (Boston, MA) has introduced a concentrator capillary, the Waters AccuSep C/PRP, with a C18 polymeric phase to concentrate samples on a small section of the capillary

10.2. Commercially Available Gel-Filled Columns

Gel-filled capillaries are currently commercially available from several manufacturers. Beckman Instruments (Fullerton, CA) has introduced a SDS-CGE using a proprietary linear polymer network that is UV transparent at 214 nm. The hydrophilic polymer solution is used for the separation of proteins ranging from 14 to 205 kD. Coated capillaries are used in conjunction with the gel-matrix to improve reproducibility by reducing protein adsorption. A rinsing procedure used between runs to replace the gel matrix greatly increases reproducibility for proteins. The gel matrix can be replaced at least 100 times. Beckman also produces a fixed, polyacrylamide/urea-containing gel, which can be used in the analysis of RNA and single-stranded DNA ranging in size from 10 to 100 bases.

Applied BioSystems produces Micro-Gel 100 columns that are 50 cm in length with 50 μm i.d. These columns provide single-base resolution of synthetic oligonucleotides as long as 120 bases. Temperature rather than buffer additives can be used to denature the oligonucleotides because the proprietary gel matrix is stable to 50°C.

Bio-Rad has also introduced a sieving buffer system for separation of PCR products. The PCR analysis buffer contains a linear hydrophilic polymer in a Tris-borate-EDTA buffer. A buffer containing 7 M urea is also available to provide denaturing conditions. The system is designed for the separation of PCR products up to 600 base pairs.

J & W Scientific produces mPAGE columns using "dimension compensating polymerization" that yield bubble-free polyacrylamide gels for high resolution separations. The columns are available in two different concentrations that cover a wide range of sizing applications. The mPAGE-% column, a 5%T, 5%C gel offers single base resolution of oligonucleotides from fewer than 20 to more than 150 bases. The mPAGE-3 (3%T, 3%C) is used for fast analysis of larger oligonucleotides and DNA fragments.

10.3. Commercially Available Reagents

ABI markets a removable coating that imparts a stable, net positive charge to the capillary surface. This charge reversal on the capillary wall also means that the EOF will be reversed and a reversal of the polarity is necessary to ensure that analytes migrate toward the detector. This reagent makes possible the analysis of cationic proteins and peptides below their isoelectric points under nondenaturing conditions. The combination of ionic as well as hydrophobic interactions of the reagent with the silanols of the fused silica capillary is strong enough that it does not have to be added to the separation buffer.

A second reagent marketed by ABI is included in an analysis kit containing a replaceable sieving matrix that separates proteins based upon the same principles as classical SDS-PAGE. Molecular sieving is accomplished by a network of linear molecules, which are free to entwine themselves, in contrast to the fixed mesh of rigid pores in standard polyacrylamide gels. The reagent also contains a capillary coating to suppress EOF and improve reproducibility. The reagent yields linear separations of proteins in the molecular weight range of 14 to 205 kD. The analysis time is typically less than 30 min, and online detection permits accurate and reproducible quantitation without the staining and destaining process.

The third reagent marketed by ABI is included in a capillary isoelectric focusing kit used in conjunction with their CE system. This reagent separates proteins in the same way as traditional polyacrylamide gel isoelectric focusing, with the exception that the capillary method is performed in free solution rather than in a gel. The entire pH range of ampholytes is used to produce linear pI-migration plots with baseline resolving power better than 0.2 pH units across the entire pI range from less than 3 through 10, in a single run. A narrower range of ampholytes can be used to increase the resolution to 0.06 pH units.

Beckman offers two reagent kits that use replaceable entangled polymer solutions for the analysis of double-stranded DNA in the molecular weight range of 100 to 1000 base pairs. The second kit differs from the first in using an intercalating dye to provide improved sensitivity when used with laser-induced fluorescence detection.

11. Conclusions

The success of capillary electrophoresis will depend largely on the development of capillary columns to widen the field of applications. Advances in geometry, coatings, and sieving media have moved CE from a curiosity to a practical technique for solving real problems. The function of these column technologies is to enhance the separation and recovery of species, to stabilize EOF across a wide pH range, and to extend the useful life of capillaries.

Capillary columns have demonstrated their utility in the separation of solutes that have not been successfully resolved by chromatography and/or slab gel electrophoresis. Column technology is reaching a level of sophistication in terms of reproducibility and selectivity that makes CE a viable technique in the separation and characterization of analytes.

It is possible to control EOF by means of an external electric field or buffer additives to optimize a given separation. However, these techniques do not represent general approaches. The method of choice is the modification of the inner surface of the capillary through chemical derivatization. Reliable manufacture of capillary coatings with adequate chemical and temporal stability is critical for adequate precision and reproducibility. It should be noted that capillary column technology figures prominently on the overall success of CE, as it is directly tied to such important issues as reproducibility, suppression of adsorption, and selectivity.

12. References

Altria, K. D. and C. F. Simpson (1986). *Anal. Proc.*, **23**, 453–454.

Baba, Y., T. Matsuura, K. Wakamoto and M. Tsuhako (1991). *Chem. Lett.*, *3*, 371–374.

Beckman Instruments Inc. (1994). *Beckman Primer, 5*, 22-41.

Bentrop, D., J. Kohr and H. Engelhardt (1991). *Chromatographia, 32,* 171–177.

Birnbaum S. and S. Nilsson (1992). *Anal. Chem. 64*, 2872-2874

Bolger, C.A., M. Zhu, R. Rodriguez and T. Wehr (1991). *J. Liq. Chromatogr. 14,* 895–906.

Bruin, G., J. Chang, R. Kuhlman, K. Zegers, J. Kraak and H. Poppe (1989a). *J. Chromatogr., 471*, 429–436.

Bruin, G., R. Huischen, J. Kraak and H. Poppe (1989b). *J. Chromatogr., 480*, 339–349.

Bushey, M. M. and J. W. Jorgenson (1989). *J. Chromatogr., 480*, 301–310.

Chen, F. A., L. Kelly, R. Palmieri, R. Biehler and H. Schwartz (1992). *J. Liq. Chromatogr., 15*, 1143–1161.

Cobb, K. A., V. Dolnik and M. Novotny (1990). *Anal. Chem., 62*, 2478–2483

Cohen, A. S., A. Paulus and B. L. Karger (1987). *Chromatographia, 24*, 15–24.

Cohen, A., D. Nagarian, J. Smith and B. L. Karger (1988). *J. Chromatogr., 458*, 323–333.

Cruzado, I.D. and G. Vigh (1992). *J. Chromatogr. 608*, 421-425

Davies, J. T. and E. L. Rieal (1961). *Interfacial Phenomena*, Academic Press, New York, NY.

Dolnik, V., K. A. Cobb and M. Novotny (1991). *J. Microcol. Sep.*, 3, 155–159.

Drossman, H., J. A. Luckey, A. J. Kostichka, J. D'Cunha, and L. M. Smith (1990). *Anal. Chem., 62*, 900–903.

El Rassi, Z. and W. Nashabeh (1992). Poster 394. Sixteenth International Symposium on Column Liquid Chromatography, Baltimore, MD.

Emmer, A., M. Jansson and J. Roeraade (1991). *J. Chromatogr. 547*, 544-550

Fanali, S (1991). *J. Chromatogr. 545*, 437-444

Foret, F., S. Fanali, L. Ossicini and P. Bocek (1989). *J. Chromatogr., 470*, 299–308.

Frenz, J., S.-L. Wu and W. S. Hancock (1989). *J. Chromatogr., 480*, 379–391.

Fujiwara, S. and S. Honda (1986). *Anal. Chem., 58,* 1811–1814.

Fujiwara, S. and S. Honda (1987). *Anal. Chem., 59*, 487–490.

Gordon, J. J., K.-J. Lee, A. A. Arras and R. N. Zare (1991). *Anal. Chem., 63*, 69–72

Green, J. S. and J. W. Jorgenson (1989). *J. Chromatogr., 478*, 63–70.

Grossman, P. D.., J. C. Colburn, H. H. Lauer, R. G. Nielsen, R. M. Riggin, G. S. Sittampalam and E. C. Rickard (1989). *Anal. Chem., 61*, 1186–1194.

Grossman, P. D.., K. J. Wilson, G. Petrie and H. H. Lauer (1989). *Anal. Biochem.*, *173* 265.

Guttman, A., A. Paulus, A. S. Cohen, N. Grinberg and B. L. Karger (1988). *J. Chromatogr.*, *488*, 41–53.

Guttman, A., A. S. Cohen, D. N. Heiger and B. L. Karger (1990). *Anal. Chem.*, 62, 137–141.

Guttman, A. and N. Cooke (1991). *Anal. Chem.*, *63*, 2038–2042.

Heiger, D. M (1992). In: *High Performance Capillary Electrophoresis–An Introduction* pp. 100-101. Hewlett Packard Company, Avondale, CA.

Herren, B., S. Shafer, J. van Alstine, J. Harris and R. Snyder (1987). *J. Colloid. Interfac. Sci.*, *115*, 46–55.

Hjertén, S. and M.-D. Zhu (1985). *J. Chromatogr.*, *327*, 157–164.

Hjertén, S. (1985). *J. Chromatogr.*, *347*, 191–198.

Hjertén, S. and M.-D. Zhu (1985). *J. Chromatogr.*, *346*, 265–270.

Hjertén, S., J.-L. Liao and K. Yao (1987). *J. Chromatogr.*, *387*, 127–138.

Hjertén, S., K. Elenbring, F. Killar, J.-L. Liao, A. J. Chen, C. J. Siebert and M.-D. Zhu (1987). *J. Chromatogr.*, *403*, 47–61.

Hjertén, S. and M. Kiessling-Johanson (1991). *J. Chromatogr.*, *550*, 811–822.

Huang, M., W. P. Vorkink and M. L. Lee (1992) *J. Microcol. Sep.* *4*, 233–238.

Huang, X., J. A. Luckey, M. J. Gordon and R. N. Zare (1989). *Anal. Chem.*, *61*, 766–770

J & W Scientific. (1992) Product Catalog, 291–303.

Jorgenson, J. W. and K. D. Lukacs (1981). *J. Chromatogr.*, *218*, 209–216.

Jorgenson, J. W. and K. D. Lukacs (1981). *Anal. Chem.*, *53*, 1298.

Jorgenson, J. W. and K. D. Lukacs (1983). *Science*, *222*, 266–272.

Jorgenson, J. W. and K. D. Lukacs (1984). *Trends Anal. Chem.*, *3*, 51–54.

Josic, D., K. Zeilenger, W. Reutter, A. Bottcher and G. Schmitz (1990). *J. Chromatogr.*, *516*, 89–98.

Kapaciewicz, W., M. A. Rounds, J. Fausnaugh and F. E. Regnier (1983). *J. Chromatogr.*, *266*, 3–21.

Karger, B. L., A. S. Cohen and A. Guttman (1989). *J. Chromatogr.*, *492*, 585–614.

Kasper, T., M. Melera, P. Gozel and R. Brownlee (1988). *J. Chromatogr.*, *488*, 303–306.

Kilar, F. and S. Hjertén (1989). *J. Chromatogr.*, *480*, 351–357

Knox, J. H. and I. H. Grant (1987). in pressure and electroendoosmotically driven liquid chromatography: Some theoretical considerations. *Chromatographia*, *24*, 135–143.

Knox, J. H. and K. A. McCormick (1989). *J. Liq. Chromatogr.*, *12*, 2435–2470.

Knox, J. H. and I. H. Grant (1991). *Chromatographia, 32,* 317–328.

Lauer, H. H. and D. McManigill (1985). *Anal. Chem.*, *58*, 166–170.

Lee, C. S., W. C. Blanchard and C.-T. Wu (1990). *Anal. Chem.*, *62*, 1550–1552.

Lee, C. S., D. McManigill, C.-T. Wu and B. Patel (1991). *Anal. Chem.*, *63*, 1519–1523.

Liu, P. Z., A. Malik, M. C. Kuchar, W. P. Vorkink, and M. L. Lee (1993). *J. Microcol. Sep. 5*, 245-253.

Loo, J. A., H. K. Jones, H.R. Udseth and R. D. Smith (1989). *J. Microcol. Sep.*, *1*, 223–229.

Lukacs, K. D. and J. W. Jorgenson (1985). *J. High Resolut. Chromatogr. Chromatogr. Commun.*, *8*, 407.

Lux, J. A., H. F. Yin and G. Schomburg (1990). *J. High Resolut. Chromatogr. Chromatogr. Commun.*, *13*, 436–7.

Maa, Y. F, K. J. Hyver, and S. A. Swedberg (1991) *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 14, 65–67

MacCrehan, W. A., H. T. Rasmussen and D. M. Northrop (1992). *J. Liq. Chromatogr.*, *15*, 1063–1080.

McCormick, R. M. (1988). *Anal. Chem.*, *60*, 2322–2328.

Manz, A., D. J. Harrison, E. M. J. Verpoorte, J. C. Fettinger, A. Paulus, H. Ludi and H. M. Widmer (1992). *J. Chromatogr.*, *593*, 253–258.

Martin, M. and G. Guiochon (1984). *Anal. Chem.*, *56*, 614–620.

Mayer, S. and V. Schurig (1992). *J. High Resolut. Chromatogr. HRC 15,* 129-131.

Mazzeo, J. R. and I. S. Krull (1991). *Anal. Chem.*, *63*, 2852–2857.

Moseley, M. A., L. J. Deterding, K. B. Tomer and J. W. Jorgenson (1991). *Anal. Chem.*, *63*, 109–114.

Motsch, S. R., M. H. Kleemiss and R. Schomburg (1991). *J. High Resolut. Chromatogr.*, *14*, 629–32.

Nashabeh, W and Z. El Rassi (1991). *J. Chromatogr.*, *559*, 367–383.

Nelson, R., A. Paulus, A. Cohen, A. Guttman and B. Karger (1989). *J. Chromatogr.*, *480*, 111–128.

Nielsen, R. G., G. S. Sittampalam and E. C. Rickard (1989). *Anal. Biochem.*, *177*, 20–26.

Paulus, A., E. Gassmann and M. M. Field (1990). *Electrophoresis*, *11*, 702–708.

Pretorius, V., B. J. Hopkins and J. D. Scheike (1974). *J. Chromatogr.*, *99*, 23–30

Regnier, F. E. and R. Noel (1976). *J. Chromatogr. Science*, *14*, 316–320.

Rice, C. L. and R. Whitehead (1965). *J. Phys. Chem.*, *69*, 4017.

Rohlicek, V. and Z. Deyl (1989). *J. Chromatogr.*, *494*, 87–99.

Strege, M. and A. Lagu (1991). *Anal. Chem.*, *63*, 1233–1236.

Swedberg, S. A. (1990). *Anal. Biochem.*, *185*, 51–56.

Sydor, W., S. Gruber and D. Pennino (1989). *BioPharm.*, *2*, 41–44.

Thormann, W., J. Caslavsda, S. Molteni and J. Chmelik (1992). *J. Chromatogr.*, *589*, 321–327

Towns, J. K. (1991) Ph.D. Dissertation, Purdue University, West Lafayette, IN

Towns, J. K. and F. E. Regnier (1990). *J. Chromatogr.*, *516*, 69–78.

Towns, J. K. and F. E. Regnier (1991). *Anal. Chem.*, *63*, 1126–1131.

Towns, J. K. and F. E. Regnier (1992). Anal. *Chem.* *64,* 2473–2478.

Towns, J. K., J. J. Bao and F. E. Regnier (1992). *J. Chromatogr.*, *599*, 227–237.

Tsuda, T. (1987). *J. High Resolut. Chromatogr.*, *10,* 622–624.

Tsuda, T., J. Sweedler and R. N. Zare (1990). *Anal. Chem.*, *62*, 2149–2152.

VanOrman, B. B., G. G. Liversidge, G. L. McIntire, T. M. Olefirowicz and A. G. Ewing (1990). *J. Microcol. Sep.*, *2,* 176–180.

Vinther, A., S. E. Bjorn, H. H. Sorensen and H. Soeberg (1990). *J. Chromatogr.*, *516* 175–184.

Walbroehl, Y. (1988) Ph.D. Dissertation, University of North Carolina, Chapel Hill, NC.

Walbroehl, Y. and J. W. Jorgenson (1989). *J. Microcol. Sep.*, *1*, 41–45.

Wallingford, R. A. and A. G. Ewing (1989). *Adv. Chromatogr.*, *29*, 1–76

Wang, T., G. J. Bruin, J. C. Kraak and H. Poppe (1991). *Anal. Chem.*, *63*, 2207–2208.

Wehr, T. (1993) *LC-GC 11,* 14–20.

Widhalm, A., C. Schwer, D. Blaas and E. Kenndler (1991). *J Chromatogr.*, *549*, 446–451.

Wieme, R. J. (1975). In *Chromatography—A Laboratory Handbook of Chromatographic and Electrophoretic Methods* (E. Heftmann, ed.), Van Nostrand Reinhold, New York, 3rd Ed., Chapter 10.

Wiktorowicz, J. E. and J. C. Colburn (1990). *Electrophoresis, 11*, 769–7732.

Yamamoto, H., J. Baumann and F. Erni (1992). *J. Chromatogr.*, *593,* 313–319.

Yim, K. W. (1991). *J. Chromatogr.*, *559*, 401–410.

Yin, H. F., J. A. Lu and G. Schomburg (1990). *J. High Resolut. Chromatogr.*, *13*, 624–627.

Zhu, M., R. Rodriguez, D. Hansen and T. Wehr (1990). *J. Chromatogr.*, *516*, 123–131.

Zhu, M., D. Hansen, S. Burd and F. Gannon (1989). *J. Chromatogr.*, *480*, 311–320.

CHAPTER 3

Electrokinetic Chromatography

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1. Introduction

As discussed in Chapter 1, capillary electrophoresis (CE) is a technique that was developed for the very efficient separations of *charged* solutes, based on their differences in electrophoretic mobility, μ_{ep}, as defined in Eq. 1,

$$\mu_{ep} = \frac{q}{f} \tag{1}$$

where q is the net charge of the solute and f is its frictional drag coefficient. Note that μ_{ep} can be different for two solutes if differences exist in q, f, or both for the two solutes. As shown in Eq. 2, no separation will occur in free solution CE if the electrophoretic mobilities of two compounds are the same.

$$R_s \propto \frac{\mu_{ep,2} - \mu_{ep,1}}{\mu_{ep,avg} + \mu_{eo}} \tag{2}$$

In the above expression, μ_{eo} is the coefficient of electroosmotic flow, a bulk displacement phenomenon explained in Chapter 1. It can be larger or smaller than $\mu_{ep,avg}$ or even negligible (*i.e.*, $\mu_{eo} \approx 0$), depending on the experimental conditions.

1.1. *Conventional CE Cannot Separate Neutral Solutes, but EKC Can*

Unfortunately, for neutral solutes q = 0 in Eq. 1, hence $\mu_{ep} = 0$ for all neutral species and none of them will be separated ($R_s = 0$, Eq. 2), as shown in Fig. 1. It was for this reason that Terabe and coworkers (Terabe *et al.*, 1984) introduced micellar electrokinetic capillary chromatography (MECC) in 1984. MECC allows neutral species to be resolved using CE instrumentation by creating a second retentive phase into which they can differentially partition. The differential partitioning gives rise to differential migration rates which provide the separation.

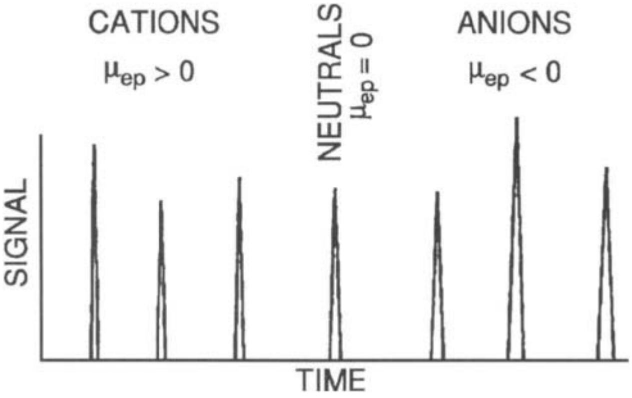


Fig. 1. Conventional CE cannot separate neutral compounds, as depicted in the hypothetical electropherogram of 3 cationic species, 3 neutral compounds, and 3 anionic species.

Soon after Terabe published his pioneering work with micelles (Terabe *et al.*, 1984; Terabe *et al.*, 1985a), he and other researchers found that separations impossible with conventional CE could also be achieved by adding substances other than micelles to the buffer (Terabe *et al.*, 1985b; Terabe, 1989; Terabe and Isemura, 1990b). In view of this, Terabe recommended the more general name "electrokinetic chromatography" (EKC) to more accurately depict the scope of these methods of separation. We follow his suggestion throughout the remainder of this chapter, and use "MEKC" to denote EKC separations performed with micelles.

1.1.1. EKC from an electrophoretic viewpoint

Micelles or other charged species added to a buffer solution impart an effective electrophoretic mobility to neutral analytes by interacting with the neutral compound to form a charged "complex". The binding constants of the analytes can be measured in a variety of ways, *i.e.*, from absorbance or fluorescence measurements, by micellar liquid chromatography (MLC), and by MEKC (Delaguardia *et al.*, 1991). A compilation of K_m values determined from MLC data was reported by Foley (1990a) for various combinations of solute and surfactant; more recently, Khaledi and coworkers compared and contrasted the determination of K_m values by MLC and MEKC (Kord *et al.*, 1991).

Assuming that only 1:1 interactions are significant, the interaction of the neutral analyte and the charged species can be described by

$$A + X^{\pm} \rightleftarrows AX^{\pm} \qquad K_f = \frac{[AX^{\pm}]}{[A][X^{\pm}]} \tag{3}$$

where A is the neutral analyte, $X^{\pm}$ is the positively or negatively charged species (micelle, cyclodextrin, etc.) that interacts with the analyte, $AX^{\pm}$ is the resulting complex, and K_f is the formation (binding) constant of the analyte with $X^{\pm}$.

The effective electrophoretic mobility of a neutral analyte can be described phenomenologically as (Foley and May, 1987)

$$\mu_{ep,A,eff} = \left(\frac{K_f[X]}{1+K_f[X]}\right) \mu_{ep,AX^{\pm}} \tag{4}$$

When micelles are employed as the charged species, the effective electrophoretic mobility is usually expressed as (Ghowsi *et al.*, 1990)

$$\mu_{ep,A,eff} = \left(\frac{k}{1+k}\right) \mu_{ep,AX^{\pm}} \tag{4a}$$

because the micelles are viewed as a separate, (pseudostationary) chromatographic phase into which neutral analytes can partition.

If the formation constants (K_f) or retention factors (k) of two analytes differ, their effective electrophoretic mobilities will differ (Eqs. 4 and 4a), and they can then be resolved by EKC, provided that X is present in the buffer at an appropriate concentration.

1.1.2. EKC from a chromatographic viewpoint

Because of the key role played by the chromatographic-like separation mechanisms in EKC, it is more customary and beneficial to describe EKC (and particularly MEKC) in terms of the partitioning mechanism below (see also Fig. 2):

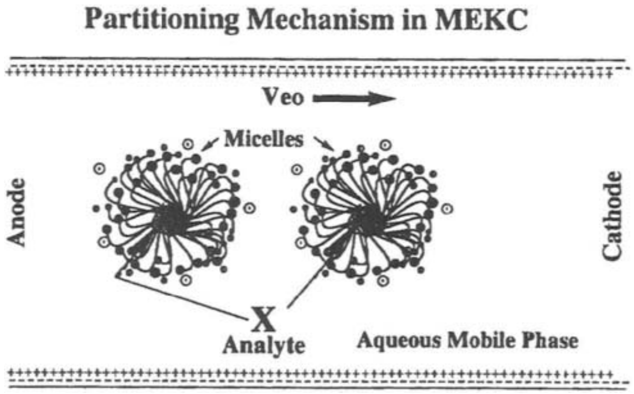


Fig. 2. Partitioning mechanism of electrokinetic chromatography (EKC). Consistent with Eq. 5, X represents the virtual (pseudophase) phase established by the interactive species.

$$[S]_{aq} \rightleftarrows [S]_x \qquad P_{wx} = \frac{[S]_x}{[S]_{aq}}, \tag{5}$$

where P_{wx} is the partition coefficient, and $[S]_{aq}$ and $[S]_x$ are the concentration of the solute, S, in the aqueous phase and the virtual (pseudophase) phase established by the micelle or other interactive species, respectively. If we now define a phase ratio β as the volume of pseudophase (V_x) divided by the volume of the aqueous mobile phase (V_{aq})

$$\beta = \frac{V_x}{V_{aq}}, \tag{6}$$

then a retention factor k can be defined as:

$$k = P_{wx}\,\beta = \frac{n_x}{n_{aq}}, \tag{7}$$

where n_x and n_{aq} are the number of moles of solute in the micellar and aqueous phases, respectively. The retention factor determines, to a large extent, the migration time of a neutral analyte (vide infra).

1.2. EKC is also Useful for Charged Molecules

Soon after the micellar-mediated EKC separation of *neutral* compounds was reported, Terabe and coworkers (Cohen *et al.*, 1987; Nishi *et al.*, 1989b,c,d) began investigating the separations of *charged* substances that were not adequately resolved by CE. In most cases, they had success similar to that with neutral molecules. In retrospect, EKC and in particular MEKC are obviously not restricted to the separation of neutral solutes. For charged substances, EKC provides a second separation mechanism that is often complementary to that provided by conventional CE, frequently enhancing the overall selectivity and enabling additional compounds to be resolved that were not by traditional CE.

Assuming a 1:1 interaction between analyte and interactive agent, the effective electrophoretic mobility of a *charged* analyte can be described as

$$\mu_{\mathrm{ep,A,eff}} = \left(\frac{1}{1+K_f[X]}\right)\mu_{\mathrm{ep,A}} + \left(\frac{K_f[X]}{1+K_f[X]}\right)\mu_{\mathrm{ep,AX}} \qquad (8)$$

or

$$\mu_{\mathrm{ep,A,eff}} = \left(\frac{1}{1+k}\right)\mu_{\mathrm{ep,A}} + \left(\frac{k}{1+k}\right)\mu_{\mathrm{ep,AX}} \qquad (8a)$$

where the charges on A, X, and AX are not specified to maintain generality.

2. Basic Terminology

2.1. A Broader View of Mobile and Stationary Phases

Chromatography is usually thought of as a method of separation involving two phases, the first of which moves with respect to the coordinate system (the mobile phase), and the second of which does not (the stationary phase). A more general view of chromatography, however, allows for the possibility that the second phase is mobile, but to a lesser degree than the first phase. Thus, the second phase functions as a pseudostationary phase.

Such a two-phase model is easy to appreciate in EKC when the second phase is micellar. On the other hand, there are a number of separation mechanisms involving buffer additives discussed earlier that do not, strictly speaking, constitute a second phase. Nevertheless, if we view the interactive species as a second phase and the aqueous buffer as the first phase, we can employ a useful chromatographic formalism that facilitates (i) the development of a theory that provides a clear explanation of the power of EKC and (ii) a theoretical unification of four, formerly distinct separation modes—LC, CE, EKC, and electrochromatography.

2.2. Electrokinetic Concepts and Parameters

In electrokinetic chromatography, we are concerned with several concepts, many of which are traced back to chromatography with a true stationary phase. Several sources discuss conventional chromatography in greater detail (Snyder and Kirkland, 1979; Giddings, 1991).

First, we define as $\mathbf{t_R}$, $\mathbf{t_o}$, **and** $\mathbf{t_{mc}}$, respectively, the **migration times** of the **analyte**, the aqueous **buffer** (mobile phase), and the **micelle** or other species that interacts with the analyte (pseudostationary phase). Whereas t_R is measured directly, t_o and t_{mc} are usually determined indirectly. The buffer migration time (t_o) is obtained from the migration time of a neutral compound (*e.g.*, organic solvent (Ahuja *et al.*, 1992) that does not interact with the retentive species; the migration time of the micelle is estimated from the migration time of an analyte that binds it strongly or from an iterative analysis of the migration times of three or more late-eluting neutral homologs (Bushey and Jorgenson, 1989a,b). Note that for neutral molecules, $t_o \le t_R \le t_{mc}$.

A hypothetical EKC separation of neutral molecules is shown in Fig. 3. The **elution range** is defined either as t_{mc}/t_o or its reciprocal. We prefer the former (Eq. 9),

$$\text{elution range} = t_{mc}/t_o \tag{9}$$

since it can be more directly related to the peak capacity (Eq. 14). In Fig. 3, the elution range is 8.8, which was estimated by measurement with a ruler.

The **degree of separation or resolution ($\mathbf{R_s}$)** of two solutes is commonly defined as

$$R_s = \frac{\Delta t_R}{W_{avg}} = \left(\frac{t_{R1} - t_{R2}}{\frac{W_1 + W_2}{2}}\right) = \left(\frac{t_{R1} - t_{R2}}{2\,(\sigma_1 + \sigma_2)}\right), \tag{10}$$

where Δt_R is the difference between the migration times of the solutes and W_{avg} is the average peak width. More detailed discussions of resolution are available (Snyder and Kirkland, 1979; Giddings,

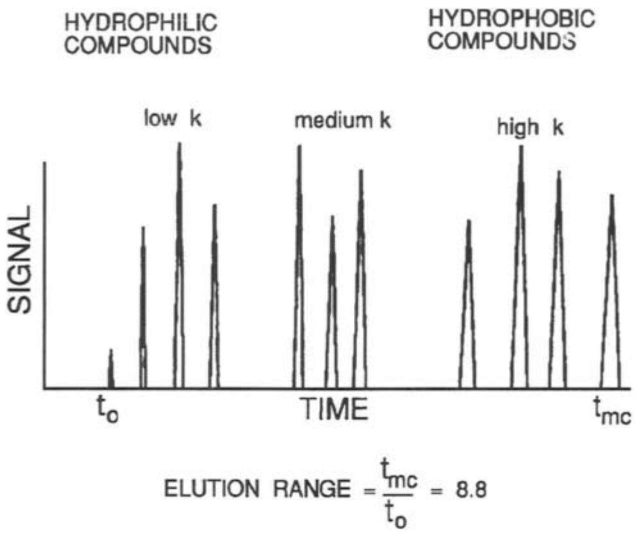


Fig. 3. Typical separation of neutral compounds in EKC and illustration of relevant migration parameters.

$$R_S = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right) f(k, t_{mc}/t_o) \tag{10a}$$

1991), and a review on the uses of various R_S equations in conventional column chromatography has recently been published (Foley, 1991). As we shall show in more detail later, the resolution in EKC can also be expressed in electrophoretic and/or chromatographic parameters, as shown in Eq. 10a, where **N** is the **column efficiency** (number of theoretical plates), α is the **selectivity**, and **f** is a function (specified later) that depends on the **retention factor** (see Eqs. 7, 26) and **elution range** (Eq. 9).

Column efficiency, N, is a measure of peak width and is given by

$$N = \frac{L}{H}, \tag{11}$$

where L is the capillary length (inlet to point of detection) and H is the height equivalent to a theoretical plate (HETP). Large values of N (small values of H) indicate high efficiency and better separating power. Experimentally, the number of theoretical plates for a peak can be calculated from:

$$N = 5.54\left(\frac{t_R}{W_{0.5}}\right)^2, \tag{12}$$

where $W_{0.5}$ is the peak width at half height, t_R is the migration time, and a Gaussian peak profile is assumed. Other, similar Gaussian-based equations can also be employed (Bidlingmeyer and Warren, 1984), but if peaks are tailed and/or the peak shape varies widely over the results to be compared, use of such equations can result in errors in excess of 100% (due to the underestimation of peak variance), and it is better to use

$$N = \frac{41.7\,(t_R/W_{0.1})^2}{b/a + 1.25}, \qquad (12a)$$

where b/a is an empirical asymmetry factor measured at 10% (Foley and Dorsey, 1983; Bidlingmeyer and Warren, 1984; Jeansonne and Foley, 1991). Although Eq. 12a is based on the exponentially modified Gaussian, it is also fairly accurate for log-normal and other asymmetric profiles. And as recently described, it can also be applied to fronted peaks (Jeansonne and Foley, 1992), thus extending the asymmetry range over which it is applicable to 0.36–2.76.

Selectivity, α, is the ratio of retention factors of two compounds, *i.e.*,

$$\alpha = k_2/k_1 \qquad (13)$$

As the selectivity increases from unity (no separation), the spacing between the two compounds increases, and the number of theoretical plates required to achieve a given resolution decreases exponentially. Because of this, values of $\alpha > 1.05$ are usually desirable.

In EKC, the selectivity can be varied by changing the identity and/or concentration of one or more of the following: (i) interactive species (surfactant, bile salt, etc.); (ii) buffer; (iii) counter ion(s) of the surfactant and/or buffer; and (iv) organic modifier. For neutral compounds, the selectivity in MEKC has been found to be approximately independent of the surfactant concentration; for charged species the selectivity changes somewhat due to changes in electrostatic effects.

Finally, the **peak capacity n_c**, is the number of solutes that can be placed side-by-side along the separation axis within the constraints of the specific separation. As such, it represents an upper limit to the number of peaks that can be resolved; this limit is seldom realized in practice due to the random distribution of analytes along the separation axis. For chromatographic techniques, the peak capacity, n_c, is given by the following:

$$n_c = 1 + \frac{\sqrt{N}}{4} \ln \frac{t_\omega}{t_\alpha}, \qquad (14)$$

where t_α and t_ω are the retention times of the first and last eluting peaks. Capillary GC and gradient LC methods have very large peak capacities, sometimes into the hundreds of solutes. But EKC as usually practiced does not have as large a peak capacity due to the finite elution range (t_{mc}/t_o), which is essentially equivalent to t_ω/t_α. In this regard EKC is much like size exclusion chromatography (SEC) in that there is a limited elution range (time window) over which solutes can be separated.

Figure 4 illustrates the effect of the elution range on the peak capacity. As shown, many more compounds can be resolved with a larger elution range, although there is also a proportional increase in the analysis time.

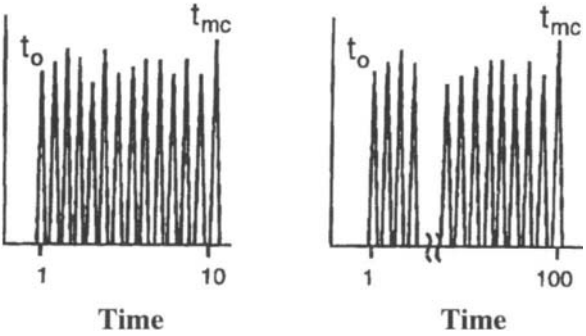


Fig. 4. Effect of the elution range (t_{mc}/t_o) on the peak capacity and analysis time.

A micellar EKC separation of neutral alkylphenones is shown in Fig. 5. The first (differential) peak corresponds to the elution of the solvent marker (t_o), whereas the elution of the last compound, dodecanophenone (a very hydrophobic molecule), provides a reasonable estimate of t_{mc} that can be refined if necessary using the homolog method noted earlier.

Importantly, the elution order in Fig. 5 is from lowest to highest homolog, the same as in reversed phase liquid chromatography (RPLC). For neutral compounds, we expect the elution order and methylene electivity (α_{CH_2}) to be similar in MEKC and RPLC because both employ a polar mobile phase and a nonpolar stationary phase into which analytes may partition. With charged compounds, significant differences in selectivity between MEKC and RPLC may be observed due to the additional charge-dependent transport (electrophoretic motion) and electrostatic interactions with the stationary "phase" that they experience in EKC.

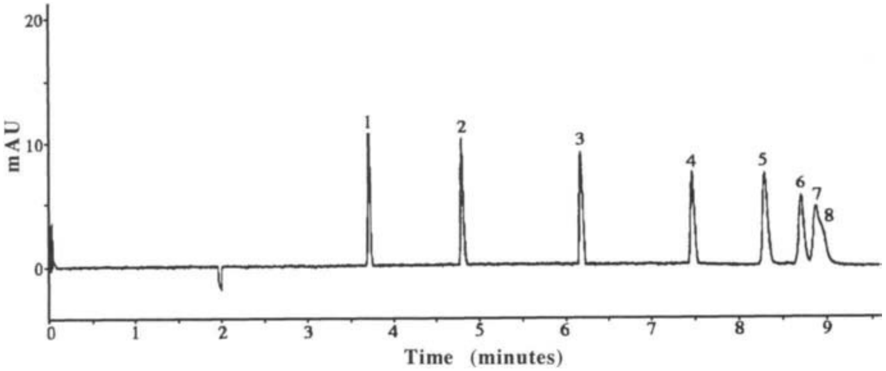


Fig. 5. Micellar EKC separation of neutral alkylphenones. Experimental conditions: 50 mM sodium dodecyl sulfate (SDS); 10 mM phosphate buffer (pH 7.0); capillary 30 cm from injection to detection, 1 s hydrodynamic injection, applied voltage 15 kV, operating current < 35 μA; detection wavelength, 254 nm. Peaks: 1, acetophenone; 2, propiophenone; 3, butyrophenone; 4, valerophenone; 5, hexanophenone; 6, heptanophenone; 7, octanophenone; 8, dodecanophenone.

3. Overview of Separation Mechanisms

It is important to appreciate that the separation of charged solutes by EKC involves a combination of chromatographic and electrophoretic separation mechanisms. This results in biases or differences in migration behavior for various types of analytes; such biases do not normally exist in conventional chromatographic techniques such as LC or GC. For example, in LC the mobile phase velocities of cationic, neutral, and anionic compounds are equal, whereas in EKC they are not, due to the additional electrophoretic velocity component of the cations and anions. Although our focus here is the chromatographic aspects of EKC, one should not forget the important role played by electrophoretic considerations.

Perhaps the best way to view any of the numerous possibilities in EKC is to determine the role of the interactive species (X, Eq. 3) in the separation. This will depend primarily on the presence and magnitude of electroosmotic flow. Electroosmotic flow is useful in EKC because it provides the bulk transport that is necessary to guarantee flow of all species past the detector. For simplicity, we consider two limiting cases: (i) electroosmotic flow that is sufficient to transport all analytes toward the detector regardless of their charge ($\mu_{eo} > |\mu_{ep,\text{ all ions}}|$); and (ii) no electroosmotic flow ($\mu_{eo} = 0$).

3.1. Separations with Electroosmotic Flow

If the electroosmotic flow is from anode to cathode, the elution order in CE is cations, neutrals, and anions, and vice-versa if the flow is from cathode to anode (see Chapter 1). Because the migration times of most, if not all, substances are already small due to large electroosmotic flow, the interactive species chosen is usually one that opposes electroosmotic flow (*i.e.*, its electrophoretic velocity is upstream), although its *net* velocity is normally still downstream ($\mu_{eo} > |\mu_{ep,mc}|$). In this context, the interactive species acts in a "retentive" fashion, *i.e.*, it serves to increase the migration time and resolution by acting as a pseudo-*stationary* phase. The buffer medium, of course, serves as the *mobile* phase.

3.2. Separations without Electroosmotic Flow

Conventional CE separations without electroosmotic flow are uncommon because two of the three kinds of potential molecules in a sample (neutral and either anionic or cationic species) would not migrate to the detector. With EKC, this problem is alleviated somewhat by the association of the analytes and the interactive species (X, Eq. 3), which now serves as a mobile phase and tends to transport all analytes toward the detector, provided that X is sufficiently mobile and the analyte•X associations are sufficiently strong. Obviously, the aqueous buffer solution, immobile by definition, now serves as a *true stationary* phase. Moreover, if the detector is located near the *cathodic* end of the capillary, X needs to be a rapidly migrating *cationic* species, and vice-versa. Assuming the associations of all analytes with X are sufficiently strong, the order of analyte migration is cations, neutral, and anions with cathodic detection and anions, neutral, and cations with anodic detection.

3.3. Description of Interactive Species

Several types of interactive species can be employed individually or in combination to facilitate the separation of both neutral and charged compounds: ion-pairing agents (Nishi *et al.*, 1989a,c; Terabe and Isemura, 1990b; Lukkari *et al.*, 1992a; Ng *et al.*, 1992a; Terabe, 1992), metal ions (Zhang *et al.*, 1992), ligands (Kuhn and Hoffstetter-

Kuhn, 1992; Kuhn *et al.*, 1992), surfactants (Terabe *et al.*, 1984; Otsuka *et al.*, 1985a,b; Terabe *et al.*, 1985a; Nishi *et al.*, 1989c) and mixtures of surfactants (Wallingford *et al.*, 1989; Little and Foley, 1992), bile salts (Terabe *et al.*, 1989a; Cole *et al.*, 1991), cyclodextrins (Terabe *et al.*, 1985b; Kuhn *et al.*, 1992), and even some types of polymers (Terabe and Isemura, 1990a).

3.3.1. Ion-pairing

Ion-pairing agents can be used to slow the migration of poorly retained ionic species (Nishi *et al.*, 1989a,c; Terabe and Isemura, 1990b; Lukkari *et al.*, 1992a; Ng *et al.*, 1992a) by the equilibrium formation of neutral (n = 1) and, possibly, charged complexes (n > 1).

$$A^+ + nX^- \rightleftarrows AX_n^{-(n-1)} \qquad K_{ip} = \frac{[AX_n^{\pm}]^{n\pm 1}}{[A^+][X^-]^n} \tag{15}$$

Assuming n = 1, the effective mobility of the analyte is

$$\mu_{ep,A^+,eff} = \left(\frac{1}{1+K_{ip}[X]}\right)\mu_{ep,A^+} \tag{16}$$

since the mobility of the neutral ion-pair is zero by definition.

The greatest shifts in migration time will occur when both the mobility of the ion-pairing agent and the formation constant are large. Selectivity changes (relative shifts in migration times of analytes) are more difficult to predict but can be very dramatic as the ion-pairing agent is switched. Potential ion-pairing agents for anionic analytes include: (i) di- and trialkylamines (*e.g.*, octyldimethylamine), (ii) tetraalkylammonium salts such as tetramethylammonium chloride, tetrabutyl ammonium hydrogen sulfate (Ng *et al.*, 1992a), and (iii) cetyltrimethylammonium bromide (CTAB), (iv) optically active amines such as quinine, quinidrine, cinchinidine; and (v) polymer cations such as diethylaminoethyl (DEAE)-dextran or poly(diallyldimethylammonium chloride) (Terabe and Isemura, 1990b). For cationic analytes, potential reagents include (i) inorganic agents such as sodium perchlorate, (ii) alkylphosphate salts (*e.g.*, sodium butyl phosphate), (iii) alkyl sulfonates such as sodium octanesulfonate or benzene sulfonic acid (ionized at pH >3), (iv) alkyl sulfates such as sodium dodecyl sulfate (SDS), and (v) optically active compounds such as di-*n*-butyltartrate and 10-camphorsulfonic acid. Surfactants such as CTAB and SDS can be used as ion-pairing agents as long as their concentration is kept below the cmc, the critical micelle concentration.

3.3.2. Hydrophobic interaction

Although little research has been conducted in this area, the same interactive species that form ion-pairs with ions of opposite charge will also interact with neutral compounds via hydrophobic interactions (Eq. 3). (Interactions with ionic species of like charge is unlikely due to electrostatic repulsion).

$$A + nX^{-} \rightleftarrows AX_n^{n-} \quad (n \geq 1) \qquad K_{hy} = \frac{[AX_n^{n-}]}{[A^{+}][X^{-}]^{n}} \tag{17}$$

Assuming n = 1, the effective mobility of the *neutral* analyte is determined by the mobility of the hydrophobic-interaction generated complex:

$$\mu_{ep,A,eff} = \left(\frac{1}{1+K_{hy}[X]}\right) \mu_{ep,AX^{-}} \tag{18}$$

Hydrophobic interactions as depicted in Eq. 17 may turn out to be extremely useful for the separation of compounds that bind too strongly to micelles and thus co-elute at t_{mc}. For example, even when the maximum concentration of an organic modifier is employed in an MEKC separation, very hydrophobic compounds such as higher alkylbenzenes, alkylphenones, and polyaromatic hydrocarbons (PAH) with four or more rings (including the buckminsterfullerenes) will still co-elute at t_{mc}, assuming that they are even soluble in the running buffer. Shown in Fig. 6 is an HI-EKC separation of dodecanophenone and tetradecanophenone that has not been achieved in MEKC with any surfactant system. Because the 50% acetonitrile used in conjunction with the 50 mM SDS is well above the concentration needed to inhibit micelle formation, the separation in Fig. 6 is the result of hydrophobic interactions between the analytes and SDS monomers (Ahuja and Foley, 1994b).

Although the resolving power of HI-EKC will generally be much larger for neutral analytes if the interactive species serves as a *stationary* phase, the separation of five PAHs (benzo[ghi]perylene, perylene, pyrene, 9-methylanthracene, and naphthalene, in order of elution) was achieved using 25 mM tetrahexylammonium perchlorate (50% acetonitrile) as a *mobile* phase (Walbroehl and Jorgenson, 1986). Note that in Fig. 6, dodecanophenone and tetradecanophenone differ by two methylene groups, whereas the five PAHs differ by at least an aromatic ring.

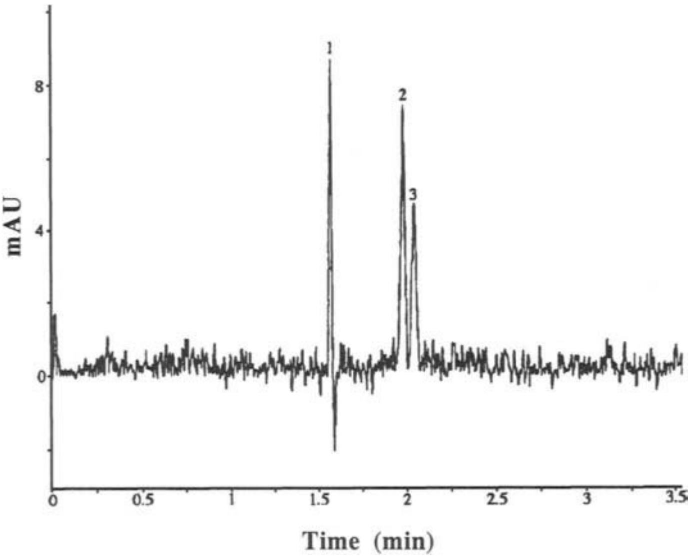


Fig. 6. Separation of very hydrophobic solutes using hydrophobic interaction EKC (EKC with charged surfactants but no micelles). Experimental conditions: applied voltage, 30 CV; operating current, 106 mA; capillary length, 30 cm from injection to detection; hydrodynamic injection time, 1 s; surfactant concentration, 50 mM SDS with 50% acetonitrile, pH 7.0 sodium phosphate buffer. Peak identification: 1 t_0 marker (solvent artifact); 2, dodecanophenone (C_{18}); 3, tetradecanophenone (C_{20}).

3.3.3. Complexation

For analytes that can form complexes, the addition of metal ions and/or ligands to the mobile phase can yield improved separations. As with the various ion-pairing examples above, the mobility of metal ion-ligand complexes will be significantly different from the mobility of the analyte itself. More importantly, the complexation constants are highly dependent on the metal-ligand ion combination, and this results in good selectivity.

Assuming that the analyte is a *metal ion* of unspecified charge that binds to no more than 2 ligand species (Eqs. 19 and 20),

$$M + L \rightleftarrows ML \qquad K_{f1} = \frac{[ML]}{[M][L]} \tag{19}$$

$$ML + L \rightleftarrows ML_2 \qquad K_{f2} = \frac{[ML_2]}{[ML][L]} \tag{20}$$

the effective mobility is

$$\mu_{ep,M,eff} = \left(\frac{1}{1+K_{f1}[L]+K_{f1}K_{f2}[L]^2}\right)\mu_{ep,M} + \left(\frac{K_{f1}[L]}{1+K_{f1}[L]+K_{f1}K_{f2}[L]^2}\right)\mu_{ep,ML} + \left(\frac{K_{f1}K_{f2}[L]^2}{1+K_{f1}[L]+K_{f1}K_{f2}[L]^2}\right)\mu_{ep,ML2} \tag{21}$$

where M is the metal-ion analyte, [L] is the molar concentration of ligand, and the K_fs are the formation constants of the metal ion/ ligand complex. Similar, albeit longer, expressions can be written when the analyte is capable of binding more than two ligands. When a 1:1 complex is formed, the equation for the effective mobility is analogous to Eqs. 4 and 8 for neutral and charged analytes, respectively. Potential ligands that could be used in the separation of metal ions, include the dithiocarbamic acids, β-diketones, 8-hydroxyquinoline, and β-ketoamines (Gurira and Carr, 1982; Nickless, 1985).

Alternatively, if the analytes are ligands that will form complexes with a given metal ion, a useful approach to improve their separation is to add a small amount of that metal ion to the running buffer. Besides changing the effective mobility of a ligand, the formation of a complex sometimes enhances its detection.

With this approach, the effective mobility of a ligand is

$$\mu_{ep,L,eff} = \left(\frac{1}{1+K_{f1}[M]+K_{f1}K_{f2}[M][L]}\right)\mu_{ep,L} + \left(\frac{K_{f1}[M]}{1+K_{f1}[M]+K_{f1}K_{f2}[M][L]}\right)\mu_{ep,ML} + \left(\frac{K_{f1}K_{f2}[M][L]}{1+K_{f1}[M]+K_{f1}K_{f2}[M][L]}\right)\mu_{ep,ML2} \tag{22}$$

where L is the analyte ligand, M is the metal-ion, and the K_fs are the formation constants of the metal ion/ligand complex. If $K_{f2} < K_{f1}$ and the concentration of metal ion is great relative to the concentration of ligand ([M]/[L] < 1%), Eq. 22 can be simplified to

$$\mu_{\mathrm{ep,L,eff}} \approx \left(\frac{1}{1 + K_{\mathrm{fl}}[\mathrm{M}]}\right) \mu_{\mathrm{ep,L}} + \left(\frac{K_{\mathrm{fl}}[\mathrm{M}]}{1 + K_{\mathrm{fl}}[\mathrm{M}]}\right) \mu_{\mathrm{ep,ML}} \qquad (22a)$$

The choice of metal ion(s) will, of course, depend on the sample as well as on its compatibility with the pH and buffer ions. For neutral-to-alkaline pH separations, the majority of transition metal ions must unfortunately be excluded due to the formation of insoluble metal hydroxides. For example, in contrast to the frequent use of silver ion in LC ("argentation chromatography") to enhance the separation of unsaturated and aromatic compounds, it is not practical to do so in EKC. Even at acidic pH, some metal ions may form precipitates with common buffer anions (*e.g.*, silver phosphate).

Another potential approach in EKC, designed previously for the separation of optical isomers in LC, involves the addition of *both metal ions and ligands*, *i.e.*, labile metal ion chelates, to the mobile phase (Karger *et al.*, 1980; Lam, 1984; Wainer, 1986; Armstrong, 1987a,b). Optically active analytes can form ternary diastereomeric complexes which can then be resolved. The most commonly used metal ion/ligand combination consists of copper (II) and a chiral amino acid such as L-proline, although L-2-alkyl-4-*n*-alkyldiethylenetriamine complexes of zinc and other metals have also been used successfully (Karger *et al.*, 1980). To date, this approach has been used primarily in LC for the resolution of optical isomers of amino acids (Lam, 1984; Armstrong, 1987b).

Ligands can also be added to the mobile phase for the separation of nonionic species. An important class of ligands for this purpose are the cyclodextrins, either in their neutral native form or as a charged derivative (*e.g.*, carboxymethyl).

Cyclodextrins (CDs) are cyclic oligosaccharides of D-glucose that, like aqueous micelles, have a hydrophilic exterior and a hydrophobic interior (cavity). They typically form 1:1 inclusion complexes with many small aromatic compounds, frequently yielding different mobilities for enantiomers as well as for structural isomers and other compounds. In a few instances with larger molecules, 1:2 inclusion complexes have been observed and 1:3 inclusion complexes have been postulated (Spino and Armstrong, 1987). Complex formation depends on the complementarity of the guest molecule and the CD, as well as other characteristics of the guest:

1. its size, or ability to fit within the hydrophobic cavity, and
2. its ability to H-bond with the hydroxyls at the base of the cavity.

Cyclodextrins are commercially available in three sizes—α, β, and γ—that consist of 6, 7, and, 8 glucose residues, respectively. Both native and derivatized forms have found significant applications. They were first employed in EKC by Terabe *et al.* in 1985 as a "moving" stationary phase by using the carboxymethyl derivative of the β-form (Terabe *et al.*, 1985b). More recently, CDs have been used in conjunction with SDS micelles to separate PAHs (Yik *et al.*, 1992b) and amino acids (Ueda *et al.*, 1992). Cyclodextrin-modified MEKC has also been effectively utilized for chiral separations (Nishi *et al.*, 1991; Ueda *et al.*, 1991). Additional applications may be found in Table 8 at the end of this chapter.

In CD-EKC, the effective electrophoretic mobility of an analyte is

$$\mu_{ep,A,eff} = \left(\frac{1}{1+K_f[X]}\right)\mu_{ep,A} + \left(\frac{K_f[X]}{1+K_f[X]}\right)\mu_{ep,A\bullet CD} \tag{23}$$

where $\mu_{ep,A\bullet CD}$ is the electrophoretic mobility of the analyte•CD complex. If the presence of an analyte molecule in the CD cavity results in only a negligible change in the mobility of the uncomplexed cyclodextrin ($\mu_{ep,CD}$), then Eq. 23 can be written as

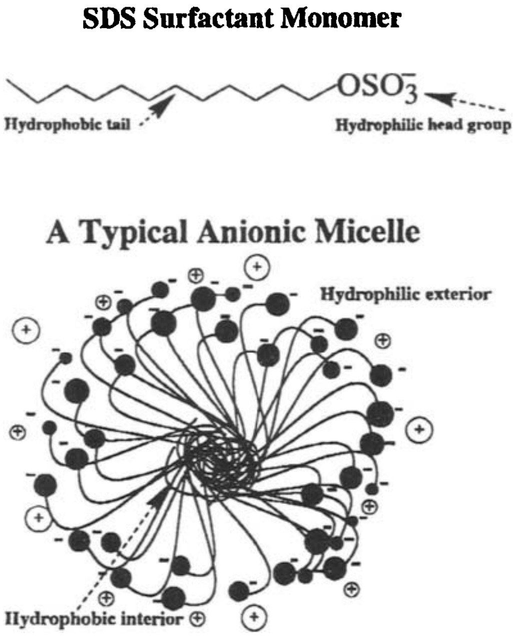
$$\mu_{ep,A,eff} = \left(\frac{1}{1+K_f[X]}\right)\mu_{ep,A} + \left(\frac{K_f[X]}{1+K_f[X]}\right)\mu_{ep,CD} \tag{23a}$$

Although neutral CDs have proven to be useful by themselves for chiral separations of *charged* enantiomers that usually *oppose* electroosmotic flow in EKC (Fanali, 1989; Fanali and Bocek, 1990; Snopek *et al.*, 1991; Sepaniak *et al.*, 1992; Ueda *et al.*, 1992; Yik *et al.*, 1992b), charged derivatives of the CDs (Terabe *et al.*, 1985b; Terabe, 1989), for reasons explained earlier, provide the mobility variation needed for *neutral* compounds. In a few cases, neutral CDs have been used in combination with MEKC (Nishi *et al.*, 1991; Ueda *et al.*, 1991). For more information, the interested reader is referred to a recent chapter on chiral separations (Okafo and Camilleri, 1993) as well as other chapters in the present work.

3.3.4. Micellar interactions

Last, but certainly not least, surfactants can also be employed as the interactive species. When used at concentrations below the cmc, they interact with the analytes in a similar fashion as the more traditional ion-pairing agents. When employed above the cmc, micelles are formed and a dramatic increase in both the migration time and separation mechanism is observed for nearly all analytes.

Micelle formation results from the dynamic aggregation of individual surfactant monomers. Each surfactant monomer generally consists of a hydrophilic head group and a hydrophobic tail (Fig. 7). The hydrophilic segment of a surfactant monomer is an ionic or very polar group, while the hydrophobic group is frequently a long chain hydrocarbon (8 carbons or more), and less frequently an oxygenated or halogenated hydrocarbon chain. The minimum concentration of surfactant monomers necessary to produce micelles is known as the *critical micelle concentration* (cmc). Once the cmc has been reached, surfactant monomers in aqueous media will be oriented such that their hydrophilic groups are facing the aqueous phase and their hydrophobic tails are facing away from it (Fig. 7). The number of surfactant monomers required to form a micelle is known as the aggregation number. Classification of surfactants is based on the charge characteristics of their hydrophilic head group. Table 1 lists and classifies some common surfactants along with their respective cmc values and aggregation numbers. Note that the data typically reported were obtained for aqueous solutions at near-infinite dilution,



SDS Surfactant Monomer

A Typical Anionic Micelle

Fig. 7. Cartoon of an anionic micelle.

and that ionic strength, organic solvents, temperature, etc. may have a significant effect on the cmc and aggregation number. It is also important to realize that micellization is a dynamic equilibrium and that micelles themselves have finite lifetimes (Almgren *et al.*, 1979).

Table 1. Common Surfactants in Micellar Electrokinetic Chromatography

	Critical Micelle Concentration (cmc), mM	Micelle Aggregation Number	Reference
Anionic			
Sodium dodecyl sulfate (SDS)	8.2	62	(Rosen, 1989)
Lithium dodecyl sulfate (LiDS)	8.9	63	(Rosen, 1989) (Mysels and Princen, 1959)
Potassium dodecyl sulfate (KDS)	7.8	N/A	(Rosen, 1989)
Magnesium dodecyl sulfate ($Mg(DS)_2$	1.2	95	(Nielsen and Foley, 1993)
Cationic			
Cetyltrimethylammonium bromide (CTAB)	1.3	78	(Love *et al.*, 1984)
Hexadecyltrimethylammonium bromide (DTAB)	0.026	169	Sigma Chemical Company, 1993)
Nonionic			
Polyoxyethylene (23) dodecanol (Brij 35)	0.1	40	(Love *et al.*, 1984)
Polyoxyethylene (4) dodecanol (Brij 30)	0.064	N/A	(Love *et al.*, 1984)

cont'd.

Table 1 cont'd.

	Critical Micelle Concentration (cmc), mM	Micelle Aggregation Number	Reference
Zwitterionic			
3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)	8	10	(Sigma, 1993)
N-dodecyl-N,N-dimethylammonium-3-propane-1-sulfonic acid (SB-12)	3	55	(Love *et al.*, 1984)

Micelles of ionic surfactants are by far the most widely utilized pseudophase in EKC. The micelles are part of a dynamic equilibrium that can be affected by many experimental parameters. Temperature can affect the cmc, but many common surfactants exhibit a minimum in their cmc between 20°C and 30°C and, thus, in those cases, the cmc is not very temperature dependent. On the other hand, the cmc is often fairly sensitive to electrolyte concentration. An increase in electrolyte concentration causes an increase in the aggregation number and a decrease in the cmc (Hinze and Armstrong, 1987). In general a lower cmc is better because there will be a lower free monomer concentration and this will reduce operating currents. From this point of view, SDS, the most popular surfactant at present for MEKC, is far from ideal, with a cmc of 8.1 mM in very dilute solutions.

In micellar EKC, the effective electrophoretic mobility of an analyte is

$$\mu_{ep,A,eff} = \left(\frac{1}{1+k}\right) \mu_{ep,A} + \left(\frac{k}{1+k}\right) \mu_{ep,A \bullet mc} \qquad (24)$$

where $\mu_{ep,A \bullet mc}$ is the electrophoretic mobility of the analyte•micelle complex. Usually the presence of an analyte molecule in the micelle results in only a negligible change in the mobility of the latter ($\mu_{ep,mc}$), and therefore Eq. 24 can be written as

$$\mu_{ep,A,eff} = \left(\frac{1}{1+k}\right) \mu_{ep,A} + \left(\frac{k}{1+k}\right) \mu_{ep,mc} \qquad (24a)$$

Bile salts such as sodium deoxycholate have a cholesterol-like backbone and form aggregates of 4–6 monomers that are sometimes loosely referred to as micelles. These 4–6 membered assemblies are chiral like their monomers, and have been used to separate several kinds of enantiomers (Cole *et al.*, 1991; Cole and Sepaniak, 1992). Bile salts are considerably more hydrophilic than conventional surfactants like SDS, and can be employed in situations where hydrophobic analytes would bind too strongly to SDS micelles because of their greater nonpolarity and aggregation number.

Shown in Fig. 8 is a comparison of the separation of several NBD-derivatized *n*-alkyl amines with two surfactants, SDS and sodium cholate. In part (a), in which SDS is employed as the stationary "phase", only methyl, ethyl, and butyl amine are completely resolved. In part (b), the addition of the trihydroxy bile salt sodium cholate improved the resolution of almost all of the components in the mixture by increasing the polarity of the stationary "phase" which resulted in lower k values for the NBD-derivatized *n*-alkyl amines. Reduction in k values through the use of bile salts improves resolution and can aid in the separation of hydrophobic analytes.

4. The Theory of EKC and its Relationship to CE, LC, and Electrochromatography

In this section we present a general theory for resolution in electrokinetic chromatography (EKC), hopefully one much broader than early works that focused on neutral separations based only on differences in retention (Terabe *et al.*, 1985a; Foley, 1990b). Other important considerations are described below:

1. We assume that one type of interaction of the analyte and interactive species is predominant. Secondary interactions can be neglected relative to the first, or they can be incorporated together with the primary interaction into one associative (binding) constant, *e.g.*, Eq. 3. For example, ion-pairing can be neglected in MEKC, since the analyte-micellar interactions are much stronger than ion-pairing (S. Terabe, personal communication; M. Khaledi, personal communication).
2. For purposes of discussion only, we assume a moderate to high electroosmotic flow such that the second phase (interactive species) is either stationary or moves more slowly toward the outlet (detector) side of the capillary than the buffer medium. In general, however, the theory described below will also be valid when the interactive species serves as the more mobile of the two phases (little electroosmotic flow).

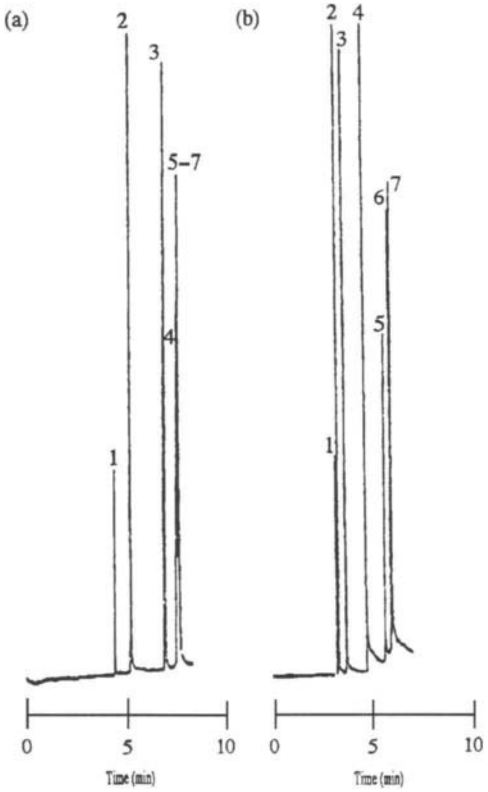


Fig. 8. Comparison of the MEKC separation of NBD-derivatized *n*-alkyl amines using (a) SDS and (b) SDS with an added bile salt (sodium cholate). Column dimensions: 50 cm × 25 μm (4 cm to detector); mobile phase: 0.01 M disodium phosphate, 0.006 M disodium borate, 0.05 M surfactant; applied voltage: 25 CV; detector: laser-based fluorescence (λ_{ex} 488 nm, λ_{em} 540 nm). Peaks: 1, methylamine-NBD, 2, ethylamine-NBD, 3, propylamine-NBD, 4, pentylamine-NBD, 5, octylamine-NBD, 6, dodecylamine-NBD, 7, quadradecylamine-NBD. (Reproduced with permission from Cole and Sepaniak, 1992.)

3. As the theory will show, from among the separation techniques of EKC, CE, LC, and electrochromatography, EKC is the broadest and most versatile.
4. Although we shall initially ignore secondary equilibria, *i.e.*, acid-base or other equilibria of the analyte that are unrelated to the association of the analyte with the interactive species (Eq. 3), we shall show how it can be accounted for at the end of our discussion.

4.1. Equation for Analyte Migration Time

The average velocity of a solute in EKC is simply the weighted average of the net solute velocity in the first and second phases (v_{mp} and v_{sp}). For consistency with the relatively well-developed nomenclature of MEKC, the most widely used separation mode within EKC, we use "mc" to denote the second phase, even when a non-micellar additive is employed in the buffer. Accordingly, we have

$$v_{avg} = F_{mp}\, v_{mp} + F_{mc}\, v_{mc}, \tag{25}$$

where F_{mp} and F_{mc} are the mole fractions of analyte in the aqueous and micellar phases. By expressing the mole fractions in terms of the solute retention factor ($k = n_{sp}/n_{mp}$), and by showing explicitly (Eq. 25a) the electroosmotic and electrophoretic velocity components (v_{eo} and v_{ep}) of v_{mp},

$$v_{avg} = \left(\frac{1}{1+k}\right)(v_{ep,A} + v_{eo}) + \left(\frac{k}{1+k}\right)(v_{ep,mc} + v_{eo}) \tag{25a}$$

Eq. 25a can be converted to (assuming a non-zero electroosmotic flow)

$$t_R = \left(\frac{(1+k)}{1 + \mu_r + t_o/t_{mc}\, k}\right) t_o \tag{26}$$

where t_R, t_o, and t_{mc} are the observed migration times of the analyte, first phase, and second phase. The quantity μ_r is the electrophoretic mobility of the "uncomplexed" analyte relative to electroosmotic flow ($\mu_r = \mu_{ep,A}/\mu_{eo}$); for moderate to high electroosmotic flow, $-1 < \mu_r < 1$. For neutral solutes, μ_r is zero since μ_{ep} is zero.

4.2. Equation for Retention Factor

Equation 26 may be solved for the retention factor to yield

$$k = \frac{t_R\,(1 + \mu_r) - t_o}{t_o(1 - t_R/t_{mc})}\,, \tag{27}$$

a more useful expression than Eq. 7 for the measurement of k from migration times. Note that the relative electrophoretic mobility of the analyte (μ_r) must be known or measured prior to the measurement of k in the presence of surfactant monomers but in the absence of micelles (Vindevogel and Sandra, 1992).

4.3. Master Resolution Equation (MRE)

The above equation for the migration time can be employed to derive a "master resolution equation" (MRE) that is valid for CE, EKC, electrochromatography, and LC (Foley, manuscript submitted). Using the commonly accepted definition of resolution (Eq. 10), and the number of theoretical plates (Foley, 1991), the following equation can be derived for the resolution of two solutes:

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{(1 - t_o/t_{mc})(k_2 - k_1) + (\mu_{r,1} - \mu_{r,2}) + (\mu_{r,1}\,k_2 - \mu_{r,2}\,k_1)}{1 + t_o/t_{mc}k_1k_2 + (1+t_o/t_{mc})\,\dfrac{(k_1+k_2)}{2} + \dfrac{(\mu_{r,1} + \mu_{r,2} + \mu_{r,1}k_2 + \mu_{r,2}k_1)}{2}}\right) \tag{28}$$

where, for simplicity, we have assumed Gaussian peak shapes and $N_1 = N_2 = N$.

To the best of our knowledge, the above equation is the first expression to appear that predicts the resolution resulting from simultaneous differences in retention (k) and relative electrophoretic mobility ($\mu_r = \mu_{ep}/\mu_{eo}$) of two solutes. It shows that resolution is proportional to: (i) differences in k; (ii) differences in μ_r; and (iii) a cross term that is positive, negative, or zero depending on whether the effects of k and μ_r on resolution are cooperative, neutral, or in opposition to one another.

4.3.1. Simplification of the master resolution equation

The denominator of the above MRE can be simplified by defining the arithmetic means k_{avg} and $\mu_{r,avg}$, and using the approximations

(i) $k_1k_2 \approx k_{avg}^2$ and (ii) $\frac{(\mu_{r,1}k_2 + \mu_{r,2}k_1)}{2} \approx \mu_{r,avg}\, k_{avg}$. This yields the simplified MRE

$$R_s \approx \frac{\sqrt{N}}{4}\left(\frac{(1 - t_o/t_{mc})(k_2 - k_1) + (\mu_{r,1} - \mu_{r,2}) + (\mu_{r,1}k_2 - \mu_{r,2}k_1)}{(1 + \mu_{r,avg} + t_o/t_{mc}k_{avg})\,(1 + k_{avg})}\right) \tag{28a}$$

Given the usual practice of expressing differences in chromatographic retention (k) in terms of selectivity ($\alpha = k_2/k_1$), Eq. 28a can be written

$$R_s \approx \frac{\sqrt{N}}{4}\left(\frac{(1 - t_o/t_{mc})(\alpha - 1)k_1 + (\mu_{r,1} - \mu_{r,2}) + (\mu_{r,1}k_2 - \mu_{r,2}k_1)}{(1 + \mu_{r,avg} + t_o/t_{mc}k_{avg})\,(1 + k_{avg})}\right) \tag{28b}$$

Although the separation mechanisms of CE and EKC are largely orthogonal, Eqs. 28a and 28b show that the simultaneous presence of both mechanisms is sometimes undesirable when only one of them contributes to the separation. When the separation is accomplished strictly by CE ($\mu_{r,1} \neq \mu_{r,2}$ and $k_1 = k_2 = k_{avg} > 0$), the EKC mode serves only to lower resolution by increasing each of the terms in the denominator of Eq. 28a (or 28b) via k_{avg}. Likewise, when the separation is accomplished strictly by EKC ($0 < k_1 < k_2$ and $\mu_{r,1} = \mu_{r,2} = \mu_{r,avg}$), the CE mode decreases resolution for analytes that move downstream ($\mu_{r,avg} > 0$) by increasing the first term in the denominator of Eq. 28a (or 28b). On the other, the CE mode increases resolution somewhat for analytes that move upstream ($\mu_{r,avg} < 0$) by decreasing the first term in the denominator.

Equations 28–28b unify the fields of LC, CE, EKC, and electrochromatography since they are applicable to all of these separation modes. The more familiar resolution equations for each of these techniques can be obtained by use of the appropriate conditions and assumptions, as we will show below.

4.3.2. Limiting cases of the master resolution equation

In the next four sections, we will consider several important limiting cases of the MRE (Eq. 28a), with no restrictions on the parameters except that we assume positive values for the elution range ($t_{mc}/t_o > 1$). This ensures that the second phase is either immobile ($t_{mc} = \infty$) or moving in the same direction as the mobile phase. The situation in which the second phase migrates in the opposite direction of the first is beyond the scope of our treatment and will be considered elsewhere.

4.4. Electrokinetic Chromatography (EKC).

If no restrictions are placed on any of the parameters in Eqs. 28, 28a and 28b, we have electrokinetic chromatography (EKC), a separation technique which, in its broadest form, utilizes both differences in retention (k) and relative electrophoretic mobility (μ_r) among solutes in achieving a separation. Included in this category are techniques that utilize micelles (MEKC), derivatized (ionic) cyclodextrins, ion-pairing agents, and other species that interact with and move more slowly than the analyte.

4.4.1. Resolution based solely on differences in retention $\{k_1 \neq k_2, \mu_{r,1} = \mu_{r,2} = \mu_r\}$.

While at first glance the number of compounds that fall into this category might appear to be low, this category includes the separation of enantiomers by chiral second phases (micelles, bile salts, ionic cyclodextrins).

Use of the above conditions and the assumption $k_{avg} = \frac{k_2+k_1}{2} \approx \sqrt{k_1k_2}$ in Eq. 28a yields

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{k_2-k_1}{1}\right)\left(\frac{k_2/k_1}{k_2/k_1}\right)\left(\frac{(1+\mu_r - t_o/t_{mc})}{1+t_o/t_{mc}k_{avg}{}^2 + (1+t_o/t_{mc})k_{avg} + \mu r(1+k_{avg})}\right) \quad (29)$$

Employing the usual definition of chromatographic selectivity, $\alpha = k_2/k_1$, the above is transformed into an equation that resembles the one developed by Terabe *et al.* (Eq. 30) for neutral solutes (Terabe *et al.*, 1985a).

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k_2}{1+k_{avg}}\right)\left(\frac{1-t_o/t_{mc}}{1+(t_o/t_{mc})k_{avg}}\right). \quad (30)$$

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k_2}{1+k_{avg}}\right)\left(\frac{1+\mu_r-t_o/t_{mc}}{1+\mu_r+(t_o/t_{mc})k_{avg}}\right). \quad (31)$$

The key difference between Eqs. 30 and 31 is that Eq. 31, by virtue of the μ_r term that appears in both the numerator and denominator of the last term, is valid for both charged and neutral solutes whereas Eq. 30 (Terabe's expression) is applicable only to neutral ones. Equation 30 is in fact a limiting case of Eq. 31, *i.e.*, the separation of neutral solutes by EKC (all μ_rs = 0).

It will be shown later via Eq. 31 that the resolving power of EKC is superior for analytes that swim downstream, assuming that their efficiencies are comparable.

4.4.2. *Resolution based solely on differences in electrophoretic mobility* $\{\mu_{r,1} \neq \mu_{r,2}, k_1 = k_2 = k\}$.

When applied to Eq. 28a, the above conditions yield

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{(1+k)(\mu_{r,1}-\mu_{r,2})}{1+(1-\sqrt{t_o/t_{mc}})^2+(1-\sqrt{t_o/t_{mc}})^2\,k+(1+k)\,\mu_{r,avg}}\right) \quad (32)$$

This equation shows the effect of non-selective solute-micelle interactions ($k_1 = k_2 = k$) and the associated finite elution range ($t_{mc}/t_o < \infty$) on resolution.

4.5. *Electrochromatography: Resolution obtained with a stationary second phase* $\{t_{mc}/t_o = \infty\}$.

Although a consistent definition for electrochromatography may not exist, we use it here to describe an electroosmotically-pumped, chromatographic separation that employs an *immobile* second phase that may or may not be bonded to a support packing or capillary. (This category thus includes those micellar EKC separations with an infinite elution range.)

Substitution of $t_{mc}/t_o = \infty$ ($t_o/t_{mc} = 0$) into the MRE yields

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{(k_2-k_1)+(\mu_{r,1}-\mu_{r,2})+(\mu_{r,1}k_2-\mu_{r,2}k_1)}{(1+\mu_{r,avg}+t_o/t_{mc}k_{avg})(1+k_{avg})}\right) \quad (33)$$

4.5.1. *Resolution achieved via differences in retention* $\{k_1 \neq k_2, \mu_{r,1} = \mu_{r,2} = \mu_r\}$.

Again, although few compounds may at first glance appear to fall into this category, the reader is reminded that this case includes the separation of enantiomers by chiral stationary phases.

Given the above constraints, Eq. 33 reduces to

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{k_2-k_1}{1+k_{avg}}\right) \quad (34)$$

An important result of this limiting case is that resolution does not depend on μ_r when $\mu_{r,1} = \mu_{r,2}$. Secondly, it would appear that to maximize R_s, we want k_{avg} to be as small as possible. But since k_{avg} is by definition constrained to be ≥ 0, it is detrimental to have small (near zero) values of k_{avg} since this also implies that $\Delta k = k_2 - k_1$ be small. This can be seen by substituting $\alpha = k_2/k_1$ into Eq. 31 to obtain

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\frac{k_2}{1 + k_{avg}} \tag{35}$$

For a given selectivity (α), it is clear that the resolution increases asymptotically with retention (k).

Although the resolution equation is the same for electrochromatography as for conventional GC and LC (Eq. 42), the corresponding equations for the migration (or retention) time are not.

$$t_R = \left(\frac{1 + k}{1 + \mu_r}\right) t_o \quad \text{(electrochromatography, case IIA)} \tag{36}$$

$$t_R = (1 + k)t_o \quad \text{(LC)} \tag{37}$$

Clearly, the relative electrophoretic mobility (μ_r) in the electrochromatography migration equation will affect the analysis time and hence the resolution per unit time. Also, there will likely be some dependence of N on μ_r and k; to consider that is beyond our scope here.

4.5.2. Resolution achieved via differences in electrophoretic mobility $\{(\mu_r)_1 \neq (\mu_r)_2\ ,\ k_1 = k_2 = k\}$.

With these constraints, the MRE becomes

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\mu_{r,1} - \mu_{r,2}}{1 + \mu_{r,avg}}\right) = \frac{\sqrt{N}}{4}\left(\frac{\mu_{ep,1} - \mu_{ep,2}}{\mu_{eo} + \mu_{ep,avg}}\right) \tag{38}$$

Since $\mu_{r,avg}$ may be positive or negative, the above equation shows that resolution can be made almost infinite by letting $\mu_{r,avg} \rightarrow -1$. This will maximize $\Delta v/v_{avg}$ which is important for resolving power, but again at the expense of migration time (below),

$$t_R = \left(\frac{1 + k}{1 + \mu_r}\right) t_o = \left(\frac{1 + k}{1 + \mu_r}\right)\left(\frac{L_d\, L_t}{\mu_{eo}\, V}\right), \tag{39}$$

where L_t is the total length of capillary and L_d is the capillary length from inlet to on-column detector.

Importantly, although chromatographic retention has no effect on R_s (Eq. 38) when $k_1 = k_2 = k$, it does affect the analysis time (Eq. 39), and hence the resolution per unit time. Also, as in Case IIA, there will likely be some dependence of N on μ_r and k to consider.

4.6. Capillary Electrophoresis (no second phase)

If the second phase is eliminated, then by definition $k_1 = k_2 = 0$; in addition, $t_o/t_{mc} = 0$. Substitution of these values into Eq. 28a yields a familiar equation previously derived for CE (Jorgenson and Lukacs, 1981).

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\mu_{r,1} - \mu_{r,2}}{1 + \mu_{r,avg}}\right) = \frac{\sqrt{N}}{4}\left(\frac{\mu_{ep,1} - \mu_{ep,2}}{\mu_{eo} + \mu_{ep,avg}}\right) \tag{40}$$

Although the expressions for resolution in case IIB and III are identical (Eqs. 38 and 40), the expressions for the migration time are different (Eqs. 39 and 41), as are the respective expressions for the resolution per unit time (not shown).

$$t_R = \left(\frac{1}{1 + \mu_r}\right) t_o = \left(\frac{1}{1 + \mu_r}\right)\left(\frac{L_d\, L_t}{\mu_{eo}\, V}\right), \tag{41}$$

Compared to the present result of Eq. 40, Eq. 32 can be thought of as a more general CE equation in which other, non-selective interactions (*i.e.*, chromatographic) are taken into account.

4.7. Separations without an Electric Field (Modern Column Chromatography: LC, GC, SFC).

Under these conditions, there is no electroosmosis and no electrophoretic motion and the second phase is stationary (all μs are 0; conversely, t_{mc} is infinite). Substitution of these values into Eq. 28a yields the well known fundamental resolution equation for conventional column chromatography {(Foley, 1991)}.

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{k_2 - k_1}{1 + k_{avg}}\right) = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\frac{k_2}{1 + k_{avg}} \tag{42}$$

4.8. Secondary Equilibria

In addition to binding with the interactive species (Eq. 3), analytes can interact with other substances in solution (S) or gain or lose protons in aqueous buffers. Acid-base and other secondary equilibria (Foley and May, 1987) can be taken into account by the defining of an apparent electrophoretic mobility $\mu_{ep}{}^*$ in terms of the electrophoretic mobility of each form of the analyte. For example, the apparent electrophoretic mobility for a monoprotic weak acid ($HA = H^+ + A^-$) is

$$\mu_{ep,HA}{}^* = \left(\frac{[H^+]}{[H^+] + K_a}\right) \mu_{ep,HA} + \left(\frac{K_a}{[H^+] + K_a}\right) \mu_{ep,A^-} \tag{43}$$

where $\mu_{ep,HA}$ is the electrophoretic mobility of the unionized acid (= 0 if the acid is neutral), and μ_{ep,A^-} is the electrophoretic mobility of the ionized acid, K_a is the acid dissociation constant and $[H^+]$ is the hydrogen ion concentration.

A similar procedure should also be used to define an apparent partition coefficient, $P_{wx}{}^*$, so that the retention factor can then be calculated using [defined by] $k = P_{wx}{}^* \beta$ (Eq. 7). Assuming the same monoprotic weak acid, the apparent partition coefficient is (Khaledi *et al.*, 1991)

$$P_{wx}{}^* = \left(\frac{[H^+]}{[H^+] + K_a}\right) P_{HA} + \left(\frac{K_a}{[H^+] + K_a}\right) P_{A^-} \tag{44}$$

where P_{HA} and P_{A^-} are the partition coefficient for the unionized and ionized forms of the acid, respectively. Equation 44 indicates that there is a sigmoidal relationship between $P_{wx}{}^*$ and pH; depending on the nature of the interactive species, $P_{wx}{}^*$ could increase or decrease as pH is increased.

For analytes that undergo acid-base equilibria, one additional consideration to be addressed is their potential shift in pK_a in MEKC due simply to the exposure of the analytes to micelles. The effective K_a of a compound in a micellar solution is (Khaledi and Rodgers, 1990)

$$K_{a,eff} = K_a \left(\frac{K_{mA-}\,[M] + 1}{K_{mHA}\,[M] + 1}\right), \tag{45}$$

where K_{mA^-} is the micellar binding constant for the ionized form of the acid, K_{mHA} is the micellar binding constant for the unionized form of the acid and [M] is the concentration of the micelles. As seen from Eq. 45, $K_{a,eff}$ will be a function of micelle concentration and the physical characteristics of the analyte and micelle that control binding to each other, K_m. More important than the absolute shift in pK_a is the relative shift in pK_a of two analytes with very similar aqueous pK_a values. When placed in a micellar solution, their pK_a values may shift by different magnitudes and/or in different directions. At a given pH their charge differences may be larger in a buffered micellar solution than in aqueous medium without micelles; consequently, the selectivity will be greater in MEKC than CE for these analytes. Conversely, two solutes with very different pK_a values in aqueous solution may have similar pK_a values in a micellar solution, and this would result in a loss of selectivity (Khaledi *et al.*, 1991).

4.9. Comparison of EKC with CE, LC and Electrochromatography

Shown in Table 2 is a summary comparison of the four techniques, with some additional comments provided below; a more detailed comparison is beyond the scope of this chapter.

Assuming $1 < t_{mc}/t_0 < \infty$, and identical values for other variables, a direct comparison of Eqs. 31 and 42 shows that resolution will always be greater with conventional column chromatographic techniques (LC, GC, etc.) than with EKC. Why then, should one bother with EKC? The answer is that all factors other than t_{mc}/t_0 are <u>not</u> equal. Many solutes, due to their limited volatility and/or thermal stability, are not amenable to GC. Moreover, selectivities in EKC, although sometimes similar to reversed phase liquid chromatography (RPLC), can also be quite unique. Furthermore, much higher column efficiencies can be achieved in EKC than in LC under average conditions, *i.e.*, 50,000 to 400,000 for EKC vs. 5,000 to 20,000 for LC. Finally, as methods for increasing the elution range beyond the typical range of $3 < t_{mc}/t_0 < 8$ are discovered and become widely practiced, the superior resolving power of EKC will become more obvious.

As shown in Table 2, the separation of charged solutes by EKC involves a combination of chromatographic and electrophoretic separation mechanisms that result in potential biases manifested in the differences in migration behavior for various types of analytes. Such biases do not normally exist in conventional chromatographic techniques such as LC. For example, in LC the mobile phase velocity of cationic, neutral, and anionic compounds are equal, while in EKC, CE, and electrochromatography they are different due to the additional electrophoretic velocity component of the cations and anions. Moreover, although both chromatographic and electrophoretic separation mechanisms are operative for charged analytes in EKC and electrochromatography, these techniques are not truly multidimensional since the resolution provided by either separation mechanism can be affected by the other (enhanced <u>or</u> diminished). If the resolution provided by one mechanism was somehow unaffected by the other, then

Table 2. Summary Comparison of Electrokinetic Chromatography (EKC), Electrochromatography, Capillary Zone Electrophoresis (CZE), and High Performance Liquid Chromatography (HPLC).

	EKC	Electrochromatography	CZE	HPLC (reversed phase)
Efficiency ranking (1=best)	2	3	1	4
Elution range	Usually finite, < 10	Infinite	Not applicable	Infinite
General elution problem	Yes, when elution range is large.	Yes	Not applicable	Yes
Ease of gradient elution	– – –	– – –	Not applicable	+++
Separation parameters to manipulate	N, α, μ_r, k, t_0/t_{mc}	N, α, μ_r, k	N, μ_r	N, α, k
Velocity of analytes in mobile phase	$v_+ \neq v_- \neq v_0 = v_{eo}$	$v_+ \neq v_- \neq v_0 = v_{eo}$	$v_+ \neq v_- \neq v_0 = v_{eo}$	$v_+ = v_- = v_0 = v_{eo}$
Optimum column diameter for separation (open tubular)	50–200 μm	2–10 μm	50–200 μm	2–10 μm
<u>Separation mechanisms</u>				
chromatographic (α)	Yes	Yes	No	Yes
electrophoretic ($\Delta\mu_{ep}$)	Yes	Yes	Yes	No
Preferred method for changing chromatographic selectivity (neutral solutes)	Change surfactant and/or counter ions	Change organic modifier?	Not applicable	Change organic modifier

cont'd.

Table 2. cont'd.

	EKC	Electrochromatography	CZE	HPLC (reversed phase)
Chromatographic selectivity is a function of retention (k)	No if k varied via [surf]; Yes if k varied with organic solvent	No if k varied via [surf]; Yes if k varied with organic solvent	Not applicable	Yes
Primary method(s) of controlling retention (k)	phase ratio β ([surf]), partition coefficient K	phase ratio β ([surf]), partition coefficient K	Not applicable	partition coefficient K
Comments	Limited separation window analogous to size-exclusion chromatography	Packed capillary mode should also be considered.	Cannot separate neutral solutes; efficiency highest for large molecules	Packed column mode preferred
Temperature control	−−−	−−−	−−−	+++

EKC and electrochromatography would qualify as novel multidimensional techniques in which both separation mechanisms operated simultaneously.

A recent example of competing chromatographic and electrophoretic mechanisms appeared in a comparison of the separation of alkylaromatic sulfonates by CE and MEKC (Desbene *et al.*, 1992). Using CE, with only an electrophoretic separation mechanism, the elution order was C_{12}, C_{10}, C_8, C_7, C_6, C_5, C_4, C_3, and C_2, where the subscript refers to the carbon number of the alkyl chain. Using MEKC, with both electrophoretic and chromatographic separation mechanisms operating simultaneously and competitively, the elution order was C_4, C_3, C_5, C_2, C_6, C_7, C_8, C_{10}, and C_{12}. Importantly, the first 4 compounds eluted in MEKC were not as well-resolved because the chromatographic and electrophoretic separation mechanisms were in opposition to one another. Once the chromatographic mechanism began to dominate ($C_6 - C_{12}$), the resolution was much better.

5. Optimization of Separations in EKC

"Optimization" means different things to different scientists. In the present section, we shall focus on optimization via fundamental principles. In a later section, we will summarize results of empirical studies, some of which are quite elegant. The advantage of fundamental optimization over empirical strategies is that the former can be applied to any system, whereas the latter may be limited to a specific type of sample or set of experimental constraints. On the other hand, the latter may yield better results for a specific sample.

Since at least one other chapter in this book is devoted to the case where electrophoretic separation mechanisms predominate, we focus here on EKC separations via chromatographic mechanisms (Case A1), and we repeat Eq. 31 here for convenience.

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2}{1 + k_{avg}}\right)\left(\frac{1 + \mu_r - t_o/t_{mc}}{1 + \mu_r + (t_o/t_{mc})k_{avg}}\right) \qquad (31)$$

5.1. *General Overview*

With GC and LC (Eq. 42), it is frequently possible to optimize N, α, and k independently of one another. On the other hand, in EKC (Eq. 31) there are five parameters to consider— N, α, k, μ_r, and t_{mc}/t_o. Two of them (k and t_{mc}/t_o) are strongly linked, and another (μ_r) introduces a bias in k_{opt} (and possibly in N) for cationic, neutral, and anionic species.

In recent years there have been many investigations of how resolution and/or one or more of its components (N, α, etc. in Eq. 31) is affected by various experimental parameters in MEKC. Selectivity has been examined extensively, and the effects of applied voltage, pH, organic modifier, surfactant identity, and surfactant concentration are well-documented for neutral (Foley, 1990b; Little and Foley, 1992), ionizable (Khaledi *et al.*, 1991), and charged analytes (Terabe, 1992; Corran and Sutcliffe, 1993; Holland and Sepaniak, 1993). Other variables, such as k and t_{mc}/t_o, have been considered less frequently but are equally if not more important in some cases (Vindevogel and Sandra, 1992).

It is appropriate to summarize how the four main adjustable parameters in Eq. 31—N, α, k, and t_{mc}/t_o—affect resolution {a detailed quantitative discussion follows immediately}, assuming that any effects of the first three on each other can be neglected (Foley, 1990b). For N and α, as in conventional GC or LC (Eq. 42), an increase in either or both parameters results in a predictable increase in resolution. For k, however, in distinct contrast to LC or GC, an increase in k does not always lead to a corresponding increase in resolution. The functional dependence of R_s on k in Eq. 31 is such that an increase in k of a pair of solutes beyond an optimum value (see Eq. 57) results in a decrease in the resolution. Finally, an increase in t_{mc}/t_0 (infinite for LC, GC) always increases resolution, but at the expense of the analysis time.

5.2. Efficiency

Although resolution increases only by the square root of N, this parameter should not be ignored in EKC, particularly with regard to experimental conditions that exert adverse effects. Efficiencies of 50,000 to 200,000 plates are typically realized in EKC, somewhat lower than that usually achieved in CE due to some additional band broadening processes that we will discuss shortly.

Column efficiency, N, can be expressed more fundamentally as L_{det}/H, where L_{det} is the capillary length from inlet to detection, and H is the plate height or band spreading index (Giddings, 1991). Many phenomena contribute to the total plate height in EKC, including several factors common to CE (item 1, unavoidable; items 1–4, nonideal) that are discussed in more detail elsewhere in this book. These are listed in Table 3.

Table 3. Contributions to Plate Height in EKC

Unavoidable	*Nonideal*
1. Longitudinal diffusion	1. Wall interactions
2. Resistance to mass transfer	2. Thermal overload (excessive Joule heating)
	3. Extracolumn effects—sample introduction, detection
	4. Hydrostatic (laminar) flow during the separation (Grushka, 1991)
	5. Radial dependence of k (or K_f) due to thermal effects
	6. Polydispersity of interactive species

In many instances, nonideal effects unnecessarily limit efficiency in both CE and EKC and can often be reduced far below what is commonly achieved today via a judicious choice of experimental conditions. For example, hydrostatic flow can be avoided completely by careful filling and/or positioning of the inlet and outlet buffer reservoir levels.

$$H_{LD} = \frac{2\,D_{app}}{v_{eo}}, \tag{46}$$

where D_{app} is the apparent diffusion coefficient of the analyte, and v_{eo} is the linear velocity of electroosmotic flow. In EKC the apparent diffusion coefficient is

$$D_{app} = \left(\frac{1}{1+k}\right) D_A + \left(\frac{k}{1+k}\right) D_{AX} \tag{47}$$

or

$$D_{app} = \left(\frac{1}{1+K_f[X]}\right) D_A + \left(\frac{K_f[X]}{1+K_f[X]}\right) D_{AX} \tag{47a}$$

where D_A and D_{AX} are the diffusion coefficients of the free and bound analyte, respectively. In MEKC, $D_{A\bullet mc} \approx D_{mc} << D_A$ for small molecules and is sometimes neglected.

Several additional band-broadening phenomena have been postulated for EKC. Some are fairly well established, such as the resistance to mass transfer, which is well known from GC and LC. Others are more tentative, such as the radial dependence of k. It is beyond the scope of this chapter to provide a detailed description of each; instead, only the more significant ones are briefly summarized (Vindevogel and Sandra, 1992).

By resistance to mass transfer, we mean the band broadening due to three processes: (i) the finite rate of transport of analyte to the interactive species (*e.g.* intermicelle diffusion in MEKC); (ii) the finite rate of interaction of the analyte with the interactive species {*e.g.*, ion-pair formation, sorption/desorption (MEKC); inclusion complex formation (cyclodextrin-EKC); etc.}; and (iii) the finite rate of analyte transport in the mobile phase in the radial direction. All three components of the mass transfer plate height term are directly proportional to mobile phase velocity (the slower the rate, the larger the proportionality constant), and thus we can write

$$H_{RMT} = (C_i + C_{ii} + C_{iii})\, v_{eo} = C_{RMT}\, v_{eo} \tag{48}$$

The C_{iii} term, radial transport, is important whenever there is a radial dependence of one or more physical properties of the mobile phase such as viscosity or temperature.

In MEKC, there has been considerable debate on the importance of the various mass transfer terms, particularly the contribution of intermicellar transfer to plate height (C_i term). Sepaniak and Cole have reported that at surfactant concentrations close to the cmc, as the surfactant concentration is increased there is a dramatic increase in N (Sepaniak and Cole, 1987). They propose that as the surfactant concentration increases the number of micelles increases such that the distance between micelles decreases and improved mass transport results. But other researchers have presented evidence, both theoretical and experimental, that the observed effects under these conditions are due to overloading the micelles with solute. The second mass transfer mechanism (C_{ii} term) in MEKC is the partitioning of the solute into and out of the micelle (C_{ii}), or "micellar mass transfer." This source of band broadening was found to be virtually insignificant for SDS under the experimental conditions examined (Davis, 1989; Terabe *et al.*, 1989b), but could be important for other surfactant systems. Finally, the third mass transfer term (C_{iii}) arises from the radial dependence of k that is postulated to cause band broadening (Davis, 1989) occurs because of the slight, but well known temperature gradient that exists across a capillary due to Joule heating and heat transfer effects. Davis' treatment of this band broadening phenomenon shows the proper dependence of H on column radius as previously observed (Sepaniak and Cole, 1987).

Polydispersity effects in EKC, also linearly proportional to electroosmotic flow velocity, are thought to be relatively unimportant (Vindevogel and Sandra, 1992),

$$H_{pd} = C_{pd}\, v_{eo} \tag{49}$$

but may be observed when the species (X) that interacts with the analyte varies in its size, shape, or chemical structure, giving rise to local differences in P_{wx}, β, and/or $\mu_{ep,X}$. For example, at any given instant in an MEKC solution, a range of micelles exist in which the aggregation number varies significantly about an average. Or, perhaps an ion-pairing agent that is nominally sodium octanesulfonate contains significant amounts (> 1%) of shorter or longer chain sulfonates. Obviously, $C_{pd} = 0$ for a pure reagent or a solution of monodisperse (identical) micelles.

Although we have so far considered only intracolumn sources of band broadening in detail, a significant amount of extracolumn band broadening can occur due to the injection and detection processes. The broadening due to the former can be modeled for electrokinetic injection as

$$H_{inj} = \frac{l_{inj}^2}{12} + \frac{2\, D_{app}\, t_{inj}}{L_{det}} \tag{50}$$

and for hydrodynamic (pressure-induced) injection as

$$H_{inj} = \frac{l_{inj}^2}{12} + \frac{2\,D_{app}\,t_{inj}}{L_{det}} + \frac{r_c^6\,\Delta P_{inj}^2\,t_{inj}}{1536\,L_{det}\,L_{tot}^2\,\eta^2\,D_{app}} \tag{51}$$

where a third term was added to account for the broadening due to the laminar flow that occurs in hydrodynamic injection.

In Eqs. 50 and 51, l_{inj} is the length of the injection "plug" and t_{inj} is the time of injection. Plug length can be expressed as $v_{inj} \bullet t_{inj}$, where v_{inj} is the analyte velocity during injection. In electrokinetic injection, $v_{inj} = (\mu_{eo} + \mu_{ep})\,E$, and is thus analyte dependent; in hydrodynamic injection, $v_{inj} = \frac{r_c^2\,\Delta P_{inj}}{8\,L_{tot}\,\eta}$, and is thus independent of the analyte.

Limited numbers of studies have been done on the effects of injection technique on plate height in MEKC. One study on electrokinetic injection illustrated that N can be maximized by using low voltages and short injection times. This results in a narrow sample plug being introduced at the beginning of the column, and hence a better efficiency (Burton *et al.*, 1986b; Row *et al.*, 1987; Terabe *et al.*, 1989b). Obviously, the general principle that narrow injection plugs yield greater efficiencies also applies to hydrostatic and vacuum injection, and implies that short injection times are desirable with these methods as well. Unfortunately, restrictions on injection plug width limit the amount of sample that can be introduced into the capillary.

Band-broadening due to detection was first examined by Terabe and coworkers (1989b). More recently, Nielsen and Foley have derived the following useful expression (Nielsen and Foley, manuscript submitted):

$$H_{det} = \frac{l_{det}^2}{12\,L_{det}^3}\left(\frac{t_o\,(1+k')}{1+\mu_r+k'\left(\frac{t_o}{t_{mc}}\right)}\right)^2 \tag{52}$$

where l_{det} is the length of the detector window, and L_{det} is the length of the capillary from inlet to detector.

When all of the contributions to plate height are summed to get H_{total}, the result is

$$H_{total} = H_{inj} + H_{det} + \frac{2\,D_{app}}{v_{eo}} + (C_{RMT} + C_{\Delta T} + C_{pd})\,v_{eo} \tag{53}$$

Importantly, if we treat H_{inj} and H_{det} as constants for a given CE instrument and set of experimental conditions, Eq. 53 can be simplified to

$$H_{total} = A + \frac{B}{v_{eo}} + C\,v_{eo} \tag{54}$$

which is the same general relationship observed in GC and LC. This means that for every solute, there will be an optimum velocity at which H will be a minimum and N = L_{det}/H will be a maximum. By minimizing H, we can optimize N for a given length of capillary.

The theory described above for efficiency (plate height) in EKC is only in its infancy. Although qualitatively it is probably accurate, it is nevertheless difficult to predict the optimum velocity for a given sample. {To date, the velocities typically employed (not necessarily optimal) range from 0.5–4 mm/s.} Instead of making risky predictions, we summarize in Table 4 several experimental procedures, based on our experience, that will help the practitioner achieve maximum efficiency. In Table 5 the primary effects of common experimental variables on efficiency are reviewed.

Table 4. Suggestions for Obtaining the Most Efficiency in CE and EKC

1. The use of organic solvents should be kept to a minimum.
2. Sample solvent and running buffer should be matched as closely as possible.
3. Inlet and outlet buffer reservoir levels should differ at most by 0.5 mm and, ideally, should be exactly the same to avoid any hydrostatic flow effects (Grushka, 1991).
4. To minimize H_{inj} (Eq. 50 or 51), the length of the injection plug should not exceed 1% of the total capillary length, unless sample stacking is utilized. Injection times should be relatively short.
5. When choosing a buffer system and interactive species, select those that minimize the overall conductance or ionic strength.
6. For a given buffer system, keep the applied voltage at the upper limit of the linear portion of an Ohm's law plot (a graph of current vs. voltage for several voltages).
7. Capillary cuts should be as square as possible to avoid peak tailing, and samples should be filtered.
8. Interactive agents should be of the highest purity, in order to minimize H_{pd} (Eq. 49).
9. When possible, use field amplified sample stacking (FASS) (see 6. Recent Developments).
10. If the length of the detector-illuminated volume of the capillary can be varied, it should be made as short as possible (*e.g.*, ≤ 0.5 mm) to minimize H_{det} (Eq. 52).
11. Judicious choice of the interactive species and/or its counter ions can sometimes increase N significantly (see 6. Recent Developments). Although this effect has been reported often in CE, it has received little attention in EKC.

Table 5. Effect of Common Experimental Variables on Efficiency

Applied voltage, V_{appl}	As V_{appl} (E_{appl}) increases, N increases to a maximum and then begins to decrease as excessive Joule heating occurs.
Injection time, t_{inj}	N increases dramatically as t_{inj} decreases, but this also results in a decrease in the amount of sample introduced to the capillary.
Capillary radius, r_c	As r_c increases, N decreases due to increases in the radial mass transfer term(s) and, when hydrodynamic injection is employed, increases in laminar flow broadening.
Temperature, T	At low analyte velocities, N decreases with increasing T because of enhanced diffusion coefficients of the analytes. At higher velocities (mass transfer limited conditions), N increases with increasing temperature because of enhanced mass transfer due to larger diffusion coefficients.
Capillary length, L	For constant field strength, the efficiency of the separation is directly proportional to the capillary length; for constant applied voltage, it is independent of capillary length.

5.3. *Selectivity*

Selectivity in EKC is defined as the ratio of analyte partition coefficients (P_{wm}) or retention factors (k), the same as in GC and LC, and it is essentially the ability of a chromatographic system to separate two or more compounds that differ by one or more chemical groups. It is often convenient to distinguish between two types of selectivity, hydrophobic and polar group selectivity, depending on whether the functional groups that differ among the compounds being separated are nonpolar or polar.

Hydrophobic selectivity can be defined for quantitative purposes as the methylene selectivity, or the selectivity between adjacent homologs. It is most easily measured from the ratio of retention factors of adjacent homologs, or from the slope of a ln k vs. carbon number (homolog) plot. The hydrophobic selectivity of most EKC systems has not been extensively studied, and most, if not all, data obtained to date are with MEKC systems. Table 6 summarizes some results obtained with C_1–C_7 alkylphenones in a pH 7 phosphate buffer/SDS

Table 6. Methylene Selectivity ($\log_{10} \alpha_{CH_2}$) in MEKC

Surfactant identity and concentration	No organic modifier	15% MeOH (v/v)	15% MeCN (v/v)	15% 1-PrOH (v/v)
25 mM SDS	0.367 (±0.004)	0.349 (±0.009)	0.310 (±0.005)	0.314 (±0.005)
40 mM SDS	0.374 (±0.011)	0.353 (±0.009)	0.331 (±0.008)	0.311 (±0.005)
55 mM SDS	0.378 (±0.012)	0.343 (±0.011)	0.337 (±0.009)	0.311 (±0.005)
70 mM SDS	0.382 (±0.014)	0.356 (±0.013)	0.341 (±0.009)	0.325 (±0.005)

micellar system with various organic modifiers (Ahuja *et al.*, manuscript in preparation). The results show that hydrophobic selectivity is comparable to that achieved in reversed phase liquid chromatography (RPLC), typically 0.20–0.50 in $\log_{10} \alpha_{\text{-}CH_2\text{-}}$ units.

Polar group selectivity has been studied more extensively, but unfortunately no general conclusions can be drawn because of the difficulty in accurately predicting differences in P_{wm} for polar solutes under any given set of experimental conditions. Table 7 compares the group selectivity obtained with aqueous SDS and SDS/Brij 35 micellar solutions, using benzene as the parent compound (Eq. 55).

$$\alpha_{\text{group}} = \frac{k_{\text{benzene derivative}}}{k_{\text{benzene}}} \tag{55}$$

A more detailed discussion of selectivity is beyond the scope of this chapter. Some general principles can be outlined, however.

1. For neutral analytes, both nonpolar and polar group selectivity is independent of surfactant concentration, assuming there are no analyte-specific interactions with the buffered micellar media. For charged solutes, there is usually a slight to moderate dependence of polar group selectivity on surfactant concentration due to electrostatic effects.
2. Selectivity is independent of applied voltage, assuming that Joule heating is not excessive, *i.e.*, the temperature of the solution at all voltages is very similar.

Table 7. Comparison of Functional Group Selectivity (Eq. 55) for Brij 35/SDS and SDS Micellar Systems

Micellar			Benzene derivative			
medium	ϕ-$COCH_3$	ϕ-OH	ϕ-NO_2	ϕ-OCH_3	ϕ-CH_3	ϕ-Cl
SDS						
40 mM	1.601	0.501	1.274	1.601	2.750	3.560
60 mM	1.593	0.500	1.271	1.593	2.694	3.458
80 mM	1.575	0.494	1.262	1.575	2.691	3.450
100 mM	1.567	0.495	1.249	1.567	2.673	3.433
mean	**1.584**	**0.498**	**1.264**	**1.584**	**2.702**	**3.475**
% RSD	**0.992**	**0.706**	**0.888**	**0.992**	**1.23**	**1.65**
Brij 35 / SDS[a]						
30 mM	0.598	0.773	1.122	1.366	2.725	4.519
40 mM	0.598	0.808	1.127	1.363	2.667	4.408
50 mM	0.595	0.827	1.125	1.360	2.653	4.331
60 mM	0.598	0.858	1.128	1.347	2.615	4.229
mean	**0.597**	**0.816**	**1.126**	**1.359**	**2.665**	**4.372**
% RSD	**0.251**	**4.36**	**0.235**	**0.616**	**1.71**	**2.84**

[a] SDS concentration in mixtures of Brij 35 was 20 mM.

3. Selectivity in EKC can be varied by causing disproportionate changes in analyte partition coefficients. Analyte partition coefficients may be changed by adding organic modifier, changing the pH (ionizable solutes) or temperature, or by using mobile phase additives such as cyclodextrins, crown ethers, metal ions, or co-surfactants in addition to the primary interactive species (X). A less common but useful approach in MEKC is to change one or more aspects of the surfactant system: (i) the counter ion composition (*e.g.*, a blend of Na^+ and another cation instead of just pure Na^+); (ii) the surfactant itself (*e.g.*, dodecylsulfonate vs. dodecylsulfate); or (iii) the relative proportion of surfactants if a mixed surfactant system is employed.

Finally, although the a priori prediction of selectivity is not yet possible, it is easy to illustrate the importance of selectivity. Figure 9 illustrates the dependence of resolution on selectivity and the elution range. As selectivity increases, the resolution increases dramatically for a given elution range. Also, for a given selectivity, the resolution increases significantly as the elution range (t_{mc}/t_0) increases.

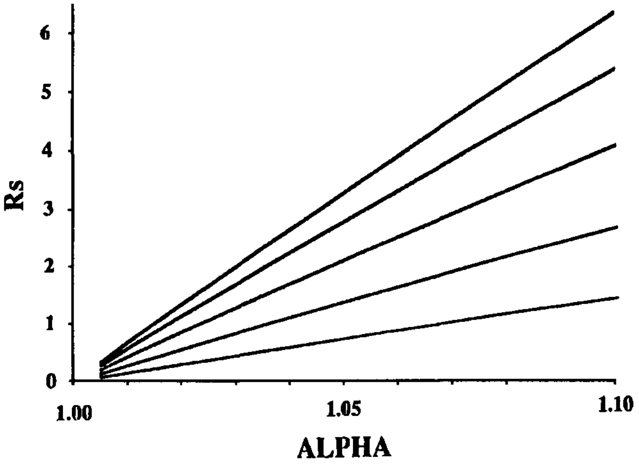


Fig. 9. Resolution as a function of selectivity (α) and elution range (t_{mc}/t_o). Values of t_{mc}/t_o for each curve (top to bottom): 100, 30, 10, 4, 2. Other conditions: $N = 100{,}000$; $k = k_{opt,Rs} = \sqrt{t_{mc}/t_o}$; and $\mu_r = 0$.

5.4. Retention

5.4.1. Theory

The optimum degree of analyte "retention" or binding of neutral solutes with the interactive species X was reported in 1990 (Foley, 1990b). Based on the EKC resolution equation for neutral solutes (Eq. 30) and assuming that N and α are independent of k, the theory linked quantitatively the optimum degree of retention with the elution range and established the existence of an optimum k range within which good resolution would be obtained. It also suggested upper and lower bounds for this range in terms of the retention needed for the best resolution and resolution per unit time (Eqs. 43 and 44).

$$k_{opt}\ (\text{for maximum } R_s) = \sqrt{\frac{t_{mc}}{t_o}} \tag{56}$$

$$k_{opt}\ (\text{for maximum } R_s/t_R) = \frac{-(1 - t_o/t_{mc}) + \sqrt{(1 - t_o/t_{mc})2 + 16(t_o/t_{mc})}}{4(t_o/t_{mc})} \tag{57}$$

If the same approach is employed, but Eq. 31 that is valid for charged solutes is used instead of Eq. 30 that is valid only for neutral solutes, then the following results are obtained:

$$k_{opt(Rs)} = \sqrt{\frac{t_{mc}}{t_o}(1 + \mu_r)} \tag{58}$$

$$k_{opt(Rs/tR)} = \frac{-(1 + \mu_r - t_o/t_{mc}) \pm \sqrt{(1 + \mu_r - t_o/t_{mc})^2 + 16(t_o/t_{mc})(1+\mu_r)}}{4(t_o/t_{mc})} \tag{59}$$

It is interesting that the optimum degree of interaction or binding is determined by both a chromatographic parameter, t_{mc}/t_o, and an electrophoretic parameter, μ_r. Note that the relative influence of these parameters will depend to a larger degree on the magnitude of electroosmotic flow.

In MEKC, as the retention factors (k) of solutes begin to exceed $k_{opt,Eq.\ 58}$, the resolution will suffer. This possibility exists because compounds with $k \gg k_{opt,Eq.\ 58}$ may elute close to or even co-elute with the micelle ($t_R = t_{mc}$). Such a situation is not encountered in GC or LC, where there is no upper limit in the separation window and, according to Eq. 42, no retention factor is too large for good resolution, although in practice the peak width and analysis time become excessive when $k > 20$.

For $\mu_r = 0$, the k_{opt} given by Eq. 59 ranges between 1.2 and 2 for t_{mc}/t_o values from 1 to infinity. For conventional column chromatographic methods such as GC, LC, and SFC, the optimum k for best R_s/t_R is 2 (Karger *et al.*, 1973).

Using the results of Eqs. 58 and 59, we can define an optimum range of the retention factors, *i.e.*, $k_{opt.\ range} = k_{opt}\ (R_s/t_R)$ to $k_{opt}\ (R_s)$. This range widens as the elution window (t_{mc}/t_o) becomes larger, and is consistent with the effect of t_{mc}/t_o on the peak capacity (Eq. 14). For near-infinite elution ranges ($t_{mc}/t_o \geq 1000$), $k_{opt}\ (R_s)$ is very large ($\geq \sqrt{1000} \approx 32$) and the upper limit for k may need to be lowered arbitrarily to keep analysis times reasonable (Eq. 17).

Alternatively, we can define optimum k ranges for the best R_s and R_s/time as the range over which the resolution and resolution/time are within 90% of their maximum values. Shown in Fig. 10 are optimum k ranges, expressed in normalized time units (t_R/t_o), for t_{mc}/t_o = 100, 30, 10, 4, and 2 (top to bottom). Although the magnitude of R_s depends on N and α, the k ranges themselves depend only on t_{mc}/t_o and the relative electrophoretic mobility of the analytes, and are independent of efficiency and selectivity. Visual inspection of Fig. 10 reveals that the optimum range of k is ca. 50 times wider for t_{mc}/t_o = 100 than for t_{mc}/t_o = 2. Moreover, for a constant t_o, the analysis time

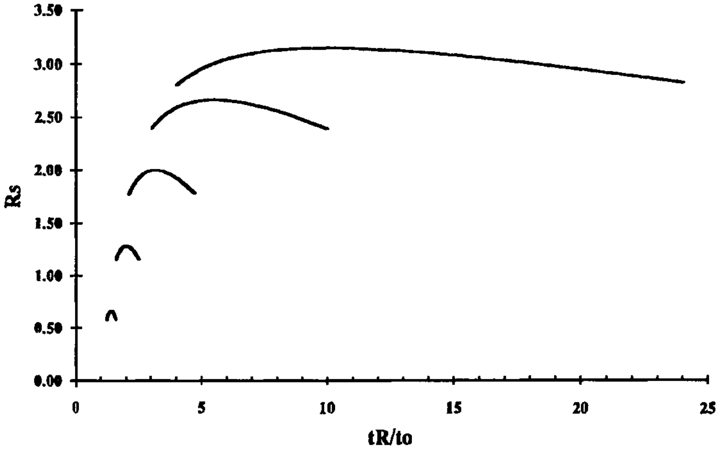


Fig. 10. Dependence of the optimum range of the retention factor (k) on the elution range (t_{mc}/t_o). Values of t_{mc}/t_o for the curves (top to bottom): 100, 30, 10, 4, 2. Other conditions: N = 100,000; $\alpha = 1.05$; and $\mu_r = 0$.

is about 12.5 times longer. Finally, in agreement with Fig. 9, the resolution increases significantly as the elution range (t_{mc}/t_o) increases.

Given the dependence of k_{opt} on μ_r shown in Eqs. 58 and 59, we can re-write Eq. 31 using $k = k_{opt}$ to emphasize the dependence of resolutionon μ_r.

$$R_{s,opt} = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_{opt}(\mu_r)}{1 + k_{opt}(\mu_r)}\right)\left(\frac{1 + \mu_r - t_o/t_{mc}}{1 + \mu_r + (t_o/t_{mc})\, k_{opt}(\mu_r)}\right). \tag{60}$$

The most important result of Eq. 60 is that a charge bias exists in the resolving power of EKC, unless there is a dependence of N on μ_r that exactly cancels the dependence of the "retention term" on μ_r (last two terms of Eq. 60). Assuming that the retention term is more dependent on μ_r than efficiency, it will be easier to separate analytes that "go with the flow" (Foley, 1992). In other words, when electroosmotic flow is from anode to cathode (the usual case in MEKC with SDS), cations will be better resolved than neutral species, and neutral species will in turn be better resolved than anionic compounds. Shown in Fig. 11 are calculated results for the retention term using μ_r = 0.8, 0.4, 0, –0.4, and –0.8 (assuming no dependence of N or α on μ_r).

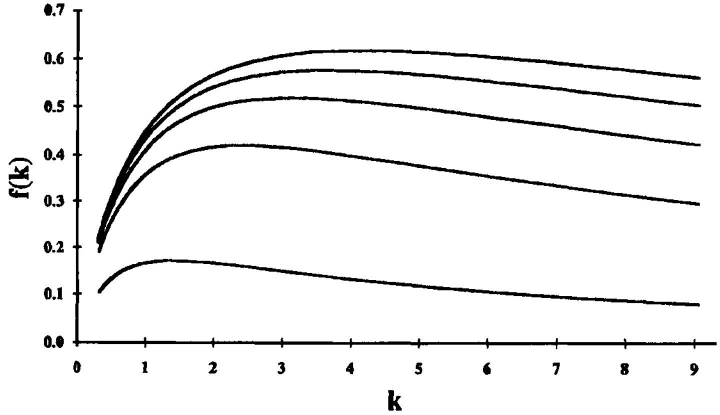


Fig. 11. Dependence of the retention terms in Eq. 60 on k and the relative electrophoretic mobility ($\mu_r = \mu_{ep,A}/\mu_{eo}$), illustrating the thermodynamic bias in the resolving power of EKC for analytes that swim upstream, "midstream", or downstream. Values of μ_r for the curves (top to bottom): 0.8, 0.4, 0, –0.4, –0.8.

After measuring or estimating the elution range, the optimization of retention (k) in MEKC can be accomplished by optimizing either (or both): (i) the phase ratio β by adjusting the surfactant concentration; or (ii) the analyte partition coefficients (P_{wm}s) by choosing a simple or mixed micellar system of appropriate hydrophobicity or modifying a readily available surfactant system (*e.g.*, SDS) with an organic solvent as necessary. With regard to (ii), although the main effect of organic solvent is to lower the polarity of the aqueous phase, many solvents will also cause changes in the micelles themselves. When possible, option (i) is preferred because of its minimal effect on selectivity (vide infra).

5.4.2. Control

The control of R_s and R_s/t_R can be accomplished by proper manipulation of any variable that controls retention, but typically it will be the surfactant concentration, organic modifier, or possibly the temperature. As we show below, k can be related directly to surfactant concentration, and an important consequence of is that the surfactant concentration that provides the best R_s or R_s/t_R can be predicted from theory when a couple of key surfactant and solute parameters are available or can be measured. In short, one easy way to optimize R_s or R_s/t_R is to optimize the surfactant concentration.

Equation 7 is an expression for the retention factor. The phase ratio (Eq. 6) can be written explicitly in terms of surfactant concentration [SURF], the critical micelle concentration (cmc), and the partial molar volume (V) of the surfactant.

$$\beta = \frac{V([\mathrm{SURF}] - \mathrm{cmc})}{1 - V([\mathrm{SURF}] - \mathrm{cmc})} \tag{61}$$

Substitution of Eq. 61 into Eq. 7 and solving for the surfactant concentration yields

$$[\mathrm{SURF}] = \frac{k + V\,\mathrm{cmc}\,(k + P_{wm})}{V\,(k + P_{wm})} \tag{62}$$

Equation 62 shows the one-to-one relationship between surfactant concentration and retention factor. Since the parameters on the r.h.s. of Eq. 62 are either experimentally measurable or physical constants, a priori prediction of an optimum surfactant concentration is possible for a given set of analytes. Assuming that $P_{wm} >> k$ (universally true except for very hydrophilic compounds), a convenient approximation to Eq. 62 is

$$[\mathrm{SURF}] \approx \frac{k}{P_{wm}V} + \mathrm{cmc} \tag{62a}$$

It is a simple matter to substitute either of the expressions for k_{opt} (Eqs. 58 or 59) into either Eq. 62 or 62a to obtain an expression for the optimum surfactant concentration. Since Eq. 62a is simpler, we use it to obtain the following:

$$[\mathrm{SURF}]_{\mathrm{opt,best\ Rs}} = \frac{k_{\mathrm{opt\ (Eq.\ 58)}}}{P_{wm}V} + \mathrm{cmc} \tag{63}$$

$$[\mathrm{SURF}]_{\mathrm{opt,best\ Rs/tR}} = \frac{k_{\mathrm{opt\ (Eq.\ 59)}}}{P_{wm}V} + \mathrm{cmc} \tag{63a}$$

Surfactant concentration cannot be varied over an infinitely wide range. The lowest usable concentration will be determined by the need for reproducible concentrations of micelles in order to obtain reproducible retention times. This lower limit will be dictated by the cmc and the precision with which it is known, but for our discussion we will assume it is 10% above the cmc, *i.e.*, $[\mathrm{SURF}]_{min} = 1.1 \times \mathrm{cmc}$. The upper limit of the surfactant concentration is likely to be governed by such adverse solution properties as high viscosity, or high current. From our own experience and that reported in the literature, the highest tolerable concentration for monovalent surfactants (SDS, CTAB, etc.) is probably about 0.5 M. Surfactant contributions to the total current in MEKC can be virtually eliminated by using nonionic or zwitterionic surfactants, particularly if the pH is close to their pI (isoelectric point) of the latter. The drawback of using only net zero charge surfactants as the interactive species, however, is that they do not migrate at a velocity different from electroosmotic velocity, meaning they will be ineffective for the separation of neutral analytes.

The optimization of i via surfactant concentration was recently demonstrated experimentally in MEKC for three pairs of phenylthiohydantoin (PTH) derivatized amino acids, using two types of aqueous micellar systems: plain micelles of SDS and mixed SDS/ Brij 35 micelles. There were several advantages to using the mixed surfactant system: (i) lower operating currents for a given degree of solute retention; (ii) smaller variation in t_{mc}/t_0 as the concentration of Brij 35 was varied, making the optimization of k easier; and (iii) 2 to 3 times the efficiency of the SDS system. On the other hand, neither surfactant system without organic solvent was ideally suited for all the amino acids. The aqueous SDS system was better suited for moderately hydrophobic PTH-AAs, whereas the aqueous Brij 35/SDS system was more retentive and better suited for hydrophilic solutes.

Solutes of moderate to high hydrophobicity are difficult to analyze by MEKC using totally aqueous buffer systems. These types of solute elute near or with the micelle at t_{mc}. One way to increase the affinity of the solute for the mobile phase is to add an organic modifier to the buffer system. The use of an organic modifier will alter the retention, k, of the solutes. The mechanisms by which an organic modifier alters retention in MEKC are similar to those in reversed phase liquid chromatography (RPLC). In RPLC, the addition of an organic modifier alters the affinity of the analyte for the stationary phase by changing: (i) the polarity of the mobile phase; and (ii) the retention characteristics of the stationary phase, usually to a much lesser degree. There are four predominant interactions between molecules in the liquid phase: (1) dispersion or London forces, (2) induction forces, (3) hydrogen bonding, and (4) orientation forces (Giddings, 1991). The "polarity" of a molecule is its ability to interact using these four mechanisms. The total interaction of a solute molecule with a solvent molecule is described by all four interactions in combination. Solvent strength is directly related to polarity, thus "polar " solvents have an affinity for "polar" analytes. In RPLC, solvent strength increases with decreasing polarity; this is also true with MEKC. Since an organic modifier is less polar than water, any combination of organic modifier and water will be less polar than pure water, hence the mobile phase will be stronger and retention will be reduced. The following relationship is often used in RPLC to describe k as a function of organic modifier:

$$\log k = \log k_{aq} - S\,\phi \qquad (64)$$

Equation 64 shows how k is reduced as the percentage of organic modifier in the mobile phase is increased. And as k is reduced, so is t_R. But one should use caution in comparing RPLC and MEKC. In MEKC the addition of organic modifier not only alters the partitioning coefficient, P_{wm}, but also the phase ratio, β, surfactant cmc, the zeta potentials of the capillary wall and the micelles, and viscosity (Gorse *et al.*, 1988; Sepaniak *et al.*, 1990). Nevertheless, it seems appropriate to employ the RPLC organic solvent analogy. Qualitatively it is valid for MEKC, even for solvent gradient elution (Balchunas and Sepaniak, 1988). Note, however, that the amount of organic solvent that can be employed in MEKC is typically limited to ≤ 35% for reasons explained below.

The effect of organic solvent on micelle formation and solute migration reproducibility govern the upper limit of organic solvent that can be used in MEKC. A fixed surfactant concentration (*e.g.*, 100 mM SDS) can accommodate only a certain percentage of organic modifier before the micellization equilibria is eliminated (or adversely affected), resulting in the absence of micelles or poor reproducibility, respectively. If micelles are eliminated by the addition of organic solvent to the buffer, the separation reverts to ion-pairing for analytes of opposite charge to the surfactant monomers, and hydrophobic interaction for neutral solutes, both of which were discussed earlier.

5.5. *Elution Range*

As the **elution range (t_{mc}/t_o)** increases, so does the peak capacity, albeit in a logarithmic fashion. In effect the path length over which solutes can be distributed is increasing. The peak capacity can also be increased in a square root fashion by increasing N.

Although the effect of the elution range on peak capacity is easy to appreciate both on an intuitive level and by inspection of Eq. 14 and Fig. 4, its effect on resolution (Eq. 31) is less obvious and is illustrated in Fig. 9. The results are striking and show rather dramatically just how much the resolving power of EKC can be increased merely by increasing the elution range. (Most MEKC practitioners achieve an elution range between 3 and 8.)

A very significant limitation to the application of EKC to complex samples is the low peak capacity due to the limited elution range (t_{mc}/t_o, Eq. 9). From a practical standpoint, it is best to use methods that do not decrease the electroosmotic flow (*i.e.*, do not increase t_o, by independently increasing t_{mc} and/or decreasing t_0). While it is possible to increase t_{mc}/t_0 by decreasing electroosmotic flow, it is not as desirable because the analysis time increases.

The problem of increasing the elution range can be solved in several ways. One approach has been the use of a different surfactant whose micelles can more effectively swim upstream. As $\mu_{ep,mc}$ approaches $-\mu_{eo}$, the micelles become "stationary" and the elution range becomes infinite. One such surfactant so employed was sodium decyl sulfate (STS), which forms small micelles with a more negative $\mu_{ep,mc}$. Unfortunately, the cmc increases as the chain length of the surfactant decreases and higher surfactant concentrations are needed to generate STS micelles. Mobile phases with high surfactant concentrations generate large currents that enhance Joule heating and result in poor reproducibility (Balchunas and Sepaniak, 1987).

A less desirable, but more easily achieved method for increasing t_{mc}/t_0 is to decrease the electroosmotic flow by reducing the ζ (zeta) potential of the capillary wall. One parameter that can be used to manipulate ζ is pH (Balchunas and Sepaniak, 1987; Otsuka and Terabe, 1989). This may be undesirable because by changing pH, the charge and retention/migration of acidic and/or basic analytes will be altered. Surface modification of the capillary is another method that can be used to reduce the ζ potential (Hjertén, 1985); however, this method can be both tedious and time consuming. In addition, some solutes might interact significantly with the capillary wall and be severely broadened, requiring an organic modifier to restore the efficiency (Balchunas and Sepaniak, 1987).

A better, albeit not yet commercially available method for controlling the ζ potential is the application of an additional electric field in a radial direction across the capillary (Ghowsi, *et al., 1990*; Lee *et al.*, 1990; Wu *et al.*, 1992a; Hayes and Ewing, 1992, 1993). This method for controlling the ζ potential has been useful in protein and peptide separations in CE (Wu *et al.*, 1992b) and could just as easily be used in EKC.

One of the easiest ways to increase t_{mc}/t_0 somewhat (by a factor of 1.5 to 2) is to add an organic modifier such as methanol, acetonitrile, 2-propanol, etc. to the buffer. This is a simple and effective way to significantly increase t_{mc}/t_0, although it usually results in reduced electroosmotic flow, thereby increasing the analysis time proportionally. In buffered systems without surfactant, the smallest effect on t_0 was observed with acetonitrile (Gorse *et al.*, 1988).

The elution range is normally independent of applied voltage and, for some surfactant systems, moderately dependent on surfactant concentration. In MEKC using SDS, at concentrations of SDS below 150 mM the elution range varies only slightly, but this dependence increases somewhat over the range 100–300 mM.

5.6. Practical Strategies

Several reports on the optimization of resolution have appeared. Unfortunately, the term "optimization" means different things to different researchers. One should be aware that different methods of optimization, including those based on fundamental resolution theory (k optimization) or experimental statistical design, provide complementary information. When optimizing a separation, one approach should not be unduly favored over another.

The approach of McNair and coworkers (Rasmussen *et al.*, 1991) considered three ways to optimize R_s for neutral molecules (Eq. 30): (1) increase N; (2) increase α; (3) increase the f(k) term. Since α is very hard to predict and manipulate, their study focused on N and f(k). At low values of v_{eo} and k, it was possible to get infinite R_s but at the expense of longer analysis times. They concluded that R_s could be maximized most effectively by lengthening the capillary rather than decreasing v_{eo}.

Sandra and Vindevogel (Vindevogel and Sandra, 1991b) utilized a Plakett-Burman statistical design to optimize R_s for a single set of four testosterone esters. This approach appears to be good for the simultaneous optimization of two or perhaps three parameters. Other systematic approaches such as the simplex algorithm, mixture design, exhaustive or interpretive response surface mapping (window diagrams), mixed lattice, adaptive searches, and step-search designs (Berridge, 1985; Schoenmakers, 1986) may also be effective but they have not yet been investigated. Some may be less suited for the discrete changes in solution variables (pH, surfactant concentration, etc.) that are more convenient with current commercial or in-house instrumentation (cf. mobile phase composition that can be varied continuously by most LCs).

6. Recent Developments

6.1. Sample Stacking of Neutral Molecules in MEKC

Field amplified sample stacking (FASS) is a very useful technique for enhancing the detectability of solutes in CE (Burgi and Chien, 1991; Chien, 1991; Chien and Helmer, 1991; Chien and Burgi, 1992; Gebauer *et al.*, 1992). FASS can be used with both the hydrodynamic and electrokinetic modes of injection. Szucs *et al.* recently applied FASS in MEKC for the analysis of hop bitter acids (Szucs *et al.*, 1993). Until now, all applications of FASS in MEKC have been limited to charged solutes, because neutral molecules are unaffected by the amplified field since their electrophoretic mobility, μ_{ep}, is zero. In order to apply FASS to neutral molecules, an electrophoretic mobility must be imparted to the latter. This can be accomplished by using charged micelles to give neutral molecules an effective electrophoretic mobility, $\mu_{ep,eff} = k'/(1+k')\ \mu_{ep,mc}$ (see equivalent Eq. 4(a)), where $\mu_{ep,mc}$ is the electrophoretic mobility of the micelle. Solutes with a large k' will have the largest effective mobility and will experience the greatest amount of stacking, while those with a k' = 0 will have $\mu_{ep,eff} = 0$ and not be stacked at all (Nielsen and Foley, 1994). This is illustrated in Fig. 12, in which no stacking is done, and Fig. 13 where FASS has been used with the hydrodynamic mode of injection. Clearly, peaks 5 and 6 in Fig. 13 are considerably sharper, with the width of peak 6 approximately the same as the first peak. The more hydrophobic analytes experience the greatest stacking effect.

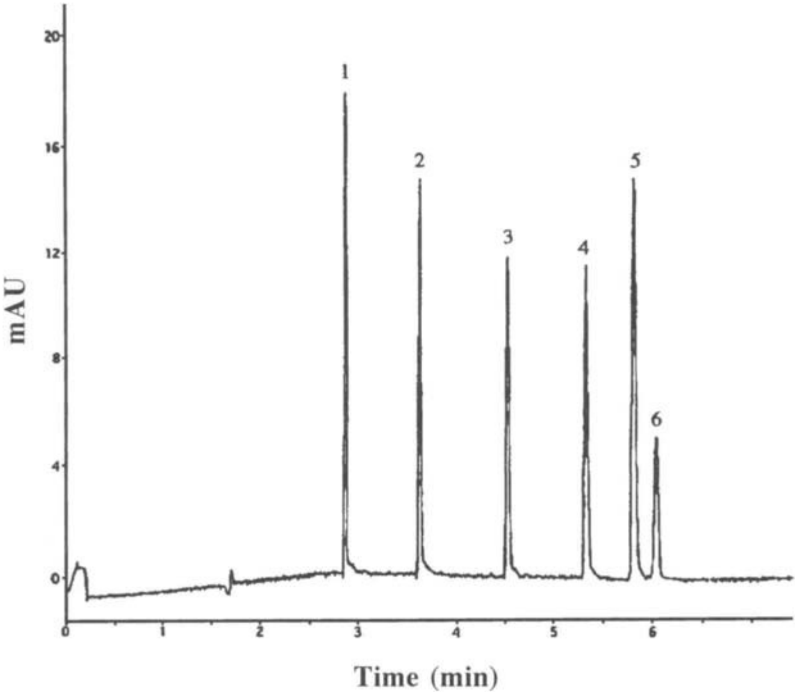


Fig. 12. Separation of alkylphenone homologous series. Hydrodynamic injection was performed with the injection buffer the same as the running buffer. Experimental conditions: 50 mM SDS; 15 mM phosphate buffer; 8 s gravity injection; capillary length, 40 cm from injection to detection; applied voltage, 20 kV; detection wavelength, 254 nm. Peak identification: 1, acetophenone; 2, propiophenone; 3, butyrophenone; 4, valerophenone; 5, hexanophenone; 6, heptanophenone.

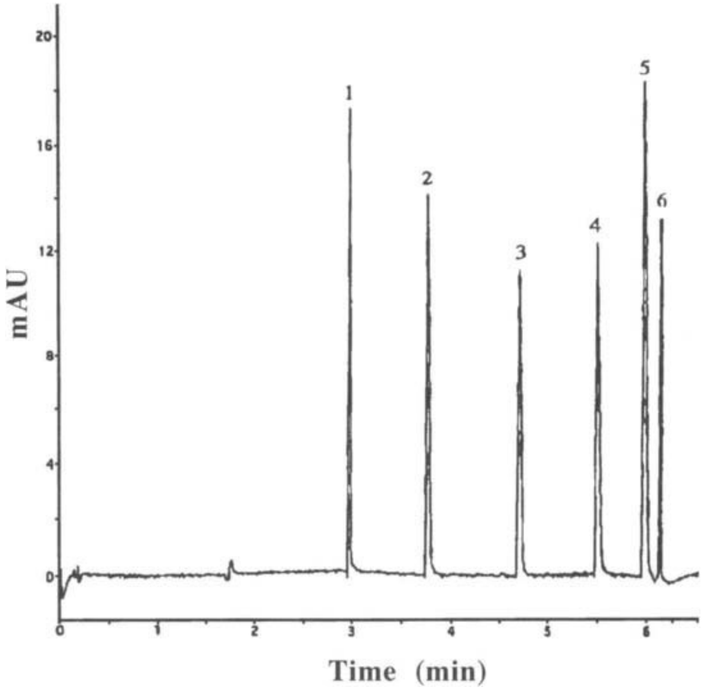


Fig. 13. FASS with hydrodynamic injection for a series of alkylphenone homologs. Injection buffer: 14 mM SDS, 1 mM phosphate buffer; 75 mM SB-12. Running buffer: 50 mM SDS, 15 mM phosphate buffer. Other experimental conditions same as in Fig. 12.

In contrast to FASS with hydrodynamic injection, where the stacking occurs only after sample introduction, in FASS with electrokinetic injection (EK-FASS) the stacking occurs both during and after sample introduction, and is potentially much more effective. Moreover, we can compensate for charge biases in electrokinetic injection by judicious choice of the micelle charge in the sample reservoir or by using polarity switching of the power supply. Figure 14 shows the effect of EK-FASS on a homologous series of alkylphenones where the charged micelles are SDS/SB-12 micelles with a net anionic charge. Here the micelles oppose electroosmotic flow upon injection when the electroosmotic flow is from anode to cathode. In this case, the anionic micelles in the sample buffer offer no significant advantage with EK-FASS; however, if the polarity is reversed, the use of anionic micelles will provide all the advantages of EK-FASS.

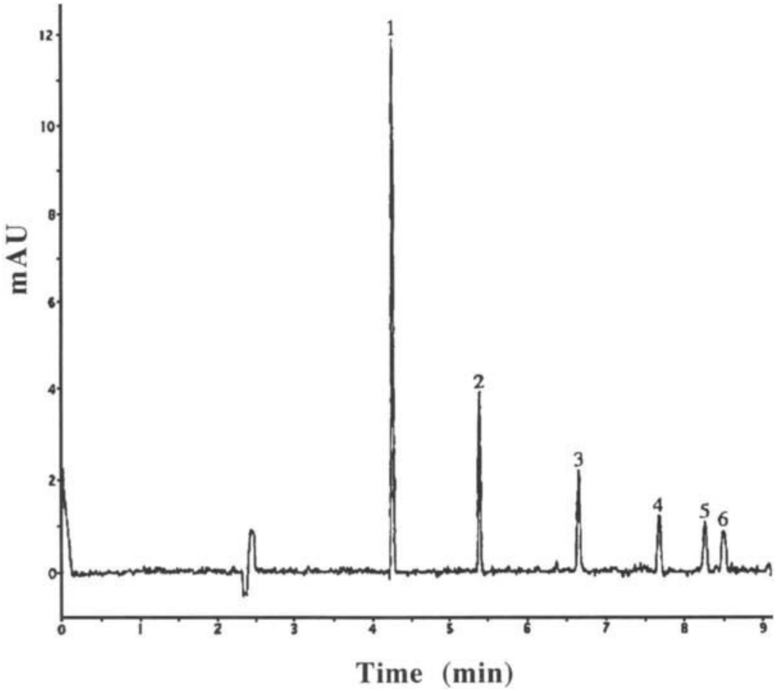


Fig. 14. Chromatogram shows separation of an alkylphenone homologous series. Electrokinetic injection has been performed with the injection buffer composition consisting of net anionic micelles. Injection buffer: 14 mM SDS, 1 mM phosphate buffer, 75 mM SB-12. Running buffer: 50 mM SDS, 15 mM phosphate buffer. Electrokinetic injection time: 6 s @ 2.5 kV; capillary length: 40 cm from injection to detection; applied voltage: 20 kV; detection wavelength, 254 nm. Peak identification: 1, acetophenone; 2, propiophenone; 3, butyrophenone; 4, valerophenone; 5, hexanophenone; 6, heptanophenone.

Figure 15 shows the improvement that can be achieved with EK-FASS when a mixed micellar system of CTAB/SB-12 is used in the sample buffer to produce a net cationic micellar system. Now, the flow of analytes is toward the cathode upon injection, hence all the peaks are sharper and detectability has been enhanced for the entire sample. In some cases the limit of detection (LOD) can be reduced by over a factor of ten with observed efficiencies in excess of 2.8 million theoretical plates per meter.

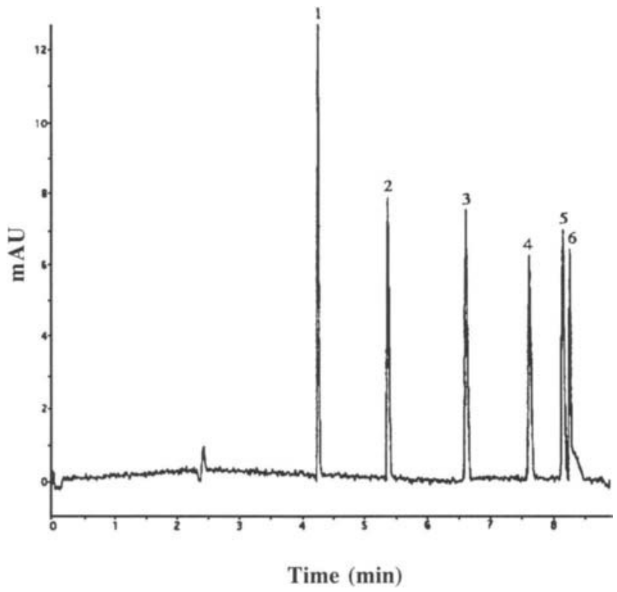


Fig. 15. EK-FASS with an injection buffer composition consisting of 14 mM CTAB, 1 mM phosphate buffer, and 75 mM SB-12. All other experimental conditions are the same as Fig. 14.

6.2. Counter Ion Effects in MEKC

In micellar electrokinetic capillary chromatography (MEKC), the most widely used surfactant for the pseudostationary phase is sodium dodecyl sulfate (SDS), although other types of surfactants have been investigated. However, little attention has been paid to the effects of counter ions on micellar structure, and the subsequent effects on resolution in MEKC. In a recent study, the magnesium salt of dodecyl sulfate ($Mg(DS)_2$) was synthesized in order to investigate how changes in the counter ion identity affect resolution as well as other important chromatographic parameters (Nielsen and Foley, 1993). Buffer solutions containing $Mg(DS)_2$ exhibited operating currents and electroosmotic velocities one-third to one-half those of a comparable SDS buffer solution, and significantly larger methylene and functional group selectivities for the various compounds that were studied. In addition, capacity factors, k', for neutral solutes were from 1.5 to 2.5 times larger for the $Mg(DS)_2$. Thus, FASS with $Mg(DS)_2$ should be better than that performed with SDS. It was also found that the elution range t_{mc}/t_0 for the $Mg(DS)_2$ system was independent of the percentage of acetonitrile in the buffer solution while the elution range for SDS increased as the percentage of acetonitrile increased. This feature of the $Mg(DS)_2$ system facilitates optimization of resolution and resolution per unit time through retention manipulation. The improvements in selectivity and increased retention resulted in increased resolution for neutral solutes studied in $Mg(DS)_2$.

While $Mg(DS)_2$ offers many advantages over SDS, one of the disadvantages of this surfactant system is the lack of commercial availability of $Mg(DS)_2$ itself. Even though the synthesis is fairly simple, widespread use of $Mg(DS)_2$ will probably not take place until this surfactant is commercially available.

Recently, Ahuja and Foley (1994a) have investigated the use of lithium and potassium dodecyl sulfate (LiDS, KDS) surfactants as possible micellar systems for MEKC. By changing the counter ion identity from sodium to lithium remarkable improvements in efficiencies were seen for a set of neutral test solutes. The hydrophobic test solutes benefited the most by the use of the LiDS micellar system as their efficiencies were one and a half times greater than that obtained with a comparable SDS based separation. A comparison of both the SDS and LiDS micellar systems is shown in Fig. 16, which shows many of the advantages of the LiDS micellar system. The elution range, t_{mc}/t_0, decreased for LiDS, but complete resolution of the neutral solutes was obtained in approximately half the time of the SDS separation. The peaks are also much narrower with LiDS, especially peaks 6–8, than in the similar SDS separation. Operating currents associated with LiDS are also lower than that obtained with either the SDS- or KDS-based separations. Once again, this example serves to illustrate how judicious choice of a surfactant before attempting an analysis can not only provide greater resolving power but can also decrease the time required for a particular analysis.

Another interesting feature of the LiDS micellar system is the effect of acetonitrile on k' and t_{mc}/t_0. As the percentage of acetonitrile is increased from 0 to 20%, k' values for a neutral test mix of varying hydrophobicity decreased continuously while similar experiments with SDS and acetonitrile show a leveling effect of k' values once the acetonitrile percentage is greater than 10. Furthermore, the increase in t_{mc}/t_0 values usually associated with increasing amounts of organic modifier was much less for LiDS than with SDS. The smaller increases in the magnitude of the elution range coupled with large changes in k' values allows for easier resolution optimization with LiDS.

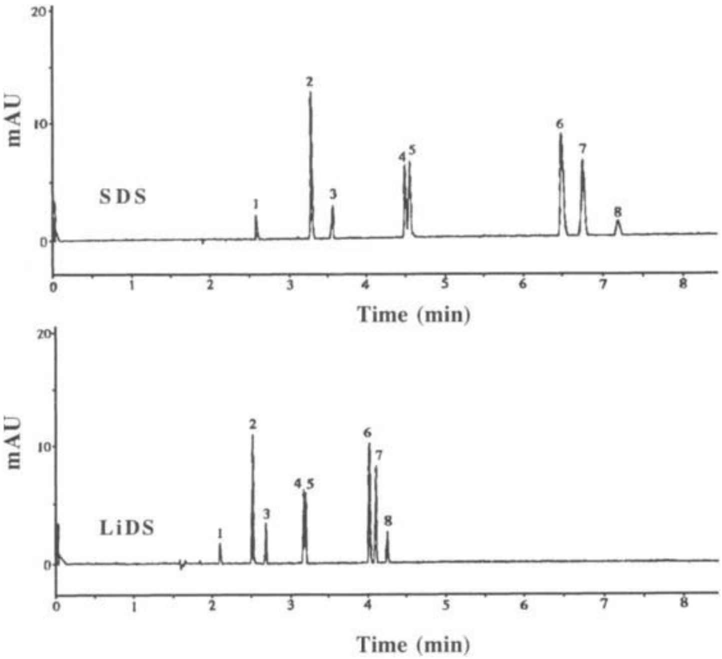


Fig. 16. Comparison of 50 mM lithium dodecyl sulfate (LiDS) and 50 mM sodium dodecyl sulfate (SDS). Experimental conditions: capillary, 30 cm from injection to detection; 1 s hydrodynamic injection; applied voltage, 15 kV; operating currents < 35 μA; 10 mM phosphate buffer (pH 7.0); detection wavelength, 254 nm. Peak identification: 1, benzyl alcohol; 2, nitrobenzene; 3, anisole; 4, *p*-nitrotoluene; 5, *m*-nitrotoluene; 6, benzophenone; 7, biphenyl; 8, decanophenone–tmc marker.

KDS has a high Krafft point and is therefore much more difficult to work with, unless the instrumentation used for the analysis is equipped with proper sample, buffer, and capillary temperature control. Initial studies with KDS involved a minimal use of 15% acetonitrile in order to avoid precipitation of KDS in either the sample and/or buffer reservoirs or the capillary itself. Separation efficiencies were approximately one-third that obtained with similar SDS separations. In addition, analysis times were also twice as long as similar SDS separations and almost four times as long as the LiDS-based separations. The elution window was largest with KDS. Therefore, the order of increasing t_{mc}/t_0 values as a function of counter ion identity is: lithium, sodium, and potassium. The KDS/acetonitrile-based separations did not offer any real advantages over similar LiDS or SDS/acetonitrile. However, further research using KDS without organic modifier, with the proper thermostating, could be advantageous in terms of increased peak capacity which would be beneficial for samples that contain a large number of analytes.

The use of mixtures of counter ions can provide some degree of resolution optimization. By varying the amounts of two or more different counter ions, one can control to a certain extent the micellar pseudophase by controlling the surface charge density on the micelle itself. Microenvironments that facilitate transfer of analytes between the micellar retentive phase and mobile phase can be achieved by choosing the right type of counter ion(s) and optimum counter ion ratio, $[M_Y]/[M_X]$.

6.3. Novel Surfactant Systems

One of the advantages of MEKC that has been relatively unexplored is the use of novel micellar systems as pseudostationary phases. There are numerous commercially available surfactants, of which, only a few have been utilized in MEKC. Of the four different types of surfactants—anionic, cationic, nonionic, and zwitterionic—anionic and cationic surfactants have been used the most. A quick review of the literature reveals that sodium dodecyl sulfate (SDS) has been the surfactant of choice for many applications. The use of mixed micelles, micelles consisting of two or more different surfactants, in MEKC has primarily been limited to nonionic/anionic mixed micelles such as Brij 35/SDS micellar systems (Rasmussen *et al.*, 1990; Rasmussen *et al.*, 1991; Little and Foley, 1992; Ahuja and Foley, 1994a). Swedburg (Swedburg, 1990) was the first to investigate the use of a zwitterionic micellar system in MEKC. Nonionic or zwitterionic mixed micelles offer some inherent advantages. The concentration of charged surfactant can be held constant while the nonionic or zwitterionic surfactant concentration can be increased or decreased. In this fashion, the operating currents are kept constant and the nonionic or zwitterionic surfactants can be viewed as stationary phase additives. Resolution and other chromatographic parameters can be optimized without having to worry about changes in operating currents that can lead to undesirable Joule heating effects.

The use of N-dodecyl-N-N-dimethylammonium-3-propane-1-sulfonic acid (SB-12) in combination with SDS as a pseudostationary phase in MEKC has shown considerable promise (Ahuja *et al.*, 1994). SB-12 is zwitterionic at all pH values and thus offers the advantage of not being limited to a small pH range. Our results show that the SB-12/SDS micellar system provides more efficient separations than that obtained with just SDS. Electroosmotic flow was virtually unchanged with the addition of increasing concentrations of SB-12. Concentrations of 10–20 mM SB-12/20 mM SDS allowed for the baseline resolution of two structural isomers, *p*-nitrotoluene and *m*-nitrotoluene, which was not possible with only SDS (Fig. 17). Table 8 offers a comparison of some of the important surfactant characteristics of SB-12 and SDS. Further research into the use of SB-12 in combination with LiDS, KDS, or $Mg(DS)_2$ will offer more insight in this unique micellar system.

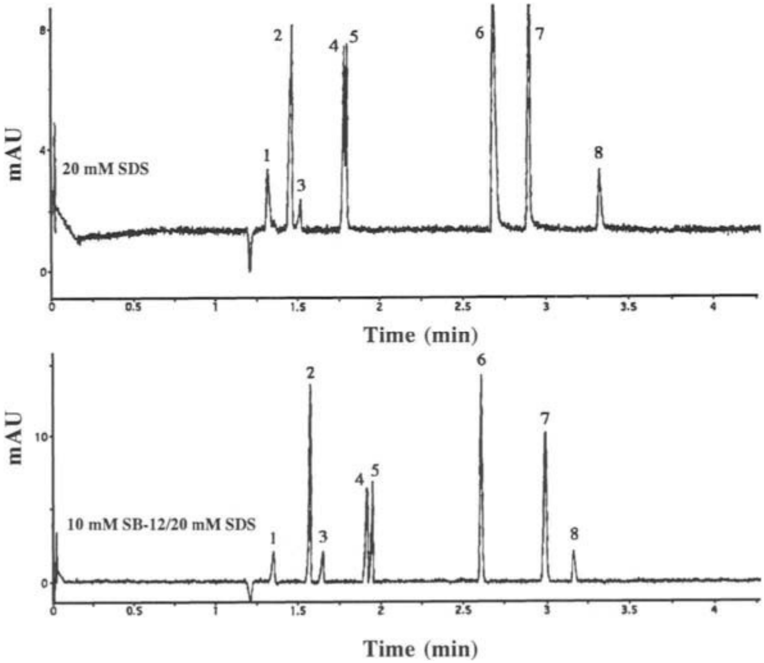


Fig. 17. Addition of 10 mM SB-12 to SDS improved resolution between two structural isomers, *p*-nitrotoluene and m-nitrotoluene. Experimental conditions: capillary, 30 cm from injection to detection; 1 s hydrodynamic injection; applied voltage –20 kV; operating currents < 35 μA; 10 mM phosphate buffer (pH 7.0); detection wavelength, 254 nm. Peak identification: 1, benzyl alcohol; 2, nitrobenzene; 3, anisole; 4, p-nitrotoluene; 5, m-nitrotoluene; 6, benzophenone; 7, biphenyl; 8, decanophenone–tmc marker.

Table 8. Characteristics of SDS and SB-12

Surfactant	Aggregation number	Krafft point (°C)
N-dodecyl-N,N-dimethyl-ammonium-3-propane-1-sulfonic acid (SB-12) $CH_3(CH_2)_{11}N^+(CH_3)_2(CH_2)_3SO_3^-$ cmc (mM) = 3	55	< 0
Sodium dodecyl sulfate (SDS) $CH_3(CH_2)_{11}OSO_3^-Na^+$ cmc (mM) = 8.1	62	9

Reference: (Love *et al.*, 1984)

Brij 35, a nonionic surfactant with 23 polyoxyethylene units, has been used to coat capillary walls (Towns and Regnier, 1991). When Brij 35 is used with SDS to form a mixed micellar pseudophase for MEKC, reproducibility of separations employing these surfactants has been rather poor due to the changing capillary surface caused by the coating effects (Ahuja *et al.*, 1995b). To counteract these wall interactions, Brij 30, a nonionic surfactant with only four polyoxyethylene units, was used with SDS to form a mixed micellar pseudophase for use in MEKC (Ahuja and Foley, manuscript submitted). Initial results indicate that the reproducibility problems seen with the Brij 35/SDS system are significantly reduced with Brij 30/SDS, while separation efficiencies were roughly the same. Other mixed micellar systems, such as the nonionic Tween or Span series surfactants, in combination with anionic or cationic surfactants, could provide additional advantages over the currently used mixed micellar systems in MEKC.

7. Applications

Since MEKC was first introduced by Terabe in 1984 (Terabe *et al.*, 1984), application of this technique to various areas of scientific and public interest has focused on taking advantage of the ability of MEKC to achieve rapid analyses and high efficiencies with the added benefits of small sample requirements (nanoliters) and relatively inexpensive experimental costs. To date, the practical applications in MEKC have been somewhat limited to high sample concentrations, primarily due to the lack of suitable detection required for trace analysis, and moderately hydrophilic solutes as a result of the finite elution range in MEKC. Most of the available commercial instrumentation employs UV detection which also limits the scope of analytes that can be studied to those that contain a UV absorbing chromophore.

One of the first applications of MEKC that demonstrated its high resolving power was the separation of a mixture of 22 phenylthiohydantoin-amino acids (Otsuka *et al.*, 1985b). Since that time, there have been numerous applications utilizing MEKC. An extensive list of some of the applications performed to date, along with their pertinent references, is shown in Table 9.

The intent here is not only to present a broad range of applications but also to offer insight into some of the areas of interest that have received a great deal of attention. A comprehensive discussion of all the applications in MEKC and EKC is beyond the scope of this book chapter. A more in depth perspective on this subject can be found by consulting the comprehensive reviews (Kuhr, 1990; Janini and Issaq, 1992; Kuhr and Monnig, 1992) and also the some of the books (Grossman and Colburn, 1992; Li, 1992) that have recently been published, including one that focuses entirely on MEKC (Sandra and Vindevogel, 1992).

The discussion that follows highlights some of the interesting applications that have appeared in the literature. The applications have been classified into separate categories and subcategories to provide quick and easy reference to a particular area of interest.

7.1. Pharmaceuticals and Drugs

Pharmaceutical analysis using MEKC has been an area of particular interest. The high efficiencies which can be generated using MEKC and the ability to separate neutral as well as charged solutes increases the separating power of this technique. Prior to the introduction of MEKC, high performance liquid chromatography (LC) was and still is the technique employed in the pharmaceutical analyses of compounds. LC has proven to be a very diverse due to the many different modes of separation possible such as ion exchange and adsorption. The main disadvantage of LC is that it provides only modest efficiencies. A thorough analysis of an intricate pharmaceutical sample or structurally similar compounds may not be possible with the efficiencies that LC generates. It is for these reasons that there has been considerable research into the analysis of pharmaceutical compounds with MEKC.

Table 9. Selected Applications in MEKC and EKC

Analyte of Interest	Comments	Reference
Amino Acids		
1. Chiral separations PTH derivatives of DL-amino acids	Sodium N-dodecanoyl-L-valinate used with SDS allowed for the separation of enantiomers and each pair was optically resolved	Otsuka *et al.*, 1991
2. Dansyl DL-amino acids		Miyashita and Terabe, 1990
3. Amino acid enantiomers peptide isomers	Derivatization with L- and D-Marfey's reagent and subsequent analysis with MEKC to confirm presence of D-amino acids in an unknown sample	Tran *et al.*, 1990
4. Derivatized amino acids vitamin metabolites		Swaile *et al.*, 1988
5. Naphthalene-2,3-dicarboxaldehyde (NDA)-derivatized amino acids	CD-MEKC shown to be effective for some chiral separations	Ueda *et al.*, 1991; 1992
6. PTH-amino acids	Optimization of resolution using a Brij 35/SDS micellar system	Little and Foley, 1992
7. Fluorescamine amino acids, 9-fluorenylmethoxycarbonyl (FMOC)-AAs and *o*-phthalaldehyde (OPA)-AAs		Albin *et al.*, 1991

cont'd

Table 9 cont'd.

Analyte of Interest	Comments	Reference
8. PTH-amino acids	Optimization of separation through the use of a weighted variable-size simplex algorithm	Castagnola *et al.*, 1993
9. Dansylated and PTH-amino acids	Glycyrrhizic acid (GRA) and beta-escin, natural chiral surfactants, are used for chiral separations	Ishihama and Terabe, 1993
10. Dansylated-DL-amino acids, polycyclic aromatic hydrocarbons, dimethylnaphthalenes	Cyclodextrin modified MEKC is used to improve resolution	Terabe *et al.*, 1993
11. Tetramethylrhodamine thiocarbamyl amino acid derivatives	Low-cost laser-induced fluorescence detection with zeptomol detection levels	Zhao *et al.*, 1992
Foods		
12. Fruit Juices	5-hydroxymethyl-2-furaldehyde and 2-furaldehyde in grapefruit juice; no sample preparation	Corradini and Corradini, 1992
13. Hop Bitter Acids		Vindevogel *et al.*, 1990; Vindevogel and Sandra, 1991a; Verschuere *et al.*, 1992
	Sample stacking and large injection volumes	Szucs *et al.*, 1993

cont'd.

Table 9 cont'd.

Analyte of Interest	Comments	Reference
Nucleic Acids		
14. Deoxyribonucleotides, deoxyribonucleosides	Deoxyribonucleotide oligomers resolved on basis of chain length, nucleic base sequence, and base modification	Griest *et al.*, 1988
15. Nucleosides	Addition of glucose to the micellar solution allowed complete separation of a mixture of nine nucleosides	Kaneta *et al.*, 1992
16. Nucleosides, nucleotides	Comparison of ion-pairing LC and MEKC; superior separation achieved with MEKC	Lahey and St. Claire, 1990
17. Nucleic Acid derivatives		Lecoq *et al.*, 1991
18. Dansylated nucleotides	Laser induced fluorescence detection	Lee *et al*, 1992
19. Phosphorylated, nucleosides	High-speed separation of mono-, di-, and triphosphorylated ribonucleosides	Liu *et al.*, 1989
20. Nucleic acid constituents	Mixture of 14 adducted and modified nucleic acid constituents resolved in < 40 min	Row *et al.*, 1987; Row, 1989
21. DNA adducts of benzo[a]pyrene		Harvey *et al.*, 1992
22. DNA adducts	Fractions collected and analyzed using MEKC and FAB-MS	Lecoq *et al.*, 1993a

cont'd

Table 9 cont'd.

Analyte of Interest	Comments	Reference
23. Nucleotide-3'-monophosphates	Influence of buffer, pH temperature, operating conditions and capillary treatments are discussed	Lecoq *et al.*, 1993b
24. Diastereomeric phosphoramidate bridged unnatural oligonucleotides	Compounds resolved with MEKC, conventional LC techniques did not provide resolution	Bevan *et al.*, 1993
Peptides		
25. Desipramine/nortriptylene, angiotensin III/[Val 4],-angiotensin	Use of zwitterionic and nonionic surfactants to aid in the separation of closely related pairs of compounds	Swedburg, 1990
26. Peptides, barbiturates	Pharmaceutical analysis of over the counter pain, cold, and allergy medications	Wainright, 1990
27. Motilins, closely related peptides	Tween 20 is used in the separation of Angiotensin II, its derivatives and motilins	Matsubara and Terabe, 1992
28. Small peptides		Issaq *et al.*, 1992
29. Closely related peptides, motilins, insulins	Study concludes that it is difficult to separate peptides consisting of more than 20 AA residues without organic modifiers	Yashima *et al.*, 1992

cont'd

Table 9 cont'd.

Analyte of Interest	Comments	Reference
30. Neurohypophysealpeptides and analogs	Separations obtained with RPLC, CE, and MEKC are compared	Sutcliffe and Corran, 1993
Pharmaceuticals, Drugs, Organic Compounds, Natural Products, Explosives, etc.		
31. Antidepressants	Separation from impurities	Altria and Smith, 1991
32. Aliphatic alcohols, phenolic compounds	Indirect fluorescence detection	Amankwa and Kuhr, 1991
33. Aflatoxins	Four common aflatoxins separated in less than 30 sec.	Cole *et al.*, 1992
34. Herbicides, polyaromatic hydrocarbons	Octylglucoside-borate micellar phase; adjustable surface charge density	Cai and El Rassi, 1992
35. Pilocarpine and degradation products	Diastereoisomers	Charman *et al.*, 1992
36. Amines (derivatized) aflatoxins, hydroxy aromatic compounds	Laser fluorometric detection; use of 2-propanol and acetonitrile for improved separation of hydrophobic solutes	Balchunas *et al.*, 1988
37. Amines (derivatized)	Stepwise solvent gradient using increasing conc. of 2-propanol and Triton X-100	Balchunas and Sepaniak, 1988
38. Sulfonamides, trimethoprim	Determination of three active ingredients in tablets	Dang *et al.*, 1992

cont'd.

Table 9 cont'd.

Analyte of Interest	Comments	Reference
39. Mycotoxins	Qualitative analysis method allowed complete identification of mycotoxins in five unknown samples without misidentification	Holland and Sepaniak, 1993
40. Chlorotriazine, herbicides in river water	Atrazine and simazine separated in less than 10 min; detection limit approx. 0.4 ppb for each herbicide	Desiderio and Fanali, 1992
41. Antipyretic, analgesic preparations	Successful application to commercial antipyretic analgesic tablets	Fujiwara and Honda, 1987
43. Water-soluble vitamins	Analysis of 7 water-soluble vitamins	Fujiwara *et al.*, 1988
44. Antiepileptic drugs in human plasma	Six antiepileptic drugs: phenobarbital, carbamazepine, valproic acid, phenytoin, primidone, and ethosuximide	Lee *et al.*, 1992
45. L-buthionine-(R,S)-sulfoximine	Assay may be used to determine the plasma pharmacokinetics of buthionine-sulfoximine over the expected therapeutic range	Lloyd, 1992
46. (bis)Amidinohydrazones	Important in human serum samples; effect of 5 different buffer solutions on elution order and separation	Lukkari *et al.*, 1992a,b

cont'd.

Table 9 cont'd.

Analyte of Interest	Comments	Reference
47. Enantiomers of amphetamine, methamphetamine	Including their hydroxyphenethylamine precursors; far superior results than that obtained using LC	Lurie, 1992
48. Glucosinates, desulfoglucosinolates	Isolated form seeds and vegetative parts of plants	Michaelsen *et al.*, 1992
49. Antibiotics, corticosteroids	Better resolution and shorter analysis times than those obtained by LC	Miyashita *et al.*, 1990
50. Benzathiazolesulfenamides	Comparison of urea and methanol modifiers in the mobile phase	Nielen and Mensink, 1991
51. Amoxycillin, clavulanic acid	Important constituents in the antibiotic augmentin	Okafo and Camilleri, 1992
52. Enantiomers of chiral compounds	Use of taurodeoxycholic acid and β-cyclodextrin; forms a 'pseudostationary' phase effective for separation of a variety of chiral compounds	Okafo *et al.*, 1992
53. Priority pollutants, substituted phenols		Ong *et al.*, 1990; 1991a
54. Water- and fat-soluble vitamins	γ-cyclodextrin in combination with SDS provided the best selectivity for separating the vitamins	Ong *et al.*, 1991b

cont'd.

Table 9 cont'd.

Analyte of Interest	Comments	Reference
55. Catechols, catecholamines		Ong *et al.*, 1991d
56. Flavanoid drugs	Compared with results from reverse-phase LC	Pietta *et al.*, 1991;1992a,b)
	UV-diode array detection	Pietta *et al.*, 1992c
	Systematic optimization	Ng *et al.*, 1992b
57. Cicletanine (enantiomers) in human plasma	Analysis with MEKC required less than half the time of the LC method	Pruñonosa *et al.*, 1992a,b
58. Alkyl paraben homologs	Low pH buffers may provide for a rapid analysis of hydrophobic solutes	Rasmussen and McNair, 1989
59. Metal complexes of acetylacetone	Micellar solution contained SDS and 100 mmol/L acetylacetone; Sc(III), V(IV), Cr(III), Mn(III), Fe(III), Co(III), Cu(II), Ti(IV), Co(II), Ni(II), and Zn(II) complexes analyzed	Saitoh *et al.*, 1991a
60. Metal chelates	Methods for metal chelate separations are presented	Saitoh *et al.*, 1989; 1991b; 1992
61. Biological separations: substituted benzenes, purines, nitro derivatives of fused-ring aromatic compounds		Sepaniak *et al.*, 1987

cont'd.

Table 9 cont'd.

Analyte of Interest	Comments	Reference
62. Cimetidine (in serum)	Preconcentrated samples determined by MEKC	Soini *et al.*, 1991
63. Barbiturates in human serum and urine	On-column, fast-scanning multiwavelength UV detection	Thormann *et al.*, 1991
	Strategies for monitoring of drugs in body fluids	Thormann *et al.*, 1993
64. Substituted purines, uric acids, methylated xanthines	Direct injection of serum or saliva samples required only μl volumes of sample for rapid analysis	Thormann *et al.*, 1992
65. Illicit drugs: heroin, cocaine, phenobarbital, methaqualone, opium alkaloids, amphetamines, barbiturates, benzodiazepines, cannabinoids	Comparisons with LC separations are made	Weinberger and Lurie, 1991
	Confirmation testing in urine samples	Wernly and Thormann, 1991; 1992a,b
66. Urinary porphyrins	Method suitable for the determination of porphyrins in clinical urine samples	Weinberger *et al.*, 1990
67. Herbicides	SDS/Brij 35 micellar system provided best separation	Wu *et al.*, 1992c
68. Nitroaromatics		Yik *et al.*, 1992a
69. B6 vitamins	Amperometric detection for a mixture of B6 vitamins	Yik *et al.*, 1991a
	Fluorescence detection	Burton *et al.*, 1986a

cont'd.

Table 9 cont'd.

Analyte of Interest	Comments	Reference
70. Environmental pollutants	Substituted phenols, polycyclic aromatic hydrocarbons, and phthalate esters	Yik *et al.*, 1990; 1991b; 1992b
71. Anionic surfactant components	Anionic components of Triton 770 are separated	Zweigenbaum, 1990
72. Antibiotics		Yeo *et al.*, 1991
	β-lactam antibiotics	Nishi *et al.*, 1989a
73. Cefpiramide	Direct injection of sample from human plasma	Nakagawa *et al.*, 1988; 1989
74. Creatinine, uric acid	Simultaneous determination in human plasma and urine	Miyake *et al.*, 1991
75. Gunshot and explosive residues	Sample collection, preparation, and quantitation	Northrop *et al.*, 1991; Northrop and MacCrehan, 1992
76. Chiral separations with bile salt micelles		Nishi *et al.*, 1989a; 1990a,b,c; 1991; Terabe *et al.*, 1989b; Cole *et al.*, 1991; Cole and Sepaniak, 1992
77. Aliphatic alcohols and carboxylic acids	Indirect fluorescence detection	Szucs *et al.*, 1991
78. Chiral separations	Overview is presented	Snopek *et al.*, 1992
	Enantiomeric resolution with chiral surfactants	Otsuka and Terabe, 1990
79. Hydroquinone (in skin-toning cream)		Sakodinskaya *et al.*, 1992

cont'd.

Table 9 cont'd.

Analyte of Interest	Comments	Reference
80. Antihistamines		Ong *et al.*, 1991c
81. Glucosinolates, desulfoglucosinolates	Cationic surfactant is used	Morin *et al.*, 1992
82. Anthracene derivatives	CD-MEKC with laser fluorimetric detection, the absolute detection limit is estimated to be at the subfemtogram level	Imasaka *et al.*, 1991; 1992
83. Dansylated methylamine and methyl-d3-amine	Methanol modified mobile phases are used to accomplish the separation	Bushey and Jorgenson, 1989b
84. Phenolic carboxylic Acids		Bjergegaard *et al.*, 1992
85. Aminoglycoside antibiotics	Indirect UV detection	Ackermans *et al.*, 1992
86. Neurochemicals, catechols	MEKC separation of catechol, 4-methylcatechol, 4-ethylcatechol, and norepinephrine with electrochemical detection	Wallingford and Ewing, 1988a
	Effect of borate complexation	Wallingford and Ewing, 1988b; 1989
	Use of mixed micelles	Wallingford *et al.*, 1989
87. Polyaromatic hydrocarbons, chlorinated benzene congeners, various isomers	Cyclodextrin added to buffer, CD-MEKC	Terabe *et al.*, 1990

cont'd.

Table 9 cont'd.

Analyte of Interest	Comments	Reference
88. Metal binding proteins		Kajiwara, 1991
89. Vitamins, caffeine		Kobayashi *et al.*, 1989
90. Polymyxins (polypeptides)	Zwitterionic surfactant used to achieve separation of closely related polypeptides	Kristensen and Hansen, 1993
91. Theophylline tablets	Separation and determination of seven active ingredients	Dang *et al.*, 1993
92. Flavanoid-O-glycosides and their flavanoid aglycones	Fast-scanning, multiwavelength UV detection is used	Morin *et al.*, 1993
93. Salicylate acetaminophen antiepileptics	Comparison of MEKC, CE, and capillary isotachophoresis (CITP) for rapid screening and confirmation of drugs in urine and serum	Caslavska *et al.*, 1993
94. Explosive residues in soils	High resolution and efficiencies of the MEKC method allowed for "fingerprinting" of complex soil extracts, comparisons made with RP-LC method	Kleibohmer *et al.*, 1993
95. Flavonol glycosides from *Tilia*	RP-LC and MEKC are used for the determination of nine different flavonol glycosides	Pietta *et al.*, 1993
96. Substituted phenols	Effect of 1-alkyl alcohols on separation performance	Aiken and Huie, 1993

cont'd.

Table 9 cont'd.

Analyte of Interest	Comments	Reference
97. RS-Chloropheniramine	Cyclodextrin-modified MEKC used for optical resolution	Otsuka and Terabe, 1993
98. β-Adrenergic blocking agents	Important in treatment of migraine and cardiovascular disorders	Lukkari *et al.*, 1993
99. Glycoconjugates		Stefansson and Westerlund, 1993
100. Alkylaromatic Sulphonates	Comparison of CE and MEKC, separation of alkylbenzene sulfonate isomers was possible only with MEKC	Desbene *et al.*, 1992

One of the early applications involved the analysis of antipyretic analgesic preparations with an emphasis on quantitative aspects (Fujiwara and Honda, 1987). The use of an internal standard allowed accurate and reproducible determination of three of these analytes. Application to a commercial antipyretic analgesic tablet showed the usefulness of their analysis method (see Fig. 18). The best separation conditions were found to be at pH 11 and a surfactant concentration of 0.05 M sodium dodecyl sulfate (SDS). It was concluded that MEKC is a viable alternative to LC. It should be noted that the precision was better than 1.4% RSD for the quantitative determination of the three ingredients studied in the commercial tablet.

Thormann *et al.* have investigated the use of MEKC with on column, fast-scanning multiwavelength UV absorption detection for the analysis of illicit drugs in human urine, direct sample injection of substituted purines in human body fluids, and various barbiturates in human serum (plasma) and urine (Thorman and Wernly, 1992; Thormann *et al.*, 1991, 1993; Wernly and Thormann, 1991; 1992a,b). They successfully demonstrated the need for multiwavelength detection over a relatively wide wavelength range as a means of peak confirmation in MEKC analyses by comparison of absorption spectra. The three-dimensional electropherograms, which are generated with this type of detection, can also provide valuable information on the requirement and appropriateness of sample pretreatment procedures.

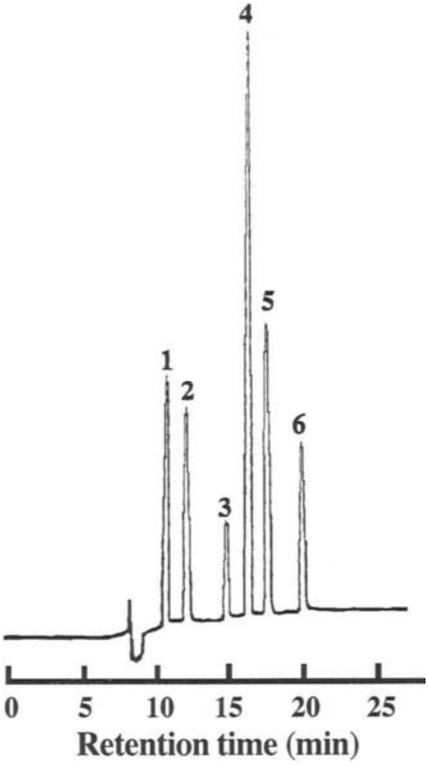


Fig. 18. Chromatogram shows the separation of the main ingredients found in antipyretic analgesics. Experimental conditions: 0.02 M phosphate buffer, 0.05 M SDS, pH 11. Capillary (0.1-mm i.d.; 0.15-mm o.d.) length: 80 cm. with detection 50 cm. from anode. Solute identification: 1, anhydrous caffeine; 2, p-acetamidophenol; 3, acetylsalicylic acid; 4, salicylamide; 5 *o*-ethoxybenzamide; 6, ethyl *p*-aminobenzoate (internal standard). (Reproduced with permission from Fujiwara and Honda, 1987.)

Figure 19 shows three-dimensional electropherograms and their respective single wavelength electropherograms for the analysis of various drugs. MEKC was found to be suitable for the confirmation testing of illicit drugs as it was highly specific, not giving false-positive results when samples were tested. In a separate study, MEKC was compared to LC for the determination of illicit drugs (Weinberger and

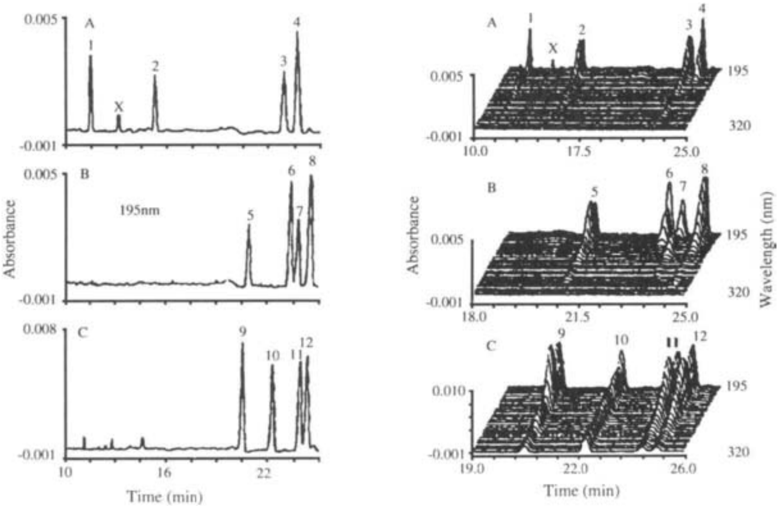


Fig. 19. Electropherograms, with single and multiwavelength UV detection, of three model mixtures of different drugs. Experimental conditions: 0.006 M sodium borate/0.010 M sodium phosphate buffer, 0.075 M SDS, pH 9.1. Capillary (75 μm i.d.) length: 90 cm. with detection 70 cm. from the anode. Sample concentrations: 20 μg/ml. Applied voltage: 20 kV (76-80 μA). Solute Identification: 1, benzoylecgonine; 2, morphine; 3, heroin; 4, methamphetamine; 5, codeine; 6, amphetamine; 7, cocaine; 8, methadone; 9, methaqualone; 10, flunitrazepam; 11, oxazepam; 12, diazepam; X, impurity of benzoylecgonine, benzoic acid. (Reproduced with permission from Wernly and Thormann, 1991.)

Lurie, 1991). It was found, for a complex mixture consisting of acidic and neutral impurities present in an illicit heroin seizure sample, that MEKC was able to resolve twice as many peaks in comparison to LC. In the same study it was also determined that illicit cocaine and its basic impurities could be analyzed by MEKC without the significant tailing that is normally seen with reversed-phase liquid chromatography (RPLC) using bonded-phase columns. Overall, MEKC gave significantly better efficiency, selectivity, and peak symmetry, as well as shorter analysis times in comparison to that obtained with LC.

The MEKC analysis of vitamins and selected metabolites has received a great deal of attention (Burton *et al.*, 1986a; Fujiwara *et al.*, 1988; Swaile *et al.*, 1988; Kobayashi *et al.*, 1989; Nishi *et al.*, 1989c; Ong *et al.*, 1991d; Swartz, 1991). Capillary electrophoresis (CE) is sufficient for the analysis of water soluble vitamins as long as the buffer pH is such that the vitamins possess a net charge. When neutral vitamins or other analytes are part of the sample, MEKC has to be used. Fat-soluble vitamins A and E were analyzed in a sample that also contained water soluble vitamins (Ong *et al.*, 1991d). The separation was accomplished using γ-cyclodextrin; this technique has been abbreviated CD-MEKC.

Drugs that are sparingly soluble in water and uncharged should be solubilized using the surfactant system employed as opposed to the use of organic solvents. The addition of organic solvent to the sample can affect the capacity factor (k'), the pseudo-effective mobilities, and the resolution of the separation (Ackermans *et al.*, 1991). Ahuja and Foley have investigated the effect of organic solvents in the sample matrix in terms of separation efficiencies and it was determined that the use of organic solvents such as acetonitrile to aid in solute solubilization can greatly diminish separation efficiencies (Ahuja and Foley, 1994c).

7.2. Nucleic Acids

Nucleic acids are a class of biopolymers that are present in nearly all cells and carry the genetic information necessary for cell reproduction. They are classified into two groups—deoxyribonucleic (DNA) and ribonucleic acids (RNA). Both DNA and RNA are polymers of a basic repeating unit called a nucleotide. Separations of these high molecular weight and long-chained polymers are generally complex and vary depending on the type of nucleic acid moiety.

MEKC was used in the separation of 14 adducted and modified nucleic acid constituents (Row *et al.*, 1987). Resolution of these analytes was accomplished in less than 40 min. The high speed separation of some mono-, di-, and triphosphorylated ribonucleosides using SDS and two cationic surfactants HTAB and DTAB (hexadecyltrimethylammonium bromide and dodecyltrimethylammonium bromide, respectively) was explored (Liu *et al.*, 1989). The effect of various surfactant structures can be seen in Fig. 20. Note the that analysis time is within 4 min. The authors concluded that the use of DTAB in the buffer phase provided for considerable improvement in resolution over CE alone and MEKC with anionic surfactants. The analysis of normal and chemically modified nucleobases, nucleosides, and nucleotides has also been studied (Lecoq *et al.*, 1991). Deoxyribonucleotide oligomers were resolved on the basis of their chain length, base sequence, and base modification using MEKC (Griest *et al.*, 1988). In 1990, Lahey and coworkers reported a superior separation of 14 nucleosides and nucleotides with MEKC as opposed to that obtained

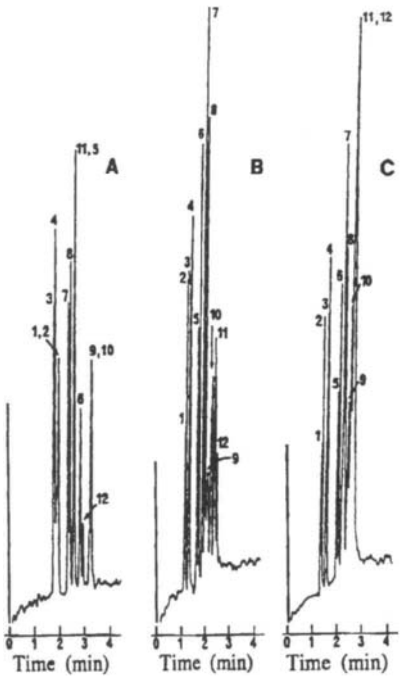


Fig. 20. Effect of surfactant on separation of phosphorylated nucleosides. Buffer: 10 mM Tris, 10 mM Na_2HPO_4, pH = 7.05, (A) 0.1 M SDS, (B) 0.1 M DTAB, (C) 0.1 M HTAB. Capillary: 45 cm in length (25 cm to detector). Injection: 5 kV, 2 s. Operating voltage: (A) +18 kV, (B) and (C) −18 kV. Best separation corresponds to buffer phase consisting of 0.1 M DTAB. Peaks: 1, CMP; 2, UMP; 3, GMP; 4, AMP; 5, CDP; 6, UDP; 7, GDP; 8, ADP; 9, CTP; 10, UTP; 11, GTP; 12, ATP. (Reproduced with permission from Liu *et al.*, 1989)

with ion-pairing liquid chromatography (IPLC) (Lahey and St. Claire, 1990). The optimized IPLC separation could not adequately resolve half of the compounds investigated while the MEKC separation of these analytes provided sufficient resolution within 40 minutes. A recent study which employed glucose as an additive to the micellar system facilitated the total separation of a mixture of nine nucleosides (Kaneta *et al.*, 1992). The addition of glucose extended the elution range and improved the separation selectivity. This was especially effective for nucleosides possessing low capacity factors. Carbohydrate additives could provide a useful means of controlling solute selectivity. Further investigations are needed to fully understand the effect and potential of these types of additives in MEKC. MEKC is somewhat limited in that the small size of the micelles does not provide for the solubilization of large biopolymers. We suggest the use of mixed micellar systems such as Brij 35/SDS where the micelles are much larger, as a possible pseudostationary phase for the analysis of larger biopolymers. Addition of bivalent cations to SDS did permit the separation of deoxypolythymidines up to the 18-mer, presumably through a micelle-cation-oligonucleotide adsorption phenomenon (Cohen *et al.*, 1987). The short analysis time afforded through the use of MEKC is an attractive alternative to other methods of analysis for these types of samples.

7.3. Peptides

The discussion here will be limited to small polypeptides. Table 9 lists numerous applications of MEKC to the analysis of derivatized amino acids. The analysis of peptides with MEKC has been primarily restricted to oligopeptides. Separation of peptides containing 20 or more amino acid residues suffers from the same limitations mentioned previously for large biopolymers. Nine oligopeptides were separated using an alkaline buffer that contained 200 mM SDS (Brownlee and Compton, 1988). Oligonucleotides that differed in structure by only their neutral amino acid residues were studied using an SDS micellar system (Wainright, 1990). These analytes could not be separated with conventional CE. Upon addition of the nonionic octyl glucoside to the buffer system, improved resolution was seen between two heptapeptides (Swedburg, 1990).

Angiotensin analogs (cationic peptides) were analyzed with two different cationic surfactants (HTAB, DTAB) (Liu *et al.*, 1990). The surfactants were added to the micellar system in quantities that were both above and below their respective cmc values and the resulting separations were compared. SDS based separations of OPA-derivatized peptides were also presented.

Polymyxins (polypeptides) were separated using a zwitterionic surfactant, PAPS (3-(N,N-dimethylhexadecylammonium)propanesulfonate), as an additive to the running buffer (Kristensen and Hansen, 1993). The proposed method was deemed useful for both qualitative and quantitative measurements on polymyxins. It was also suggested that the developed method is valuable for testing the purity of the polymyxins.

Recently, two publications have appeared in the literature that examine the separation of closely related, polypeptides. The separation of angiotensin II, its derivatives and motilins was accomplished using a micellar pseudo-stationary phase that contained the nonionic surfactant Tween 20 (Matsubara and Terabe, 1992). Polypeptides with similar electrophoretic mobilities were separated using acetonitrile modified mobile phases (Yashima *et al.*, 1992). The optimum concentration of acetonitrile was determined to be 13% (v/v) for the separation of [Met-13]motilin and [Leu-13]motilin as can be seen in Fig. 21. This particular study also examined the relationship between the size of the peptides and the strength of the interaction between thepeptides and the micelle. The authors concluded that it is difficult to separate peptides which consist of more than 20 amino acid residues without using organic modifiers.

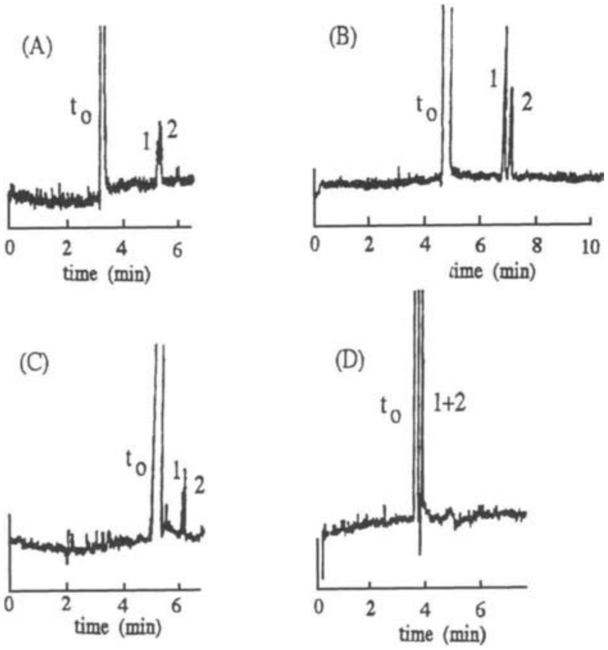


Fig. 21. Separation of motilins by MEKC with acetonitrile: (A) 5% acetonitrile; (B) 13% acetonitrile; (C) 20% acetonitrile; (D) 25% acetonitrile; (1) [Met^{13}]-motilin; (2) [Leu^{13}]motilin. Conditions: capillary, 650 mm × 0.05 mm i.d., 500 mm to detector; electrophoretic solutions, 25 mM phosphate–200 mM borate buffer (pH 7.0) containing 10 mM CTAB and acetonitrile at various concentrations; temperature, ambient; detection wavelength, 215 nm. (Reproduced with permission from Yashima *et al.*, 1992.)

7.4. Other Applications

Many applications performed using electrokinetic chromatography, particularly MEKC, are listed in Table 9. This extensive list not only serves to highlight applications in EKC but also displays the diversity of the numerous analyses. The majority of the applications performed to date have occurred in the last five to six years. As EKC continues to grow and mature as an analytical technique, it is safe to assume that the number of applications utilizing EKC will also increase.

8. Conclusions

Micellar electrokinetic capillary chromatography (MEKC) is a rapidly growing area within capillary electrophoresis, largely due to its ability to simultaneously separate charged, neutral, and/or chiral compounds in a highly efficient manner. Although the thermodynamic aspects of resolution are reasonably well understood, a comparable understanding of the kinetic aspects (band-broadening in EKC has yet to be attained. We can expect the applications of MEKC to continue to grow almost exponentially and to remain somewhat ahead of our theoretical understanding, particularly for charged solutes and/or more complex EKC systems. We look forward to the increase in our comprehension of EKC that future research will provide.

- See **Recent Developments** (added in press) following references.

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9. References

Ackermans, M. T., F. M. Everaerts and J. L. Beckers (1991). *J. Chromatogr. 585*, 123–131.
Ackermans, M. T., F. M. Everaerts and J. L. Beckers (1992). *J. Chromatogr. 606*, 229–235.
Ahuja, E. S. and J. P. Foley (1994a). *Analyst 119*, 353–360.
Ahuja, E. S. and J. P. Foley (1994b). *J. Chromatogr. A 680*, 73–83.

Ahuja, E. S. and J. P. Foley (1994c). "Effect of Sample Solvent on Reproducibility in Micellar Electrokinetic Chromatography," Fifth Annual Frederick Conference on Capillary Electrophoresis, Frederick, MD.

Ahuja, E. S., E. L. Little and J. P. Foley (1992). *J. Liq. Chromatogr. 15*, 1099–1113.

Ahuja, E. S., B. P. Preston and J. P. Foley (1994). *J. Chromatogr. B 657*, 271–284.

Aiken, J. H. and C. W. Huie (1993). *J. Microcol. Sep. 5*, 95–99.

Albin, M., R. Weinberger, E. Sapp and S. Morig (1991). *Anal. Chem. 63*, 417–422.

Almgren, M., F. Grieser and J. K. Thomas (1979). *J. Amer. Chem. Soc. 101*, 279–291.

Altria, K. D. and N. W. Smith (1991). *J. Chromatogr. 538*, 506–509.

Amankwa, L. N. and W. G. Kuhr (1991). *Anal. Chem. 63*, 1733–1737.

Armstrong, D. W. (1987a). *Chromatogr. Forum 2*, 43–48.

Armstrong, D. W. (1987b). *Anal. Chem. 59*, 84A–91A.

Balchunas, A. T. and M. J. Sepaniak (1987). *Anal. Chem. 59*, 1466–1470.

Balchunas, A. T. and M. J. Sepaniak (1988). *Anal. Chem. 60*, 617–621.

Balchunas, A. T., D. F. Swaile, A. C. Powell and M. J. Sepaniak (1988). *Sep. Sci. Tech. 23*, 1891–1904.

Berridge, J. C. (1985). *Techniques for the Automated Optimization of HPLC Separations.* Wiley, New York.

Bevan, C. D., I. M. Mutton and A. J. Pipe (1993). *J. Chromatogr. 636*, 113–123.

Bidlingmeyer, B. A. and J. F. V. Warren (1984). *Anal. Chem. 56*, 1583A–1596A.

Bjergegaard, C., S. Michaelsen and H. Sorensen (1992). *J. Chromatogr. 608*, 403–411.

Brownlee, R. G. and S. W. Compton (1988). *Amer. Biotechn. Lab. 6*, 10.

Burgi, D. S. and R.-L. Chien (1991). *J. Microcol. Sep. 3*, 199–202.

Burton, D. E., M. J. Sepaniak and M. P. Maskarinec (1986a). *J. Chromatogr. Sci. 24*, 347–351.

Burton, D. E., M. J. Sepaniak and M. P. Maskarinec (1986b). *Chromatographia 21*, 583–586.

Bushey, M. M. and J. W. Jorgenson (1989a). *J. Microcol. Sep. 1*, 125–130.

Bushey, M. M. and J. W. Jorgenson (1989b). *Anal. Chem. 61*, 491–493.

Cai, J. and Z. El Rassi (1992). *J. Chromatogr. 608*, 31–45.

Caslavska, J., S. Lienhard and W. Thormann (1993). *J. Chromatogr. 638*, 335–342.

Castagnola, M., D. V. Rossetti, L. Cassiano, R. Rabino, G. Nocca and B. Giardina (1993). *J. Chromatogr. 638*, 327–334.

Charman, W. N., A. J. Humberstone and S. A. Charman (1992). *Pharm. Res. 9,* 1219–1223.

Chien, R. L. (1991). *Anal. Chem. 63*, 2866–2869.

Chien, R.-L. and J. C. Helmer (1991). *Anal. Chem. 63*, 1354–1361.

Chien, R.-L. and D. S. Burgi (1992). *Anal. Chem. 64*, A489–A496.

Cohen, A. S., S. Terabe, J. A. Smith and B. L. Karger (1987). *Anal. Chem. 59*, 1021–1027.

Cole, R. O. and M. J. Sepaniak (1992). *LC GC 10*, 380–385.

Cole, R. O., M. J. Sepaniak, W. L. Hinze, J. Gorse and K. Oldiges (1991). *J. Chromatogr. 557*, 113–123.

Cole, R. O., R. D. Holland and M. J. Sepaniak (1992). *Talanta 39*, 1139–1147.

Corradini, D. and C. Corradini (1992). *J. Chromatogr. 624*, 503–509.

Corran, P. H. and N. Sutcliffe (1993). *J. Chromatogr. 636*, 87–94.

Dang, Q. X., Z. P. Sun and D. K. Ling (1992). *J. Chromatogr. 603*, 259–266.

Dang, Q. X., L. X. Yan, Z. P. Sun and D. K. Ling (1993). *J. Chromatogr. 630*, 363–369.

Davis, J. M. (1989). *Anal. Chem. 61*, 2455–2461.

Delaguardia, M., E. Periscardells, J. Sancenon, J. L. Carrion and E. Pramauro (1991). *Microchem. J. 44*, 193–200.

Desbene, P. L., C. Rony, B. Desmazieres and J. C. Jacquier (1992). *J. Chromatogr. 608*, 375–383.

Desiderio, C. and S. Fanali (1992). *Electrophoresis 13*, 698–700.

Fanali, S. (1989). *J. Chromatogr. 474*, 441–446.

Fanali, S. and P. Bocek (1990). *Electrophoresis 11*, 757–760.

Foley, J. P. (1990a). *Anal. Chim. Acta 231*, 237–247.

Foley, J. P. (1990b). *Anal. Chem. 62*, 1302–1308.

Foley, J. P. (1991). *Analyst 116*, 1275–1279.

Foley, J. P. (1992). Seventh Middle Atlantic Regional Analytical Chemistry Conference (MARACC VII), Newark, DE.

Foley, J. P. and J. G. Dorsey (1983). *Anal. Chem. 55*, 730–737.

Foley, J. P. and W. E. May (1987). *Anal. Chem. 59*, 102–109.

Fujiwara, S. and S. Honda (1987). *Anal. Chem. 59*, 2773–2776.

Fujiwara, S., S. Iwase and S. Honda (1988). *J. Chromatogr. 447*, 133–140.

Gebauer, P., W. Thormann and P. Bocek (1992). *J. Chromatogr. 608*, 47–57.

Ghowsi, K., J. P. Foley and R. J. Gale (1990). *Anal. Chem. 62*, 2714–2721.

Giddings, J. C. (1991). *Unified Separation Science.* John Wiley & Sons, Inc., New York.

Gorse, J., A. T. Balchunas, D. F. Swaile and M. J. Sepaniak (1988). *HRC 11*, 554–558.

Griest, W. H., M. P. Maskarinec and K. H. Row (1988). *Sep. Sci. Technol. 23*, 1905.

Grossman, P. D. and J. C. Colburn (1992). *Capillary Electrophoresis—Theory And Practice.* Academic Press, New York.

Grushka, E. (1991). *J. Chromatogr. 559*, 81–93.

Gurira, R. C. and P. W. Carr (1982). *J. Chromatogr. Sci. 22*, 461–465.

Hayes, M. A. and A. G. Ewing (1992). *Anal Chem. 64*, 512–516.

Hayes, M. A. and A. G. Ewing (1993). *Anal Chem. 65*, 2407.

Harvey, S. D., R. M. Bean and H. R. Udseth (1992). *J. Microcol. Sep. 4*, 191–198.

Hinze, W. L. and D. W. Armstrong, Ed. (1987). *Ordered Media in Chemical Separations.* American Chemical Society, Washington, DC.

Hjertén, S. (1985). *J. Chromatogr. 347*, 191–198.

Holland, R. D. and M. J. Sepaniak (1993). *Anal. Chem. 65*, 1140–1146.

Imasaka, T., K. Nishitani and N. Ishibashi (1991). *Analyst 116*, 1407–1409.

Imasaka, T., K. Nishitani and N. Ishibashi (1992). *Anal. Chim Acta 256*, 3–7.

Ishihama, Y. and S. Terabe (1993). *J. Liq. Chromatogr. 16*, 933–944.

Issaq, H. J., G. M. Janini, I. Z. Atamna, G. M. Muschik and J. Lukszo (1992). *J. Liq. Chromatogr. 15*, 1129–1142.

Janini, G. M. and H. J. Issaq (1992). *J. Liq. Chromatogr. 15*, 927–960.

Jeansonne, M. S. and J. P. Foley (1991). *J. Chromatogr. Sci. 29*, 258–266.

Jeansonne, M. S. and J. P. Foley (1992). *J. Chromatogr. 594*, 1–8.

Jorgenson, J. W. and K. D. Lukacs (1981). *Anal. Chem. 53*, 1298–1302.

Kajiwara, H. (1991). *J. Chromatogr. 559*, 345–356.

Kaneta, T., S. Tanaka, M. Taga and H. Yoshida (1992). *J. Chromatogr. 609*, 369–374.

Karger, B. L., L. R. Snyder and C. Horváth (1973). *Introduction to Separation Science.* John Wiley & Sons, Inc., New York.

Karger, B. L., J. N. LePage and N. Tanaka (1980). In: *High-Performance Liquid Chromatography: Advances and Perspectives* (C. Horváth, ed.), Vol. 1, Academic Press, New York, pp. 113–206.

Khaledi, M. G. and A. H. Rodgers (1990). *Anal. Chim. Acta 239*, 121–128.

Khaledi, M. G., S. C. Smith and J. K. Strasters (1991). *Anal. Chem. 63*, 1820–1830.

Kleibohmer, W., K. Cammann, J. Robert and E. Mussenbrock (1993). *J. Chromatogr. 638*, 349–356.

Kobayashi, S., T. Ueda and M. Kikumoto (1989). *J. Chromatogr. 480*, 179–184.

Kord, A. S., J. K. Strasters and M. G. Khaledi (1991). *Anal. Chim. Acta 246*, 131–137.

Kristensen, H. K. and S. H. Hansen (1993). *J. Chromatogr. 628*, 309–315.

Kuhn, R. and S. Hoffstetter-Kuhn (1992). *Chromatographia 34*, 505–512.

Kuhn, R., F. Stoecklin and F. Erni (1992). *Chromatographia 33*, 32–36.

Kuhr, W. G. (1990). *Anal. Chem. 62*, R403–R414.

Kuhr, W. G. and C. A. Monnig (1992). *Anal. Chem. 64*, 389R–407R.

Lahey, A. and R. L. St. Claire (1990). *Amer. Lab. 22*, 68,70.

Lam, S. (1984). *J. Chromatogr. Sci. 22*, 416–423.

Lecoq, A. F., C. Leuratti, E. Marafante and S. Dibiase (1991). *HRC 14*, 667–671.

Lecoq, A., S. Dibiase and L. Montanarella (1993a). *J. Chromatogr. 638*, 363–373.

Lecoq, A., L. Montanarella and S. Di Biase (1993b). *J. Microcol. Sep. 5*, 105–118.

Lee. C. S., W. C. Blanchard and C. T. Wu (1990). *Anal. Chem. 62*, 1519–1523.

Lee, K. J., G. S. Heo, N. J. Kim and D. C. Moon (1992). *J. Chromatogr. 608*, 243–250.

Li, S. F. Y. (1992). *Capillary Electrophoresis—Principles, Practice, and Applications*. Elsevier Science Publishers B.V., London.

Little, E. L. and J. P. Foley (1992). *J. Microcol. Sep. 4*, 145–154.

Liu, J., J. F. Banks and M. Novotny (1989). *J. Microcol. Sep. 1*, 136–141.

Liu, J., K. A. Cobb and M. Novotny (1990). *J. Chromatogr. 519*, 189.

Lloyd, D. K. (1992). *Anal. Proc. 29*, 169–170.

Love, L. J. C., J. G. Habarta and J. G. Dorsey (1984). *Anal. Chem. 56*, 1132A–1148A.

Lukkari, P., J. Jumppanen, T. Holma, H. Siren, K. Jinno, H. Elo and M. L. Riekkola (1992a). *J. Chromatogr. 608*, 317–323.

Lukkari, P., J. Jumppanen, K. Jinno, H. Elo and M. L. Riekkola (1992b). *J. Pharm. Biomed. Anal. 10*, 561–565.

Lukkari, P., H. Siren, M. Pantsar and M. L. Riekkola (1993). *J. Chromatogr. 632*, 143–148.

Lurie, I. S. (1992). *J. Chromatogr. 605*, 269–275.

Matsubara, N. and S. Terabe (1992). *Chromatographia 34*, 493–496.

Michaelsen, S., P. Moller and H. Sorensen (1992). *J. Chromatogr. 608*, 363–374.

Miyake, M., A. Shibukawa and T. Nakagawa (1991). *J. High Resol. Chromatogr. 14*, 181.

Miyashita, Y. and S. Terabe (1990). *Chromatogram 11*, 6–7.

Miyashita, Y., S. Terabe and H. Nishi (1990). *Chromatogram 11*, 7–8.

Morin, P., F. Villard, A. Quinsac and M. Dreux (1992). *HRC 15*, 271–275.

Morin, P., F. Villard and M. Dreux (1993). *J. Chromatogr. 628*, 153–160.

Mysels, K. J. and L. H. Princen (1959) *J. Phys. Chem. 63*, 1696–1700.

Nakagawa, T., T. Oda, A. Shibukawa and H. Tanaka (1988). *Chem. Pharm. Bull. 36*, 1622.

Nakagawa, T., T. Oda, A. Shibukawa, H. Fukuda and H. Tanaka (1989). *Chem. Pharm. Bull. 37*, 707.

Ng, C. L., H. K. Lee and S. F. Y. Li (1992a). *J. Chromatogr. Sci. 30*, 167–170.

Ng, C. L., C. P. Ong, H. K. Lee and S. F. Y. Li (1992b). *Chromatographia 34*, 166–172.

Nickless, G. (1985). *J. Chromatogr. 313*, 129–159.

Nielen, M. W. F. and M. J. A. Mensink (1991). *HRC 14*, 417–419.

Nielsen, K. R. and J. P. Foley (1993). *J. Microcol. Sep. 5*, 347–360.

Nielsen, K. R. and J. P. Foley (1994). *J. Chromatogr.*, in press.

Nishi, H., T. Fukuyama, M. Matsuo and S. Terabe (1989a). *J. Microcol. Sep. 1*, 234–241.

Nishi, H., N. Tsumagari, T. Kakimoto and S. Terabe (1989b). *J. Chromatogr. 477*, 259–270.

Nishi, H., N. Tsumagari, T. Kakimoto and S. Terabe (1989c). *J. Chromatogr. 465*, 331–343.

Nishi, H., N. Tsumagari and S. Terabe (1989d). *Anal. Chem. 61*, 2434–2439.

Nishi, H., T. Fukuyama, M. Matsuo and S. Terabe (1990a). *J. Chromatogr. 515*, 233–243.

Nishi, H., T. Fukuyama, M. Matsuo and S. Terabe (1990b). *J. Chromatogr. 513*, 279–295.

Nishi, H., T. Fukuyama, M. Matsuo and S. Terabe (1990c). *J. Chromatogr. 498*, 313–323.

Nishi, H., T. Fukuyama and S. Terabe (1991). *J. Chromatogr. 553*, 503–516.

Northrop, D. M., D. E. Martire and W. A. MacCrehan (1991). *Anal. Chem. 63*, 1038–1042.

Northrop, D. M. and W. A. MacCrehan (1992). *J. Liq. Chromatogr. 15*, 1041–1063.

Okafo, G. N. and P. Camilleri (1992). *Analyst 117*, 1421–1424.

Okafo, G. N. and P. Camilleri (1993). In: *Capillary Electrophoresis: Theory and Practice* (P. Camilleri, ed.), Vol. 1, pp. 164–199. CRC, Boca Raton, FL.

Okafo, G. N., C. Bintz, S. E. Clarke and P. Camilleri (1992). *J. Chem. Soc. Chem. Commun. 17*, 1189–1192.

Ong, C. P., C. L. Ng, N. C. Chong, H. K. Lee and S. F. Y. Li (1990). *J. Chromatogr. 516*, 263–270.

Ong, C. P., C. L. Ng, N. C. Chong, H. K. Lee and S. F. Y. Li (1991a). *Environ. Monit. Assess. 19*, 93–103.

Ong, C. P., C. L. Ng, H. K. Lee and F. Y. Li (1991b). *J. Chromatogr. 547*, 419–428.

Ong, C. P., C. L. Ng, H. K. Lee and S. F. Y. Li (1991c). *J. Chromatogr. 588*, 335–339.

Ong, C. P., S. F. Pang, S. P. Low, H. K. Lee and S. F. Y. Li (1991d). *J. Chromatogr. 559*, 529–536.

Otsuka, K. and S. Terabe (1989). *J. Microcol. Sep. 1*, 150–154.

Otsuka, K. and S. Terabe (1990). *J. Chromatogr. 515*, 221–226.

Otsuka, K. and S. Terabe (1993). *J. Liq. Chromatogr. 16*, 945–953.

Otsuka, K., S. Terabe and T. Ando (1985a). *J. Chromatogr. 348*, 39–47.

Otsuka, K., S. Terabe and T. Ando (1985b). *J. Chromatogr. 332*, 219–226.

Otsuka, K., J. Kawahara, K. Tatekawa and S. Terabe (1991). *J. Chromatogr. 559*, 209–214.

Pietta, P. G., P. L. Mauri, A. Rava and G. Sabbatini (1991). *J. Chromatogr. 549*, 367–373.

Pietta, P., A. Bruno, P. Mauri and A. Rava (1992a). *J. Chromatogr. 593*, 165–170.

Pietta, P., C. Gardana and P. Mauri (1992b). *HRC 15*, 136–139.

Pietta, P., P. Mauri, R. M. Facino and M. Carini (1992c). *J. Pharm. Biomed. Anal. 10*, 1041–1045.

Pietta, P., P. Mauri, A. Bruno and L. Zini (1993). *J. Chromatogr. 638*, 357–361.

Pruñonosa, J., R. Obach, A. Diez-Cascon and L. Gouesclou (1992a). *J. Chromatogr. 581*, 219–226.

Pruñonosa, J., R. Obach, A. Diez-Cascon and L. Gouesclou (1992b). *J. Chromatogr. 574*, 127–133.

Rasmussen, H. T. and H. M. McNair (1989). *HRC 12*, 635–636.

Rasmussen, H. T., L. K. Goebel and H. M. McNair (1990). *J. Chromatogr. 517*, 549–555.

Rasmussen, H. T., L. K. Goebel and H. M. McNair (1991). *HRC 14*, 25–28.

Rosen, M. J. (1989). *Surfactants and Interfacial Phenomena.* John Wiley & Sons, New York.

Row, K. H. (1989). *Korean J. Chem. Eng. 6*, 179–184.

Row, K. H., W. H. Griest and M. P. Maskarinec (1987). *J. Chromatogr. 409*, 193–203.

Saitoh, T., H. Hoshino and T. Yotsuyanagi (1989). *J. Chromatogr. 469*, 175–181.

Saitoh, K., C. Kiyohara and N. Susuki (1991a). *Anal. Sci. 7*, 269–272.

Saitoh, T., H. Hoshino and T. Yotsuyanagi (1991b). *Anal. Sci. 7*, 495–497.

Saitoh, T., N. Ojima, H. Hoshino and T. Yotsuyanagi (1992). *Mikrochim Acta 106*, 91–99.

Sakodinskaya, I. K., C. Desiderio, A. Nardi and S. Fanali (1992). *J. Chromatogr. 596*, 95–100.

Sandra, P. and J. Vindevogel (1992). *Introduction to Micellar Electrokinetic Chromatography.* Hüthig, Heidelberg, Germany.

Schoenmakers, P. J. (1986). *Optimization of Chromatographic Selectivity: Guide to Method Development.* Elsevier, Amsterdam.

Sepaniak, M. J. and R. O. Cole (1987). *Anal. Chem. 59*, 472–476.

Sepaniak, M. J., D. E. Burton and M. P. Maskarinec (1987). In: *Ordered Media in Chemical Separations* (A. S. Ser., ed.), Vol. 342, pp. 142–151.

Sepaniak, M. J., D. F. Swaile, A. C. Powell and R. O. Cole (1990). *HRC 13*, 679–682.

Sepaniak, M. J., R. O. Cole and B. K. Clark (1992). *J. Liq. Chromatogr. 15*, 1023–1040.

Sigma Chemical Company (1993). *Catalog*, p.561.

Snopek, J., H. Soini, M. Novotny, E. Smolková-Keulemansová and I. Jelinek (1991). *J. Chromatogr. 559*, 215–222.

Snopek, J., I. Jelinek and E. Smolková-Keulemansová (1992). *J. Chromatogr. 609*, 1–17.

Snyder, L. R. and J. J. Kirkland (1979). *Introduction to Modern Liquid Chromatography.* John Wiley & Sons, Inc., New York.

Soini, H., T. Tsuda and M. V. Novotny (1991). *J. Chromatogr. 559*, 547–558.

Spino, L. A. and D. W. Armstrong (1987). In: *Ordered Media in Chemical Separations* (W. L. Hinze and D. W. Armstrong, ed.), Vol. 342. American Chemical Society, Washington, D.C., pp. 235–246.

Stefansson, M. and D. Westerlund (1993). *J. Chromatogr. 632*, 195–200.

Sutcliffe, N. and P. H. Corran (1993). *J. Chromatogr. 636*, 95–103.

Swaile, D. F., D. E. Burton, A. T. Balchunas and M. J. Sepaniak (1988). *J. Chromatogr. Sci. 26*, 406–409.

Swartz, M. (1991). *J. Liq. Chromatogr. 14*, 923–938.

Swedburg, S. A. (1990). *J. Chromatogr. 503*, 449–452.

Szucs, R., J. Vindevogel and P. Sandra (1991). *HRC 14*, 692–693.

Szucs, R., J. Vindevogel, P. Sandra and L. C. Verhagen (1993). *Chromatographia 36*, 323–329.

Terabe, S. (1989). *Trends Anal. Chem. 8*, 129–134.

Terabe, S. (1992). *J. Pharm. Biomed. Anal. 10*, 705–715.

Terabe, S. and T. Isemura (1990a). *J. Chromatogr. 515*, 667–676.

Terabe, S. and T. Isemura (1990b). *Anal. Chem. 62*, 650–652.

Terabe, S., K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando (1984). *Anal. Chem. 56*, 111–113.

Terabe, S., K. Otsuka and T. Ando (1985a). *Anal. Chem. 57*, 834–841.

Terabe, S., H. Ozaki, K. Otsuka and T. Ando (1985b). *J. Chromatogr. 332*, 211–217.

Terabe, S., M. Shibata and Y. Miyashita (1989a). *J. Chromatogr. 480*, 403–411.

Terabe, S., K. Otsuka and T. Ando (1989b). *Anal. Chem. 61*, 251–260.

Terabe, S., Y. Miyashita, O. Shibata, E. R. Barnhart, L. R. Alexander, D. G. Patterson, B. L. Karger, K. Hosoya and N. Tanaka (1990). *J. Chromatogr. 516*, 23–31.

Terabe, S., Y. Miyashita, Y. Ishihama and O. Shibata (1993). *J. Chromatogr. 636*, 47–55.

Thormann, W. and P. Wernly (1992). *J. Chromatogr. 608*, 251.

Thormann, W., P. Meier, C. Marcolli and F. Binder (1991). *J. Chromatogr. 545*, 445–460.

Thormann, W., A. Minger, S. Molteni, J. Caslavska and P. Gebauer (1992). *J. Chromatogr. 593*, 275–288.

Thormann, W., S. Lienhard and P. Wernly (1993). *J. Chromatogr. 636*, 137–148.

Towns, J. K. and F. E. Regnier (1991). *Anal. Chem. 63*, 1126–1132.

Tran, A. D., T. Blanc and E. J. Leopold (1990). *J. Chromatogr. 516*, 241–249.

Ueda, T., F. Kitamura, R. Mitchell, T. Metcalf, T. Kuwana and A. Nakamoto (1991). *Anal. Chem. 63*, 2979–2981.

Ueda, T., R. Mitchell, F. Kitamura, T. Metcalf, T. Kuwana and A. Nakamoto (1992). *J. Chromatogr. 593*, 265–274.

Verschuere, M., P. Sandra and F. David (1992). *J. Chromatogr. Sci. 30*, 388–391.

Vindevogel, J. and P. Sandra (1991a). *HRC 14*, 795–801..

Vindevogel, J. and P. Sandra (1991b). *Anal. Chem. 63*, 1530–1536.

Vindevogel, J. and P. Sandra (1992). *Introduction to Micellar Electrokinetic Chromatography*. Hüthig, Heidelberg.

Vindevogel, J., P. Sandra and L. C. Verhagen (1990). *J. High Resolut. Chromatogr. 13*, 295.

Wainer, I. W. (1986). *Chromatography Forum 1*, 55–61.

Wainright, A. (1990). *J. Microcol. Sep. 2*, 166–175.

Wallingford, R. A. and A. G. Ewing (1988a). *Anal. Chem. 60*, 258–263.

Wallingford, R. A. and A. G. Ewing (1988b). *J. Chromatogr. 441*, 299–309.

Wallingford, R. A. and A. G. Ewing (1989). *Anal. Chem. 61*, 98–100.

Wallingford, R. A., P. D. Curry and A. G. Ewing (1989). *J. Microcol. Sep. 1*, 23–27.

Weinberger, R. and I. S. Lurie (1991). *Anal. Chem. 63*, 823–827.

Weinberger, R., E. Sapp and S. Moring (1990). *J. Chromatogr. 516*, 271–285.

Wernly, P. and W. Thormann (1991). *Anal. Chem. 63*, 2878–2882.

Wernly, P. and W. Thormann (1992a). *J. Chromatogr. 608*, 251–256.

Wernly, P. and W. Thormann (1992b). *Anal. Chem. 64*, 2155–2159.
Wu, C. T., C. S. Lee and C. J. Miller (1992a). *Anal. Chem. 64*, 2310–2311.
Wu, C. T., T. Lopes, B. Patel and C. S. Lee (1992b). *Anal. Chem. 64*, 886–891.
Wu, Q., H. A. Claessens and C. A. Cramers (1992c). *Chromatographia 34*, 25–30.
Yashima, T., A. Tsuchiya, O. Morita and S. Terabe (1992). *Anal. Chem. 64*, 2981–2984.
Yeo, S. K., H. K. Lee and S. F. Y. Li (1991). *J. Chromatogr. 585*, 133–137.
Yik, Y. F., C. L. Ng, C. P. Ong, S. B. Khoo, H. K. Lee and S. F. Y. Li (1990). *Bull. Singapore Natl. Inst. Chem. 18*, 91–100.
Yik, Y. F., H. K. Lee, S. F. Y. Li and S. B. Khoo (1991a). *J. Chromatogr. 585*, 139–144.
Yik, Y. F., C. P. Ong, S. B. Khoo, H. K. Lee and S. F. Y. Li (1991b). *Environ. Monit. Assess., 19*, 73–81.
Yik, Y. F., H. K. Lee and S. F. Y. Li (1992a). *HRC 15*, 198–200.
Yik, Y. F., C. P. Ong, S. B. Khoo, H. K. Lee and S. F. Y. Li (1992b). *J. Chromatogr. 589*, 333–338.
Zhang, C. X., Z. P. Sun, D. K. Ling and Y. J. Zhang (1992). *J. Chromatogr. 627*, 281–286.
Zhao, J. Y., D. Y. Chen and N. J. Dovichi (1992). *J. Chromatogr. 608*, 117–120.
Zweigenbaum, J. (1990). *Chromatogram 11*, 9–10.

10. More Recent Developments

Since the initial writing of this chapter there have been numerous advances in EKC. From the use of liposomes as pseudostationary phases in EKC (see Fig. 22) (Ahuja and Foley, unpublished results) and the suspension of chromatographic particles as pseudophases in EKC (Bachmann *et al.*, 1994) to the advent of affinity electrokinetic chromatography (Nishi *et al.*, 1994; Tanaka *et al.*, 1994; Tanaka and Terabe, 1995) the developments have been rapid. *Commercially available chiral surfactants* (Mazzeo *et al.*, 1994) should further promote the use of MEKC for chiral separations. The elegant use of linear solvation energy relationships (LSER) by Yang and Khaledi (Yang and Khaledi, 1995a,b) to model solute-surfactant interactions in MEKC should provide important information about the nature of solute interactions with different types of surfactant aggregates and aid in characterizing the important interactions that control migration patterns in MEKC. Eventually this could lead to classification of surfactants based on their chemical selectivity.

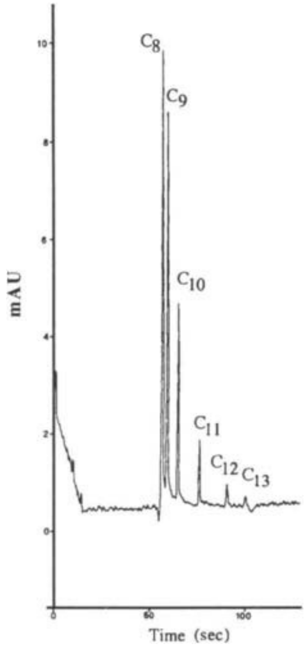

Fig. 22. Separation of alkylphenone homologs (C_8–C_{13}) using liposomes as a pseudostationary phase in EKC. Liposomes: 50:50 mixture of L-α-phosphatidyl choline (egg) and L-α-phosphatidyl glycerol (egg). Running buffer: 25 mM HEPES (pH 7); applied voltage: 30 kV; operating current: 8 mA; Hydrodynamic injection: 20 s.

Although we cannot discuss in detail the breadth and scope of these latest advancements at this time, we refer the reader to several reviews that have appeared in the literature (Deyl *et al.*, 1994; Monnig and Kennedy, 1994; Oefner and Chiesa, 1994; Rogan *et al.*, 1994; Sadecka *et al.*, 1994; Shimada and Mitamura, 1994; Smith and Evans, 1994; Szulc and Krull, 1994; Terabe *et al.*, 1994; Timerbaev *et al.*, 1994; Watzig and Dette, 1994; Bruin and Paulus, 1995; Cancalon, 1995; Issaq and Chan, 1995; Karger *et al.*, 1995; Landers, 1995; Nishi and Terabe, 1995; Tomas-Barberan, 1995; Xu, 1995) as well as other recently published texts (Camilleri, 1993; Landers, 1994; Baker, 1995).

In the table that follows, recent applications utilizing EKC are presented. This is not a comprehensive listing of all the applications in recent years, but hopefully it will provide references to several key applications.

Table 10. Selected Applications in MEKC and EKC

Analyte of Interest	Comments	Reference
1. Anti-HIV agents	MEKC separation of michellamines A and B and two other structurally related monomers found in the extract of the Ancistrocladus plants	(Chan *et al.*, 1994a)
2. Artificial sweeteners	MEKC analysis of aspartame and saccharin as well as other food additives	(Thompson *et al.*, 1995)
3. Benzodiazepines	Ten benzodiazepines baseline resolved with 25 mM SDS, 20% MeOH(v/v), 75 mM glycine-250 mM triethanolamine buffer	(Bechet *et al.*, 1994)
	Confirmation testing of drugs in human urine	(Schafroth *et al.*, 1994)
4. Cardiovascular drugs	A micellar electrokinetic chromatography method was optimized for the separation of the six cardiovascular drugs atenolol, nicardipine, nifedipine, diltiazem, verapamil, and amlodipine	(Bretnall and Clarke, 1995)
5. Cephalosporins	MEKC used in separation of cephalosporin antibiotics	(Sciacchitano *et al.*, 1994)
6. Clinical drugs	Ovomucoid used as a chiral-pseudostationary phase in EKC	(Ishihama *et al.*, 1994)
	Affinity electrokinetic chromatography	(Nishi *et al.*, 1994; Tanaka *et al.*, 1994; Tanaka and Terabe, 1995)

cont'd.

Table 10 cont'd.

Analyte of Interest	Comments	Reference
7. Cocaine and related substances	Quantitative MEKC method developed	(Trenerry *et al.*, 1994) (Krogh *et al.*, 1994)
8. Corticosteriods	Mixtures of bile salt surfactants and/or SDS used to separate a group of corticosteriods	(Bumgarner and Khaledi, 1994)
9. 4-aminobenzonitrile carbohydrate derivatives	Optimum resolution achieved with 100 mM SDS, Tris-phosphate buffer, pH 7.5	(Schwaiger *et al.*, 1994)
10. Dansylated amino acids	Low temperature separation of 23 dansylated amino acids	(Skocir *et al.*, 1994)
11. Estrogens	Sex hormone estrogens separated by MEKC	(Chan *et al.*, 1995)
	Urinary estrogens	(Ji *et al.*, 1995)
12. Fatty acids	C_8 to C_{20} fatty acids separated using MEKC with indirect detection	(Erim *et al.*, 1995)
13. FITC and DTAF Amino acids	MEKC with LIF detection	(Lalljie and Sandra, 1995)
14. Food Grade antioxidants	MEKC used to separate four major groups of food grade antioxidants	(Hall *et al.*, 1994)
15. Glycosaminoglycan disaccharides	Separation and determination of glycosaminoglycan (GAG) disaccharides units without derivatization using MEKC	(Michaelsen *et al.*, 1993)

cont'd.

Table 10 cont'd.

Analyte of Interest	**Comments**	**Reference**
16. Herbicides	Linuron, metolachlor, atrazine and metsulfuron analyzed using MEKC	(Dinelli *et al.*, 1994)
	Mega-borate complex surfactants (adjustable surface charge density)	(Smith and El Rassi, 1994a,b; Smith *et al.*, 1994)
	Microemulsion EKC and Micellar EKC	(Song *et al.*, 1995)
17. Honey flavonoids	Thirteen honey flavonoids separated by MEKC	(Delgado *et al.*, 1994)
18. Metallothionein isoforms	The charge different isoforms MT-1 and MT-2 from rats, rabbits and sheep separated in 9–12 min using MEKC (SDS)	(Beattie and Richards, 1994)
19. Non-steroidal anti-inflammatory drugs	CE and MEKC analysis of ibuprofen, indomethacin, ketoprofen, piroxicam and diclofenac	(Donato *et al.*, 1994)
	Fifteen non-steriodal anti-inflammatory drugs separated by MEKC	(Maboundou *et al.*, 1994)
	Quantitative determination	(Bechet *et al.*, 1995)
20. Peptides (small)	Mixed fluorocarbon hydrocarbon anionic surfactants in MEKC	(Ye *et al.*, 1995)
21. Phenol derivatives	Suspension of chromatographic particles used as a pseudophase in EKC	(Bachmann *et al.*, 1994)
22. Plasma samples	SDS used to complex with proteins, effect of buffer pH is evaluated	(Watzig and Lloyd, 1995)

cont'd.

Table 10 cont'd.

Analyte of Interest	Comments	Reference
23. Polycyclic aromatic hydrocarbons (PAHs)	Resorcarenes were studied as new pseudostationary phases in EKC	(Bachmann *et al.*, 1995)
24. Porphyrins	Resolution of type I, II, III, and IV isomers of coproporphyrin as well recent advancements	(Wu *et al.*, 1994a,b)
25. PTH-amino acids	CTAB-SDS mixed surfactant system improves separation	(Ong *et al.*, 1994)
26. Sunscreen agents	UV-A and UV-B determined in cosmetic products	(Pietta *et al.*, 1995)
27. Taxol and related compounds		(Chan *et al.*, 1994b)
28. Xanthine derivatives	Quantitative MEKC was applied to determine impurities (caffeine and xanthine)	(Korman *et al.*, 1994)

11. References (Recent Developments)

Bachmann, K., A. Bazzanella, I. Haag, K. Y. Han, R. Arnecke, V. Bohmer and W. Vogt (1995). *Anal. Chem., 67*, 1722–1726.

Bachmann, K., B. Gottlicher, I. Haag, K. Y. Han, W. Hensel and A. Mainka (1994). *J. Chromatogr. A, 688*, 283–292.

Baker, D. R. (1995). *Capillary Electrophoresis.* Wiley, New York.

Beattie, J. H. and M. P. Richards (1994). *J. Chromatogr. A., 664*, 129–134.

Bechet, I., M. Fillet, P. Hubert and J. Crommen (1994). *Electrophoresis, 15*, 1316–1321.

Bechet, I., M. Fillet, P. Hubert and J. Crommen (1995). *J. Pharm. Biomed. Anal., 13*, 497–503.

Bretnall, A. E. and G. S. Clarke (1995). *J. Chromatogr. A., 700*, 173–178.

Bruin, G. J. M. and A. Paulus (1995). *Anal. Methods Instrum., 2*, 3–26.

Bumgarner, J. G. and M. G. Khaledi (1994). *Electrophoresis, 15*, 1260–1266.

Camilleri, P., Ed. (1993). *Capillary Electrophoresis: Theory and Practice*. Boca Raton, CRC Press.

Chan, K. C., F. Majadly, T. G. Mccloud, G. M. Muschik, H. J. Issaq and K. M. Snader (1994a). *Electrophoresis, 15*, 1310–1315.

Chan, K. C., G. M. Muschik, H. J. Issaq and P. K. Siiteri (1995). *J. Chromatogr. A, 690*, 149–154.

Chan, K. C., G. M. Muschik, H. J. Issaq and K. M. Snader (1994b). *HRC-J.High Resolut. Chromatogr., 17*, 51–52.

Delgado, C., F. A. Tomasbarberan, T. Talou and A. Gaset (1994). *Chromatographia, 38*, 71–78.

Deyl, Z., F. Tagliaro and I. Miksik (1994). *J. Chromatogr. B, Biomed. Appl. 656*, 3–27.

Dinelli, G., A. Bonetti, P. Catizone and G. C. Galletti (1994). *J. Chromatogr. B, Biomed. Appl., 656*, 275–280.

Donato, M. G., W. Baeyens, W. Vandenbossche and P. Sandra (1994). *J. Pharmaceut. Biomed. Anal., 12*, 21–26.

Erim, F. B., X. Xu and J. C. Kraak (1995). *J. Chromatogr. A, 694*, 471–479.

Hall, C. A., A. Zhu and M. G. Zeece (1994). *J. Agr. Food Chem., 42*, 919–921.

Ishihama, Y., Y. Oda, N. Asakawa, Y. Yoshida and T. Sato (1994). *J.Chromatogr. A, 666*, 193–201.

Issaq, H. J. and K. C. Chan (1995). *Electrophoresis, 16*, 467–480.

Ji, A. J., M. F. Nunez, D. Machacek, J. E. Ferguson, M. F. Iossi, P. C. Kao and J. P. Landers (1995). *J. Chromatogr. B, Biomed. Appl., 669(1)*, 15–26.

Karger, B. L., Y. H. Chu and F. Foret (1995). *Annu. Rev. Biophys. Biomol. Struc., 24*, 579–610.

Korman, M., J. Vindevogel and P. Sandra (1994). *Electrophoresis, 15*, 1304–1309.

Krogh, M., S. Brekke, F. Tonnesen and K. E. Rasmussen (1994). *J.Chromatogr. A, 674*, 235–240.

Lalljie, S. P. D. and P. Sandra (1995). *Chromatographia, 40*, 513–518.

Landers, J. P., Ed. (1994). *Handbook of Capillary Electrophoresis*. Boca Raton, CRC Press.

Landers, J. P. (1995). *Clin. Chem., 41*, 495–509.

Maboundou, C. W., G. Paintaud, M. Berard and P. R. Bechtel (1994). *J. Chromatogr. B, Biomed. Appl., 657*, 173–183.

Mazzeo, J. R., E. R. Grover, M. E. Swartz and J. S. Peterson (1994). *J.Chromatogr. A, 680*, 125–135.

Michaelsen, S., M. Schroder and H. Sorensen (1993). *J. Chromatogr. A, 652*, 503–515.

Monnig, C. A. and R. T. Kennedy (1994). *Anal. Chem. 66*, R280–R314.

Nishi, H. and S. Terabe (1995). *J. Chromatogr. A, 694*, 245–276.

Nishi, H., K. Nakamura, H. Nakai, T. Sato and S. Terabe (1994). *Electrophoresis, 15*, 1335–1340.

Oefner, P. J. and C. Chiesa (1994). *Glycobiology, 4*, 397–412.

Ong, C. P., C. L. Ng, H. K. Lee and S. F. Y. Li (1994). *Electrophoresis, 15*, 1273–1275.

Pietta, P. G., A. Bruno, P. L. Mauri and C. Gardana (1995). *J. Pharmaceut. Biomed. Anal., 13*, 229–235.

Rogan, M. M., K. D. Altria and D. M. Goodall (1994). *Chirality, 6*, 25–40.

Sadecka, J., J. Polonsky and H. Shintani (1994). *Pharmazie, 49*, 631–641.

Schafroth, M., W. Thormann and D. Allemann (1994). *Electrophoresis, 15*, 72–78.

Schwaiger, H., P. J. Oefner, C. Huber, E. Grill and G. K. Bonn (1994). *Electrophoresis, 15*, 941–952.

Sciacchitano, C. J., B. Mopper and J. J. Specchio (1994). *J. Chromatogr. B, Biomed. Appl., 657*, 395–399.

Shimada, K. and K. Mitamura (1994). *J. Chromatogr. B, Biomed. Appl., 659*, 227–241.

Skocir, E., J. Vindevogel and P. Sandra (1994). *Chromatographia, 39*, 7–10.

Smith, J. T. and Z. El Rassi (1994a). *Electrophoresis, 15*, 1248–1259.

Smith, J. T. and Z. El Rassi (1994b). *J. Chromatogr. A, 685*, 131–143.

Smith, N. W. and M. B. Evans (1994). *J. Pharm. Biomed. Anal. 12*, 579–611.

Smith, J. T., W. Nashabeh and Z. El Rassi (1994). *Anal. Chem., 66*, 1119–1133.

Song, L. G., Q. Y. Ou, W. L. Yu and G. Z. Li (1995). *J. Chromatogr. A, 699*, 371–382.

Szulc, M. E. and I. S. Krull (1994). *J. Chromatogr. A, 659*, 231–245.

Tanaka, Y. and S. Terabe (1995). *J. Chromatogr. A, 694*, 277–284.

Tanaka, Y., N. Matsubara and S. Terabe (1994). *Electrophoresis, 15*, 848–853.

Terabe, S., K. Otsuka and H. Nishi (1994). *J. Chromatogr. A, 666*, 295–319.

Thompson, C. O., V. C. Trenerry and B. Kemmery (1995). *J. Chromatogr. A, 694*, 507–514.

Timerbaev, A., O. Semenova and G. Bonn (1994). *Chromatographia, 38*, 255–258.

Tomas-Barberan, F. A. (1995). *Phytochem. Anal., 6*, 177–92.

Trenerry, V. C., J. Robertson and R. J. Wells (1994). *Electrophoresis, 15*, 103–108.

Watzig, H. and C. Dette (1994). *Pharmazie, 49*, 83–96.

Watzig, H. and D. K. Lloyd (1995). *Electrophoresis, 16*, 57–63.

Wu, N., G. E. Barker and C. W. Huie (1994a). *J. Chromatogr. A, 659*, 435–442.

Wu, N., B. H. Li and J. V. Sweedler (1994b). *J. Liq. Chromatogr., 17*, 1917–1927.

Xu, Y. (1995). *Anal. Chem., 67*, R463–R473.

Yang, S. Y. and M. G. Khaledi (1995a). *Anal. Chem., 67*, 499–510.

Yang, S. Y. and M. G. Khaledi (1995b). *J. Chromatogr. A, 692*, 301–310.

Ye, B., M. Hadjmohammadi and M. G. Khaledi (1995). *J. Chromatogr. A, 692*, 291–300.

Part Two: Detection

CHAPTER 4

Optical Detection Strategies in Capillary Electrophoresis

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1. General Introduction

Detection is a primary concern in capillary electrophoresis (CE) due to the small dimensions associated with the capillary columns and the small injection volumes. The most practical approach for detection in capillary electrophoresis is to detect the material directly within the separation column to preserve the high separation efficiencies by minimizing extracolumn effects. Conventional high performance liquid chromatography (LC) detectors employ volumes much larger than that appropriate for CE analysis. Optical detection is particularly attractive for CE because any optical technique can be performed directly within the capillary column. The flow cell for the detector is made by simply removing a section of the polyimide coating surrounding the capillary. The polymer coating can be removed by burning, heating or chemical methods, producing an optical window which allows the interrogating light to enter the capillary column. In addition, a significant fraction of the light can be efficiently introduced into the capillary and focused to spots with diameters on the order of 5–100 µm, producing detection volumes smaller than the capillary column used for the separation.

Another advantage of optical detection is the nondestructive nature associated with most optical methods. In mass spectrometric or electrochemical detection, the analyte is altered chemically via ionization, oxidation or reduction. For optical detection, the analyte is altered only transiently and returns to its native form allowing the components to be collected and re-analyzed by ancillary techniques. However, permanent chemical alterations of the analyte can occur in some instances due to photobleaching, which involves converting a species in an excited electronic state into a different chemical form. Photobleaching is particularly prevalent when tightly focused laser beams are used in the optical detection method. There are available many different types of optical detection schemes that can be implemented to suit the type of analysis to be performed. The two most important criteria that must be addressed when considering an appropriate detection strategy are the sensitivity (limits of detection) and selectivity needed for the analysis. In addition, the linearity of the response with concentration is often a concern.

A high degree of selectivity results in the ability to detect the component of interest in a rather complex matrix with few interferences from that matrix. Fluorescence is particularly well suited, especially when visible excitation is utilized, since few compounds demonstrate intrinsic fluorescence in this region of the spectrum. A dichotomy arises between selectivity and the universality of the detection scheme, with higher selectivity resulting in few components that can be detected. Therefore, in some cases, chemical modification of the components to be analyzed must be performed in order to obtain the necessary analytical signal. If universal detection is required, one can resort to UV absorption or refractive index detection due to the fact that most compounds possess a chromophoric group that will absorb UV radiation or have a refractive index different from that of the buffered system used in the analysis. UV absorption is probably the most prevalent commercially available detection strategy associated with CE due to its far-reaching applicability.

The limit of detection is the minimal amount of material that will yield a signal significantly greater than the background and, in most cases, is a paramount concern associated with choosing a detection scheme for CE. It is typically defined as the amount of material that yields a signal 2–3 times greater than the peak-to-peak fluctuations in the background (noise). The concern for high detection sensitivity results from the fact that minute amounts of material are typically loaded onto the column. For example, if one uses a 5 s electroinjection at 5 kV onto a capillary column 50 cm in length with a diameter of 50 μm and an apparent solute mobility of 1.5×10^{-4} $cm^2/V \cdot s$, only approximately 1.5 nl of material is introduced. If the molar concentration is 1 μM, the amount of material loaded onto the column is 1.5 fmol.

Table 1 gives typical detection limits for CE using several different optical detection strategies as well as electrochemical and mass spectrometric detection. It is evident that direct fluorescence is the most sensitive detection scheme currently available for CE, with a mass detection limit in the range of 300–500 × 10^{-24} moles (several hundred molecules). Although UV absorption is the most popular detection strategy, it suffers from rather poor detection limits. This is primarily due to the short optical pathlength of the capillary column when using on-column detection. Another general trend is that detectors that are not selective (more universal) are generally less sensitive. For example, refractive index detection gives the poorest detection limits, but it is the most universal.

Table 1. Representative Detection Limits in CE Using Various Detection Strategies

Detection Mode	Mass detection limit (amol)	Reference
Refractive index	1.2×10^{4a}	Bruno *et al.* (1991)
UV absorption	1.8×10^{4}	Walbroehl and Jorgenson (1984)
Thermo-optical	5.0×10^{1}	Yu and Dovichi (1989)
Fluorescence	5.0×10^{-4}	Flanagan *et al.* (1995)
Indirect fluorescence	5.0×10^{1}	Yeung and Kuhr (1991)
Raman[b]	1.7×10^{5}	Chen and Morris (1988)
Electrochemical (amperometric)	7.0×10^{-1}	Wallingford and Ewing (1988)
Mass spectrometric	1.0×10^{-1}	Smith *et al.* (1988)

[a] Determined with no applied voltage to the capillary column. The detection sensitivity will degrade dramatically with applied voltage.
[b] Estimated using an injection volume of 67 nl.

Linearity of the observed response with concentration is often essential in order to obtain quantitative information from the electropherogram. Scott (1977) has defined a function in order to determine the linearity of the detector response with concentration:

$$\log y = \log A + r \log C \tag{1}$$

where y is the detector response, C is the concentration of the analyte in the determination, A is a constant and r represents the response index of the detector. For detectors with ideal linearity, r = 1. Detectors that give r values in the range $0.98 \leq r \geq 1.02$ were arbitrarily considered by Scott to be linear detectors. If r is not in this predetermined range, these detectors can still be used to obtain quantitative information , but calibration graphs are required.

In some instances it is beneficial to employ a detection method that will reveal structural information about unknown components in the electropherogram to aid in identification. Electronic spectra, UV absorption or fluorescence, for example, typically offer little information with regard to molecular structure due to the broad, featureless attributes of the spectra. Mass spectroscopy provides a tremendous amount of structural information not available from electronic spectra or refractive index detection. If structural information is necessary from optical detection, Raman detection is an alternative. Typically, Raman spectroscopy does not offer particularly good detection limits, but can yield vibrational information similar, in most cases, to that obtained from infrared spectroscopy without the interferences associated with the solvent that make IR intractable as a detection scheme for CE. Definitive identification of unknown components often requires information from several different spectroscopies. Optical detection, due to its nondestructive nature, can be coupled to another detection mode to yield complementary structural information required for conclusive identification of an unknown. For example, Raman detection could be used to obtain a vibrational spectrum of an unknown, followed by off-column mass spectrometric analysis.

We will, in this chapter, introduce the reader to several different optical detection strategies that have been utilized in CE. We will begin with a general theoretical description of the optical technique and then discuss instrumental requirements associated with it. Finally, design considerations and current instrumental approaches to CE detection implementing each optical spectroscopy will be presented.

2. Refractive Index Detection

2.1. Introduction

The ability to observe a signal from an analyte using refractive index detection does not require a chromophoric group on the molecule and, therefore, represents a universal optical detection strategy in CE. The only requirement for observing a response is that the sample must possess a refractive index different from that of the solvent. Since a wide range of compounds can produce a signal, chemical derivatization is not necessary, but the electropherogram is frequently complex, with many peaks present from interferences within the sample matrix. In addition, the limits of refractive index detection are poor compared to those of UV absorption or fluorescence detection.

Several different types of refractive index detectors have been implemented in HPLC detection schemes and have been reviewed by Yeung (1986). The most common type for CE applications is deflection. In this method, light rays entering the capillary column are refracted or their propagation direction altered as a result of a change in the refractive index of the solution filling the interior of the capillary tube. The angle of refraction can be calculated via Snell's law:

$$n_1 \sin \theta_1 = n_2 \sin \theta_2 \tag{2}$$

where $n_{1,2}$ are the refractive indices of the material on either side of the interface and $\theta_{1,2}$ are the angles of the beam with respect to the normal of the interface. When using a capillary tube, there are actually four interfaces that will cause refraction of the incoming light rays. These interfaces, which are defined as the junction point between two materials with difference refractive indices, occur when the light beam travels from air into the glass, then into the solution core of the capillary and through the same two interfaces on the opposite side as it leaves the capillary. When a laser beam is used as the light source and is focused to a diameter much smaller than the diameter of the capillary column, an interference pattern of the incoming light is generated (Bornhop and Dovichi, 1986). If a small-area photodiode is place on one of the fringes of the interference pattern in the far-field, a change in the refractive index will cause a physical displacement or shift in the fringe which can be measured as a decrease in intensity on a photodetector. As shown by Synovec (1987), the magnitude of the deflection as a function of a change in refractive index (dq/dn) can be determined through the expression

$$\frac{dq}{dn} = \frac{2D}{rn^2} \tag{3}$$

where n is the refractive index of the solution in the capillary, D is the offset of the light ray from the capillary center axis and r is the inside radius of the capillary. Dovichi (1988) has shown that the detection limits for the deflection type refractive index detector are independent of the capillary column diameter (pathlength independent). Although the detector response is proportional to capillary diameter, the major noise component also increases with capillary diameter, resulting in no net increase in signal-to-noise for larger capillary diameters.

Major sources of noise associated with refractive index detection in CE applications include: (1) thermal fluctuations in the capillary due to Joule heating; (2) intensity and pointing instability of the light source; and (3) capillary movement resulting from changes in the electrical double layer on the interior wall of the column. The intensity and pointing instability in the light source can, to a certain degree, be minimized by using a dual-beam arrangement by splitting off part of the interrogating beam and focusing onto a monitoring photocell and subtracting the reference signal from the analytical signal. An approach to minimize thermal noise has been suggested (Chen *et al.*, 1989) in which the applied voltage is modulated (electrical modulation), resulting in changes in the velocity of the analytes within the capillary tube and substantially reducing noise components with frequencies outside the passband of the demodulated signal.

2.2. *Applications in CE*

An example of an on-column refractive index detector for CE is shown in Fig. 1. The light source is a He-Ne laser which is focused into the flow cell using a cylindrical lens, giving a beam waist of 23 × 600 μm. Using a 50 μm i.d. capillary column, the detection volume was estimated to be 1.2 nl. The fringe chosen for the measurement was selected by a mirror which directed the refracted light rays onto a photocell. A servo system was constructed in order to autozero the response by changing the position of the photocell. In order to simplify the fringe pattern generated by the focused laser beam and to help control the temperature in the capillary, the capillary was encased in a larger bore tube with a flat front face and a circular back face and filled with a fluid with a refractive index similar to that of fused silica. Ray tracing diagrams of this flow cell and the flat front and back face cell are shown in Fig. 2. The fringe pattern is simplified by reducing the number of rays refracted at the external capillary wall by immersing the capillary in a fluid of similar refractive index. This allows the selection of a fringe which demonstrates a large shift in position with small changes in the refractive index of the inner solution. The use of a circular back face results in all rays exiting the cell perpendicular to the cell. This avoids refraction and reduces noise associated with temperature fluctuations at this interface, which alters the propagation direction of the rays.

The noise level in this system, which results primarily from temperature fluctuations, was found to be on the order of 2.5×10^{-8} refractive index units; this corresponds to a thermal stability of 2.5×10^{-4}°C obtained with pressurized flow. When high voltages were applied to the capillary, increased noise in the baseline was observed, with the noise level found to increase with higher applied voltages due to Joule heating within the capillary. The concentration detec-

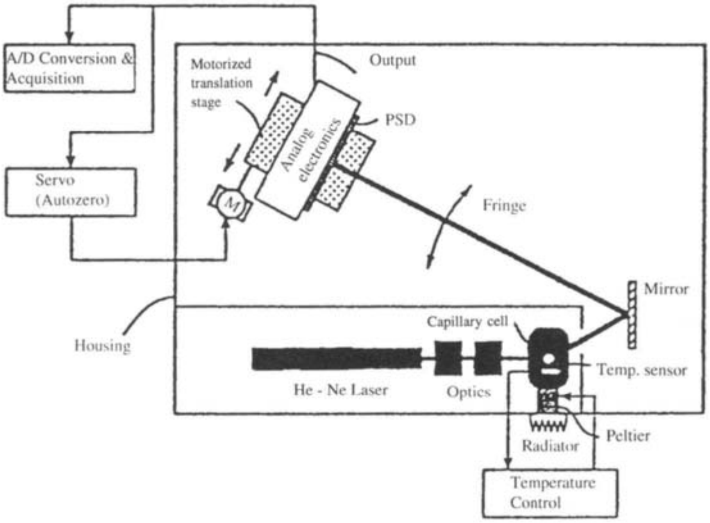


Fig. 1. Refractive index detector for CE. (Reproduced with permission from Bruno *et al.*, 1991).

tion limit for the carbohydrate saccharose was determined to be 10 μM (signal-to-noise ratio of 2) or 12 fmol for the 1.2 nl detection volume used here. However, these detection limits were reported for a pressure driven flow with no high voltage applied to the capillary. In addition, the authors state that at currents above 60 μA, corresponding to an applied voltage of approximately 15 kV for a 50 μm i.d. capillary tube and a 100 mM borate buffer, the noise levels were unacceptable for CE analysis of the saccharides using refractive index detection. Although the refractive index change increases with capillary diameter, the current within the capillary increases with the square of the capillary radius. This results in little gain in sensitivity with increasing capillary diameter.

3. UV Absorption Detection

3.1. Introduction

UV detection is one of the most commonly practiced optical detection methods in CE. Although it is not the most sensitive detection mode, most analytes possess chromophores that permit detection without the need for chemical derivatization prior to the analytical separation. As a consequence of the large number of compounds that absorb UV radiation, the electropherogram is frequently plagued

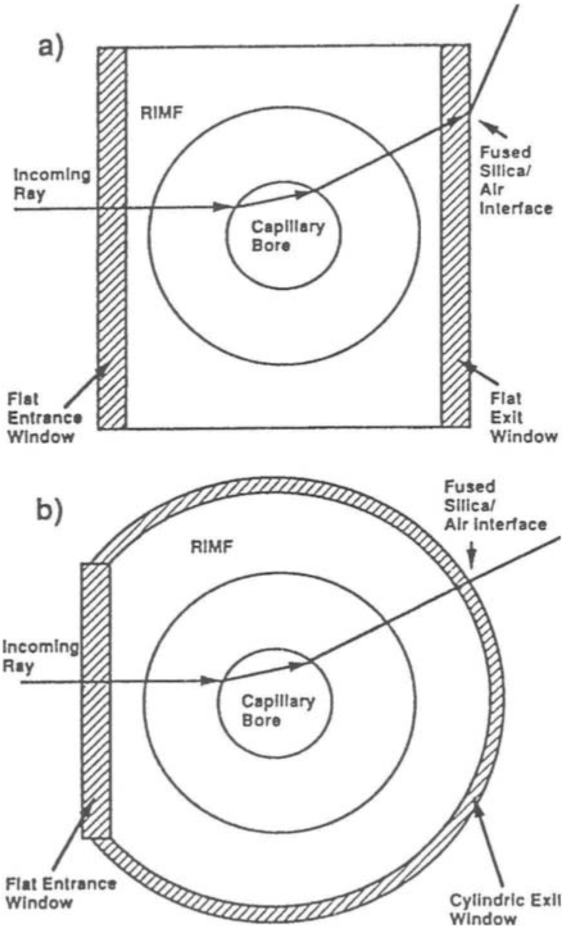


Fig. 2. Ray tracing diagram for light rays traveling through a capillary refractive index cell with (a) two flat faces and (b) one flat face and a circular back face. (Reproduced with permission from Bruno *et al., 1991*).

by interferences that may mask the components to be analyzed. Most commercial CE systems incorporate variable wavelength UV detectors due to their increased applicability. An important problem associated with UV detection is that the small dimensions of the capillary provide an optical pathlength that is exceedingly small compared to that of conventional HPLC. This results in small signals superimposed on a large background. In addition, postcolumn detection incorporating a conventional HPLC UV detector with an extended optical pathlength degrades the high separation efficiencies associated with CE.

In UV detection, photons from a light source impinge upon a sample and, if the energy of the photon corresponds to the energy difference between electronic levels of the molecule, that photon is absorbed and promotes an electron from the ground electronic state (S_0) to an excited electronic state (S_1). Most UV transitions in organic molecules depend on conjugation in the molecular structure and involve electrons from an occupied molecular orbital, typically a non-bonding p or bonding π-orbital, promoted to the next higher energy orbital, for example, an antibonding π^* or σ^* orbital. The probability of an electronic transition is related to the square of the dipole moment for that transition, which, in turn, is proportional to the change in the electronic charge distribution occurring during the excitation. The probability per second of a molecule absorbing a photon with energy $h\nu$ (h = Planck's constant and ν is the frequency of the photon) can be determined from the product of the number of photons per unit volume and the Einstein coefficient for induced absorption between electronic energy levels 0 and 1, B_{01}. B_{01} is related to the transition dipole of the molecule through the equation

$$B_{01} = \frac{8\pi^3}{3h^2c} | R_{01} | \tag{4}$$

where c is the speed of light and R_{01} is the transition dipole moment (for a transition to be allowed, R_{mn} must not equal zero). The more familiar term describing the transition probability is the absorption coefficient or extinction coefficient, which can be related to the oscillator strength (f) associated with that transition through the use of the equation

$$f_{01} = 4.32 \times 10^{-9} \int \varepsilon \, d\nu \tag{5}$$

where $\int \varepsilon \, d\nu$ is the integrated absorption band (wave-number scale) for the electronic transition between states 1 and 2. The oscillator strength, f_{01}, is related to the Einstein coefficient by the relation

$$f_{01} = B_{01} \frac{c^2 vmh}{\pi e^2} \tag{6}$$

where m is the mass of an electron and e is the charge of an electron. Typical transitions observed in organic molecules are $\pi \rightarrow \pi^*$ transitions that have large absorptivity values (ε_{max} > 10,000) and $n \rightarrow \pi^*$ transitions that are of lower energy than $\pi \rightarrow \pi^*$ transitions (occur at longer wavelengths) but display lower absorptivity values ($\varepsilon_{max} << 10{,}000$).

The analytical signal, absorption, can be determined through the use of the classical Beer-Lambert law, where the absorption is equal to the product of the concentration of the absorbing species (C), the optical pathlength (b) and the molar extinction coefficient (ε). If the concentration of the absorbing species is equal to 1 μM, and the optical pathlength is assumed to be 50 μm with a molar extinction coefficient of 20,000 cm^{-1} M^{-1}, the measured absorption would be 1×10^{-4} AU (absorption units). This simple calculation demonstrates the inherent problem associated with UV detection and capillary electrophoresis, namely the short optical pathlength. This calculation is not entirely correct, with the actual absorbance less than that calculated. The reason for this stems from the fact that the capillary columns are circular and the effective pathlength is less than the diameter of the capillary column. If the interrogating beam illuminates the entire inner region of the capillary, the effective pathlength (l_{eff}) is

$$l_{eff} = \frac{1}{2}\pi r \qquad (7)$$

where r is the radius of the capillary. Utilizing l_{eff} in the above calculation, the actual measured absorbance value is only 7.8×10^{-5} AU.

Several other parameters affect the sensitivity of UV detection, and the linearity of the response. Bruin and co-workers (1991) have recently presented theoretical equations which describe the transmission (T) of light through a small capillary tube in the presence of absorbing species, calculated using the following equation:

$$T = \frac{1}{2s}\int_{-s}^{s} \exp\{-2e'\ C\ (r^2 - x^2)^{1/2}\}\ dx \qquad (8)$$

where s is one-half the aperture width and controls the size of the light beam impinging on the capillary tube, ε' is ln 10 times the molar absorptivity and T is expressed as the ratio of N/N_0, where N is the total number of photons that leave the cell when absorption takes place and N_0 is the number of photons when no absorption takes place. The absorbance is calculated by taking the negative log of T. This equation is valid for the case where s is equal to or smaller than the inner radius of the capillary. For the case of s >> r, the following equation can be used to calculate the transmitted light:

$$T = \frac{s-r}{s} + \frac{1}{2s}\int_{-r}^{r} \exp\{-2e'\ C\ (r^2 - s^2)^{1/2}\}\ dx \qquad (9)$$

Numerical evaluation of these integrals allows one to calculate the expected absorbance as a function of concentration, aperture width or radius of the capillary column. Figure 3 shows the calculated absorbance as a function of the aperture width for a 50 μm i.d. capillary column (350 μm o.d.). As can be seen, a larger absorbance value is obtained for smaller aperture widths, due to the fact that a larger percentage of the light travels through the inner region of the cell, thus resulting in all of the light taking part in the absorption process. When the aperture width becomes larger than the inner diameter of the capillary, a significant fraction of the light is not involved in the absorption process. In the model put forth by these authors, refraction was not included. Neglect of refraction in this model will result in significant deviation of the experimental points from the predicted points at larger aperture widths.

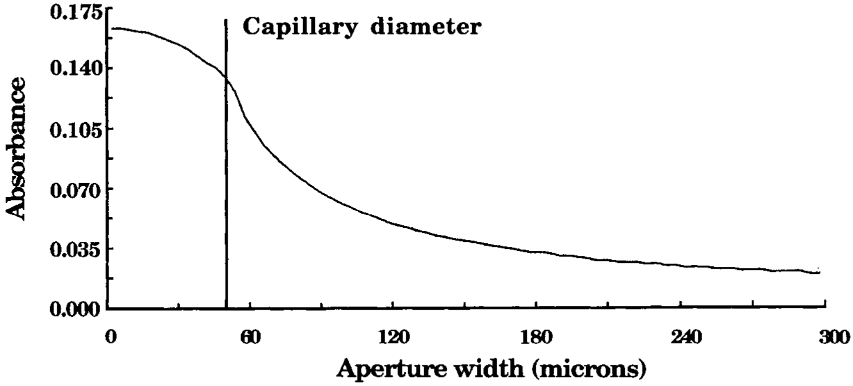


Fig. 3. Theoretical absorbance value as a function of the aperture width (s) for a 50 μm diameter capillary column. Values used in the calculation were; $C = 1 \times 10^{-4}$ M and $\varepsilon' = 75000$. For $s < r$, equation 8 was used in the calculation and for $s > r$, equation 9 was used.

Another interesting consequence of the aperture width with respect to the capillary radius is the effect on the linearity of the absorbance with concentration (Bruins *et al.*, 1991). The linearity of the absorbance occurs over a much greater concentration range for smaller aperture widths and decreases as the width gets larger. The limited linearity in the response for larger aperture widths is a consequence of the light rays being affected by the capillary tube itself, which acts as a converging lens. When $s \ll r$, the face of the capillary appears like a flat surface to the light rays impinging upon it, and the rays go through the capillary unaltered in their direction of travel. For $s \geq r$, the direction of travel is severely altered and focused to a point beyond the outer diameter of the capillary, affecting the amount of light striking the photodetector and resulting in nonlinear calibration plots.

3.2. *Instrumental considerations*

A schematic of a simple double-beam UV-vis spectrophotometer is shown in Fig. 4. The source is either a deuterium (UV) or tungsten (visible) lamp which is imaged onto a grating (monochromator) in order to select the appropriate wavelength for the measurement. After selection of the wavelength of the light, a rotating sector mirror (which is a half reflective and half transparent device) directs the light onto either the reference or sample cells. Although one can use a single-beam device, a double-beam instrument significantly reduces drift in the light source and detector. In addition, the double-beam arrangement continuously monitors the ratio I/I_0, where I is the light intensity passing through the sample and reaching the detector, and I_0 is the light intensity passing through the reference. This permits easy calculation of the absorbance by taking the negative log of this ratio. Absorption due to the solvent can also be accounted for using the double-beam arrangement. The use of a double-beam configura-

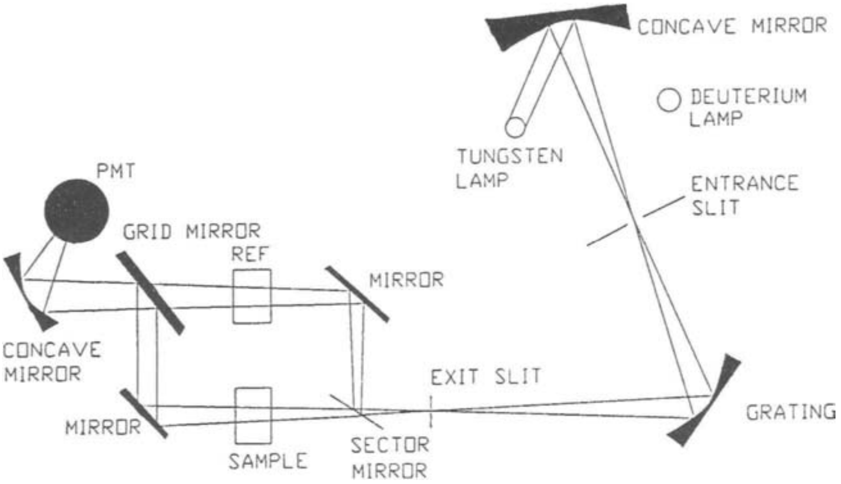


Fig. 4. Block diagram of a simple double-beam UV/vis absorption spectrometer.

tion can be very beneficial in CE applications, due to refraction and reflection of the light on the circular capillary column. The light from the reference and sample cells is detected on a single photomultiplier tube and converted into a voltage, from which the absorbance is determined from the ratio of these signals. The above configuration is typically called a double-beam in time arrangement, since the signals from the reference and sample are separated in time by the rotating sector mirror. A double-beam instrument in space uses two different photomultiplier tubes with the light from the source split evenly between the reference and sample cells.

Absorption is always measured on a large background from the source. The signal arises from absorption of a small percentage of those photons, with the number of photons absorbed determined by the concentration of the absorbing species. The major noise component in optical spectroscopies is shot noise. This noise varies with the magnitude of the current fluctuations (noise) related to the square root of the average output current (number of photons striking the detector). The larger the background, the larger these fluctuations become. For low concentration measurements, it is advantageous to lower the intensity of the lamp on the sample in order to reduce the magnitude of the current fluctuations. However, this has the effect of lowering the dynamic range of the measurement (higher concentrations of the absorbing species result in nonlinearities in the measured absorbance values).

3.3. Applications in CE

A representative design of an UV detector for on-column detection in CE is shown in Fig. 5. The light source used in these experiments was a 7 W cadmium pen lamp that emits radiation on two different sides, one side serving as the sample beam and the other the reference beam. In order to control the width of the light such that the majority of the photons impinge upon the inner region of the capillary, two 100 μm pinholes were positioned on either side of the penlamp. The capillary tube (75 μm i.d.) was located directly behind the pinholes (in this particular application, the reference capillary was not used). Two photomultiplier tubes were incorporated into the arrangement with 229 nm interference filters placed directly in front of the capillaries to select the appropriate observation wavelengths. The outputs of both photomultipliers were digitized and stored on a computer, from which the absorbance values were calculated after the electropherogram had been run. Using this system, the minimum detected amounts of the test analytes acridine, lysozyme and quinoline were 820, 18 and 120 fmol, respectively, at a signal-to-noise

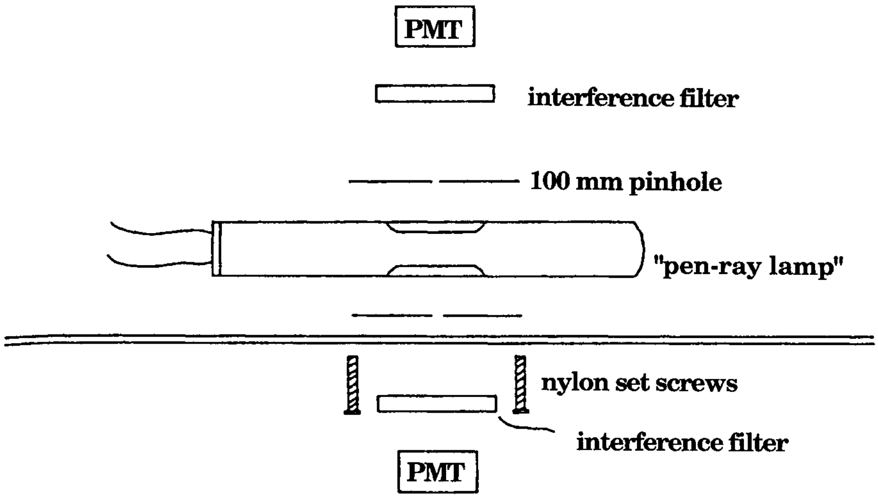


Fig. 5. Diagram of an on-column UV absorption detector for CE. (Reproduced with permissionf fromWalbroehl and Jorgenson 1984).

ratio of two. The linear dynamic range was found to be approximately four orders of magnitude for the test analytes. One interesting observation from this study was that the detection sensitivity was a function of the voltage applied to the capillary tube, with higher voltages resulting in lower detection sensitivity. For example, an applied voltage of 30 kV resulted in detection sensitivity about one-half that when no voltage was applied to the capillary. Degradation in the detection sensitivity at high applied voltages was a consequence of vibrations induced in the capillary under high voltage operation, which causes increases in the peak-to-peak fluctuations in the background. Greater precaution in mounting of the capillary tube would rectify this problem.

Bruins *et al.* (1991) have recently made a critical comparison between different optical arrangements of UV detectors in terms of the linearity of response, sensitivity and band broadening in CE. The three designs investigated included: (1) on-column detection with an aperture to control the width of the interrogating light, (2) on-column detection with a focusing lens placed directly in front of the capillary, and (3) off-column detection in a U-shaped flow cell with an optical pathlength of 8.0 mm. The U cell with extended pathlength resulted in the lowest detection limit, with a concentration detection limit of approximately 1 × 10^{-7} M, but also demonstrated severe nonlinearities in the response vs. concentration when compared to the on-column detectors due to complex reflection and refraction of the light where it enters the cell. As expected, the U-shaped cell contributed significantly to band broadening.

To increase the optical pathlength for UV detection without degrading the separation efficiencies, Tsuda *et al.* (1990) have used rectangular-shaped capillaries. Theoretical plate numbers were compared between circular (51 μm i.d.), square (50 × 50 μm i.d.) and rectangular (27 × 340 μm i.d.) capillary columns. In all cases, the number of theoretical plates for the test analyte pyridoxine was found to be approximately 2×10^5 plates. No degradation in separation efficiency was observed for square or rectangular bore capillary columns when compared to the round bore tubes. The detection sensitivity using a 50 × 1000 μm rectangular capillary (the optical pathlength defined by the 50 or 1000 μm dimension of the capillary) demonstrated that at low concentrations a 20-fold improvement in the minimum detectable concentration resulted with the 1000 μm optical pathlength. Another decisive advantage of the flat face capillary is that refraction and reflection effects inherent in circular capillaries are negligible. This results in reduction of nonlinear signals, yielding a large dynamic range, especially when the aperture width is large compared to the internal dimensions of the capillary.

Another interesting technique to increase the optical pathlength has been suggested recently by Xi and Yeung (1990, 1991). In this approach, the interrogating beam is launched down the entire length of the capillary column. Using axial-beam illumination, the pathlength is determined by the physical length of the analyte bands traveling through the capillary. A schematic of an axial-beam absorption detector for CE using a conventional light source is shown in Fig. 6. The radiation from a UV light source (deuterium lamp) is collected and imaged onto a diffraction grating to select the appropriate wavelength with the light coupled (focused) into the capillary with a small ball lens. The transmitted light was collected at the high-voltage end using an optical fiber and delivered onto the photomultiplier tube. Transmission of the light through the entire length of the capillary is based upon the same principle that allows light to propagate down optical fibers, total internal reflection. The refractive index of the core, as with optical fibers (in this case the fluid filled region of the capillary column), must be greater than the fused silica casing. Since fused silica has a refractive index of 1.458, and water has a refractive index of 1.333, water would not permit sufficient light to travel through the capillary column. Therefore, dimethyl sulfoxide (refractive index = 1.4770) is used as the solvent for the electrophoresis. The electropherogram of the test analytes acridine and 3-aminoquinoline is shown in Fig. 7. The unusual shape of the electro-

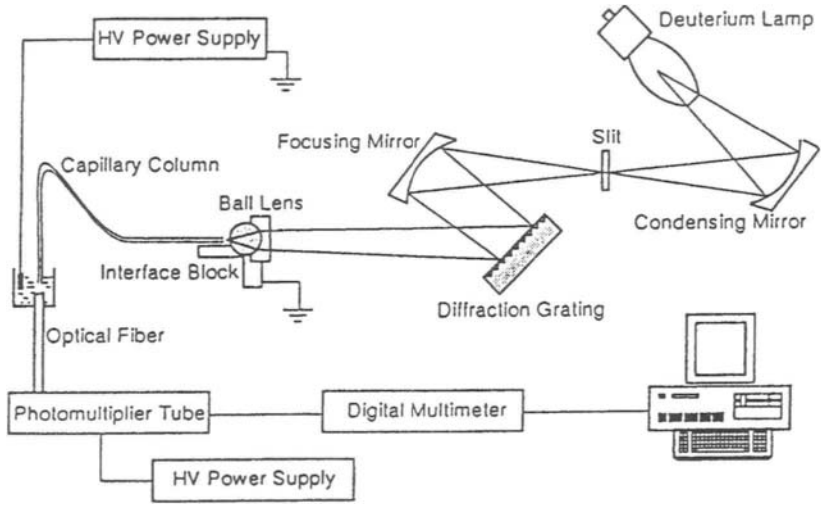


Fig. 6. Diagram of an axial-beam UV absorption detector. (Reproduced with permission from Xi and Yeung, 1991).

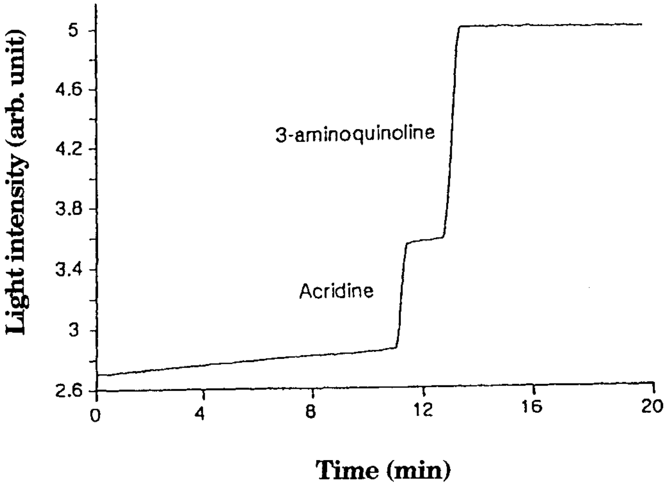


Fig. 7. Integrating electropherogram of acridine and 3-aminoquinoline using axial-beam excitation. (Reproduced with permission from Xi and Yeung, 1991).

pherogram results from the axial-beam illumination, which integrates the observed absorbances. When the analytes elute from the column, a decrease in the absorbance results. If the first derivative of the integrated electropherogram is performed, a peak-shaped electropherogram would result with a concomitant loss in signal-to-noise. The limits of detection at a signal-to-noise ratio of 3 for acridine and 3-aminoquinoline using the axial arrangement were 1.9×10^{-7} M and 1.1×10^{-6} M, respectively.

4. Thermo-Optical Detection

4.1. Introduction

The pathlength dependence for absorption measurements is a vital concern in terms of sensitivity for CE. An alternative to conventional absorption measurements is a thermo-optical detection scheme. The detection utilizes a crossed-laser beam arrangement in which one beam, the pump beam, is tuned to an electronic transition associated with the analytes under investigation and the second beam, the probe beam, is positioned 90° with respect to the propagation direction of the pump beam. In thermo-optical measurements, the pump beam generates an excited state population of analyte molecules which decay nonradiatively to the ground state, producing a rise in the temperature of the sample and a change in the refractive index of the solution. Since a change in refractive index of the solution results, the propagation direction of the probe beam is altered due to refraction. The analytical signal is determined by measuring the shift in the propagation direction and the defocusing of the probe beam by monitoring the center intensity of the probe beam in the far-field on a photodetector. The relative shift in the probe beam in the far-field is proportional to the laser power and θ, where θ is given by

$$\theta = \frac{4.606\ \varepsilon\ C\ (^{dn}/_{dT})\ Z_1}{(2\pi)^{1/2}\ \omega\ k} \tag{10}$$

where ε is the molar absorptivity, C is the concentration of the absorbing species, dn/dT is the change in solution refractive index with temperature at the probe beam wavelength, Z_1 is the distance of the probe beam waist from the intersection region of the two beams, ω is the pump laser beam waist and k is the thermal conductivity of the solvent (Dovichi *et al.*, 1984). Inspection of this relationship shows that the thermo-optical signal is independent of the optical pathlength of the probe and pump beam. Bornhop and Dovichi (1987) have demonstrated that the thermo-optical response is pathlength independent for capillaries with diameters ranging from 50 to 500 μm, which makes this detection strategy particularly attractive for on-column detection in CE.

Several factors affect the magnitude of the response and noise associated with the measured thermo-optical signal. Fluorescence of the absorbing species results in smaller temperature rises and smaller thermo-optical signals; therefore, the probe molecule should possess small fluorescence quantum yields. Since the perturbation of the probe beam depends upon the solvent as well, namely through its thermal conductivity and change in refractive index with temperature (dn/dT), some solvents will yield higher sensitivity than others. For example, methanol has been shown to yield lower detection limits for thermo-optical measurements compared to water due to superior thermo-optical properties (Nolan *et al.*, 1984). Noise contributions to the observed response result primarily from intensity fluctuations in the probe beam and air flow in the surrounding environment which perturbs the propagation direction of the probe beam and induces temperature fluctuations in the capillary column. Temperature fluctuations can be particularly troublesome in capillary columns due to the high surface-to-volume ratio and the low heat capacity of the thin capillary wall. Additionally, since the temperature changes which occur during a typical thermo-optical measurement are very small, in the range of one degree Kelvin, the capillary must be mounted on a large heat sink to reduce temperature drifts.

4.2. Applications in CE

A schematic of a thermo-optical absorbance detector for CE is shown in Fig. 8. This detector was used to detect amino acids labeled with (dimethylamino)azobenzenesulfonyl chloride (DABSYL), which forms stable and highly colored adducts that display negligible fluorescence. The pump beam was supplied by either an He-Cd laser (4 mW, 442 nm) or an argon ion laser (125 mW, 458 nm). The probe beam was a helium-neon (He-Ne) laser (1 mW, 632.8 nm). The pump beam was modulated with an optical chopper, with the reference signal originating from the chopper itself. The modulated signal results from a periodic refraction of the probe beam when the pump beam is impinging upon the capillary tube. The modulated signal is detected on a small-area photodiode. Both beams are focused into the capillary column with microscope objectives, yielding an effective probe volume, defined by the intersection of these beams, of approximately 1 pl. The detection sensitivity for the glycine-labeled adduct was 260

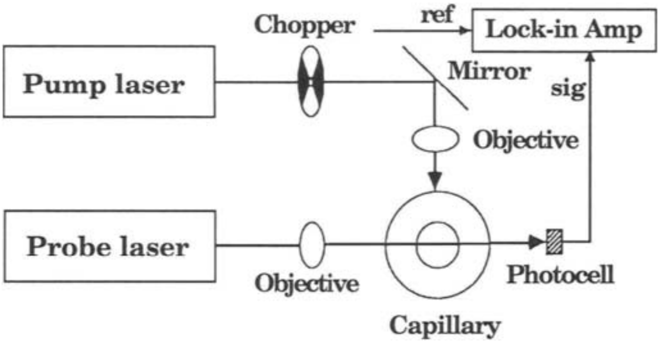


Fig. 8. Schematic of a crossed-beam thermo-optical detector for CE. (Reproduced with permission from Yu and Dovichi, 1989).

amol for the He-Cd pump beam arrangement and 40 amol for the argon ion pump configuration at a signal-to-noise ratio of three. The higher sensitivity for the argon ion pump laser results from the higher output power for this laser and from the linear dependence of the thermo-optical signal on pump power. Although it is advantageous to use a relatively high power laser for the pump beam to improve the limits of detection, a cheap, low-power He-Ne laser can be used as the probe beam.

5. Fluorescence Detection

5.1. Introduction

Fluorescence presents the practitioner of CE with a detection scheme that is both selective and sensitive. The instrumental simplicity of the technique along with the sensitivity, especially when lasers are used as the excitation source, makes fluorescence detection one of the most attractive detection schemes available for CE. The sensitivity of fluorescence detection has recently been demonstrated at the single molecule level in condensed phases (Shera *et al.*, 1990; Soper *et al.*, 1990, 1992). The necessity for ultrasensitive detection in CE becomes particularly important when reducing the diameter of the capillary column and attempting to analyze minute samples, such as the contents of single cells (Kennedy and Jorgenson, 1989; Oates *et al.*, 1990). The selectivity of fluorescence results from the fact that few compounds demonstrate intrinsic fluorescence, especially in the visible region of the spectrum. Therefore, components can be detected and analyzed in complex matrices with minimal interferences and little need for extensive sample cleanup prior to the analytical separation. Although the limited number of compounds which demonstrate intrinsic fluorescence makes detection prohibitive in many cases, this has become less of a constraint with the development of a number of different fluorescent tagging reagents. These tags are designed to convert a particular non-fluorescent species into fluorescent components in a region of the spectrum that shows little interference from the matrix. Examples of fluorescent tags include *o*-phthalaldehyde (OPA) and naphthalenedialdehyde (NDA), which react exclusively with primary amines and form isoindoles that fluoresce with high efficiencies in the UV or visible region of the spectrum.

Several references are available which describe fluorescence spectroscopy in detail from both a theoretical and experimental viewpoint (Winefordner *et al.*, 1972; Wehry, 1976; Schulman, 1977; Lakowicz, 1983; Dewey, 1991). We will present here only a brief description of fluorescence and a discussion of the instrumental components necessary to perform a fluorescence experiment. We will then discuss in detail the design considerations for construction of a fluorescence detector for CE and current instrumental arrangements used in CE analysis.

The process of fluorescence and other processes which compete with fluorescence to depopulate the excited-singlet state are shown schematically in the simplified Jablonski diagram depicted in Fig. 9. Upon excitation (k_e) of the molecule with a photon of energy corresponding to the energy difference between the ground (S_0) and first excited singlet state (S_1), the molecule relaxes to the ground state via internal conversion (k_{ic}), intersystem crossing to the triplet state (k_{is}), other non-radiative manifolds (k_{nr}) or fluorescence (k_f). Once the molecule is in the triplet state, it may return to the singlet ground state by phosphorescence (k_p) or a nonradiative route (k_{nr}). In order to compare the fluorescence efficiencies of various fluorophores, the fluorescence quantum efficiency (Φ_f), defined as the probability of fluorescence emission once the molecule has been promoted to the excited state, is measured experimentally. The quantum efficiency is defined as:

$$\Phi_f = \frac{k_f}{k_f + k_{ic} + k_{is} + k_{nr}} \tag{11}$$

where k_{nr} are additional non-radiative paths that serve to depopulate S_1, for example, excited-state reactions such as proton transfer, charge transfer or isomerizations. The larger the fluorescence quantum efficiency, the more effective (better sensitivity) is the fluorescent probe used for detection. The quantum efficiency is affected by a number of different parameters, including properties associated

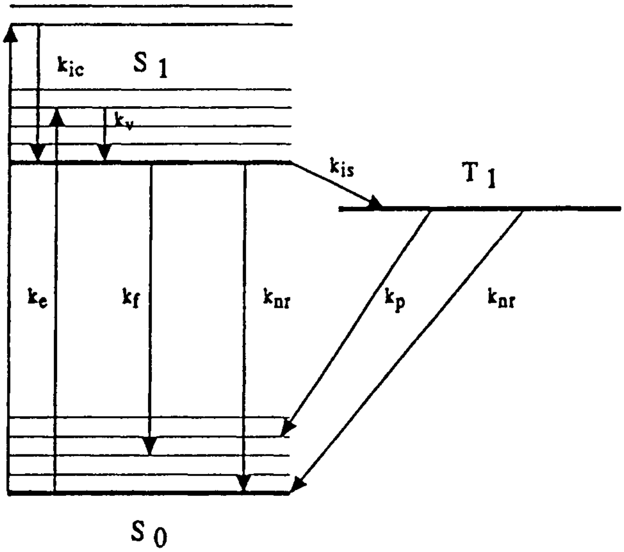


Fig. 9. Simplified Jablonski diagram which depicts the processes which depopulate the excited singlet state (see text for details). The rate of each process (k) has units of s^{-1} and is first order.

with the solvent, for example, solvent polarity, pH and the concentration of dissolved oxygen.

The intensity of a fluorescence signal (I_f), in the limit of low concentrations of the fluorescing species, can be expressed through the use of the following relation,

$$I_f = 2.3\ I_o\ b\ C\ \Phi_f \varepsilon\ F(\theta)\ G(\lambda) \tag{12}$$

where I_0 is the incident photon flux, b is the pathlength, C is the concentration of the fluorescent species, ε is the molar absorptivity of the fluorophore, $F(\theta)$ is the instrumental collection and transmission efficiency of the fluorescent photons, and $G(\lambda)$ is the quantum efficiency of the photodetector at the monitored wavelengths. Inserting typical values into the above expression ($I_o = 2 \times 10^{15}$ photons s^{-1}, $b = 50 \times 10^{-4}$ cm, $\Phi_f = 0.25$, $\varepsilon = 20{,}000\ cm^{-1}\ M^{-1}$, $F(\theta) = 0.01$, $G(\lambda) = 0.10$), the expected number of fluorescent photons per second for 600,000 molecules (1 amol) in the detection volume (50.0 pl) is 4×10^6. Equation 12 also provides information on the instrumental and photophysical parameters associated with the fluorescent compound, which may be optimized to increase the sensitivity. For example, increasing the excitation power (I_0) results in an increase in the observed fluorescence intensity. While the fluorescence intensity does scale linearly with I_0 at a certain flux, called the saturation point, the fluorescence fails to increase with further increases in intensity. The saturation arises from the finite period of time the molecule spends in the excited state, described by its fluorescence lifetime (τ_f).

The emission spectra are typically shifted to lower energy (higher wavelengths) when compared to the excitation spectra. This results from a fast relaxation process, k_V (Fig. 9) on the picosecond time scale, to the lowest vibrational state of S_1 from which the fluorescence emission occurs to excited vibrational levels of S_0. The shift between the excitation and emission spectra is called the Stokes' shift. Another feature sometimes present is band structure in the excitation and emission spectra with a mirror image relationship between these spectra. The band structure results from transitions between vibrational levels in S_1 and S_0, with the magnitude of these bands related to the probability of a particular transition between the vibrational states in S_0 and S_1 giving rise to that band (defined as Franck-Condon factors). The Franck-Condon factors for a particular transition are equally probable for absorption and emission processes, resulting in the mirror image relationship. In some cases the mirror image relationship is not observed. The breakdown in the mirror image relationship is usually a consequence of geometrical differences between nuclei in the excited and ground electronic states.

5.2. *Instrumental Considerations*

The principal components of a fluorescence spectrometer are shown in Fig. 10. A Xe arc lamp is typically used since a high intensity light source is desirable in the fluorescence analysis. A monochromator selects the appropriate excitation wavelength when a broad band source is used. If a laser is used as the excitation source, a monochromator may be omitted due to the monochromaticity of the laser, but an optical filter is often used to eliminate plasma lines from the gas discharge. The fluorescence is usually collected at 90° to the excitation light with a lens *f* matched to the emission monochromator and the photons detected with a photomultiplier tube. The primary advantage of fluorescence compared to absorption is the fact that the signal of interest is measured on a "dark" background. In an absorption measurement, the signal is measured on a high background. Since the signal-to-noise ratio in optical measurements increases inversely with the background, better sensitivity is always associated with lower background levels. The major factor limiting the sensitivity of fluorescence measurements is the ability to discrimi-

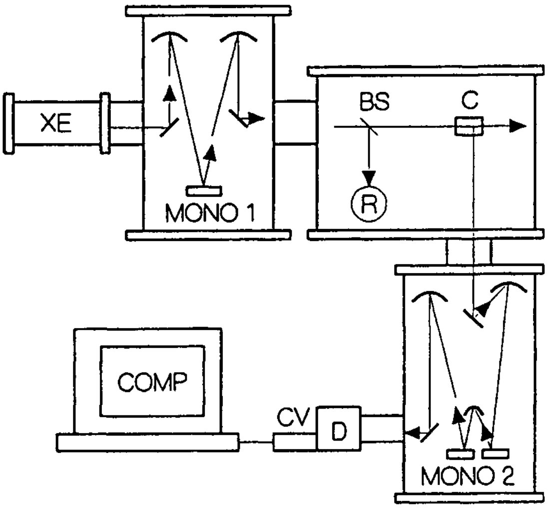


Fig. 10. Block diagram showing the principal components of a fluorescence spectrometer. XE = xenon lamp source; R = reference quantum counter; Mono 1 = excitation monochromator; C = cell; Mono 2 = emission monochromator; D = photodetector; CV = current to voltage converter; COMP = computer for data acquisition and analysis.

nate the fluorescent photons from the background scattered or stray photons created by the excitation beam and solvent molecules. Therefore, great care and effort must be devoted to effectively filtering the excitation light from the photodetector. In addition, Raman scattered photons from the solvent often overlap the emission band from the fluorophores. In this case, optical filtering is ineffective in eliminating this source of background. For example, the predominant Raman bands from water occur at 1650 and 3200 cm^{-1}. If the fluorescence is excited at 488 nm, these bands occur at 531 and 578 nm. The ideal detector would be designed such that the observation wavelengths occur between or prior to these wavelengths to minimize background. Typically, optical filtering alone cannot completely eliminate the scattering background. An approach which significantly reduces background-scattered photons is a technique called time-gating (Haugen and Lytle, 1981), in which the sample is excited by a light pulse of short duration (picosecond time regime) and the counting electronics are configured to look at only the late arriving photons. Since the scattering photons are coincident with the light pulse and a significant fraction of the fluorescent photons are delayed with respect to the light pulse, backgrounds free from scattering photons can be obtained.

The detection sensitivity in a typical CE experiment utilizing fluorescence is, in most cases, limited by background photons. The signal-to-noise ratio is given by the ratio $n_f/n_b^{1/2}$, where n_f is the number of fluorescent photons and n_b is the average number of background photons. Since the background and fluorescent photons scale linearly with the excitation intensity, the signal-to-noise ratio increases with the square root of the excitation intensity. This relationship holds to the saturation point, where the fluorescence fails to increase linearly with intensity, although the background scattering photons continue to increase with excitation intensity. The optimal intensity for the detection is selected near the saturation point. The saturation intensity can be determined, to a first approximation, from the reciprocal of the fluorescence lifetime and the calculated absorption rate (k_a) per molecule by:

$$k_a = 3.8 \times 10^{-21}\, \varepsilon\, I \tag{13}$$

where I is the laser irradiance in photons s^{-1} cm^{-2}. When $1/k_a = \tau_f$, the transition is saturated. Another process that may result in nonlinearities in the fluorescence intensity is photobleaching. Photobleaching is particularly significant when tightly focused laser beams are used to excite the fluorescence and the molecules reside within the excitation beam for extended periods of time, as is the case for slowly moving components in an electropherogram. Photobleaching the fluorescent molecules results in irreversible chemical changes, converting them from fluorescent to non-fluorescent species. When choosing the optimal intensity for detection, both saturation and photobleaching must be considered (Shear *et al.*, 1993).

When developing a fluorescence detector for CE, the excitation wavelength is chosen to match the maximum in the excitation spectrum (large ε value). Also, it is essential to inject a large number of photons into the capillary columns in order to obtain favorable sensitivity. Therefore, in most applications, lasers are used as excitation sources in CE analysis due to their superior spatial coherence and large photon flux. The principal disadvantage of a laser as an excitation source results from the high cost and complexity of the device and the narrow tuning range when compared to conventional light sources. For example, the He-Cd laser has only two operating wavelengths, 325 and 442 nm, but can deliver up to a few milliwatts of power at these wavelengths. In addition, a single lens can be used to focus the laser light to a spot of approximately 10–20 μm in diameter, efficiently coupling all of the photons into the small capillary column. Conventional light sources require elaborate optical arrangements to focus a large percentage of the photons into the capillary. Even when exercising extreme care, spot diameters smaller than those obtained with lasers are not achievable. Although the small tuning range of the laser can be a disadvantage, derivatization procedures circumvent this problem to some degree. With regard to the cost and complexity associated with lasers as excitation sources for fluorescence detectors, solid-state and diode lasers will offer viable alternatives to ion lasers and will help to reduce the operating costs associated with gas ion lasers. Diode lasers are particularly attractive due to their low cost and ease of operation. Although diode lasers operate in the near infrared (660–850 nm), frequency doubling can generate a few microwatts of blue light.

Fluorescence is typically collected at 90° to the exciting light by a lens and directed onto the phototube. Since the fluorescence emission is isotropic, collecting a significant fraction of the fluorescent photons becomes a challenging task. The best collection lens to use is a microscope objective with a high numerical aperture (low *f* number). For example, a microscope objective with a numerical aperture of 0.6 collects approximately 10% of the light, while a numerical aperture of 0.2 results in only 1% of the light being collected. In comparison, a single component lens with an *f*/# of 1.0 will collect only approximately 7% of the fluorescent light. In addition to a high collection efficiency, the imaging properties of objectives are superior to those of single component lenses. The imaging quality associated with microscope objectives allows the use of a spatial filter or slit at the secondary focal plane of the objective, which prevents a significant number of scattered photons generated by the excitation light striking the interface between air and the glass of the capillary from reaching the photodetector. Therefore, the slit can be adjusted so that the background photons generated at these interfaces impinge upon the face of the slit, while the fluorescent light generated from within the capillary is allowed to pass through the slit.

To further isolate the fluorescence photons from background photons, a monochromator or bandpass filter is placed directly in front of the phototube. The advantage of using a monochromator is the flexibility associated with the device. It can easily be tuned to the desired wavelength region, and the width of the observation window adjusted simply by changing the slit width. The cost associated with the monochromator and the low throughput of the device are definite disadvantages. A monochromator with an f/# of 4 will permit only approximately 0.3% of the light to be transmitted. On the other hand, a spectral filter is less expensive than a monochromator and transmits nearly 70–80% of the light at the maximum in the passband. The difficulty with spectral bandpass filters results from the loss of flexibility associated with their use. The width of the passband is fixed for a particular filter and can not be changed; however, the position of the passband can be controlled to a certain degree simply by changing the angle of incidence of the fluorescence light on the face of the spectral filter. Angle-tuning the filter can shift the passband to lower wavelengths by several tens of nanometers .

The final component of any fluorescence experiment is the photodetector. There are a variety of different types of detectors that may be chosen to suit the particular application. The workhorse detector has traditionally been the photomultiplier tube with a bi- or multialkali photocathode. The proper choice of photomultiplier tube is selected with respect to the observation wavelengths, since photomultiplier tubes have quantum efficiencies that are wavelength dependent. Typical quantum efficiencies of photomultipliers are in the range of 10–20%. In addition, consideration should be given to the background or "dark" counts associated with the phototube. The associated components for a phototube are a high voltage power supply and a current-to-voltage converter which permits the output of the photomultiplier to be fed directly into an analog recorder or computer after digitization of the analog signal. A diagram of a simple home-built current-to-voltage converter with variable gain and low pass filter is shown in Fig. 11. The output voltage, V_0, of the converter is given by $V_{out} = -R_{1-5}\ I_{in}$, where I_{in} is the output current from the photomultiplier and R_{1-5} is a series of resistors. The gain in the converter is determined by the value of the resistor used across the operational amplifier. For example, a 0.1 μA output current will require a 10 MΩ resistor to give an output voltage of 1 V. The low pass filter allows low frequency components of the signal to be unper-

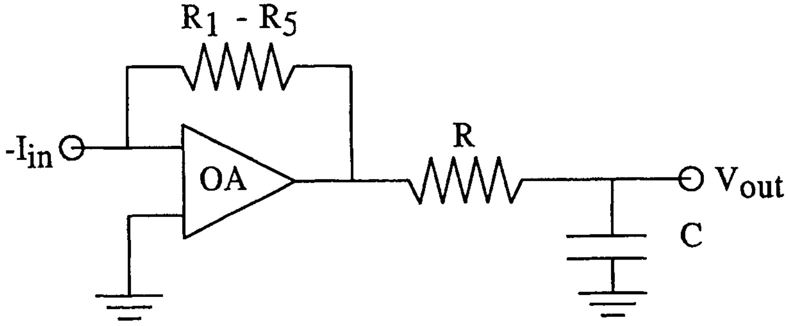


Fig. 11. Current-to-voltage converter with variable gain and a high frequency filter. OP = operational amplifier; R_{1-5} = resistors; C = capacitor of low pass filter; R = resistor of low pass filter.

turbed, but attenuates the high frequency components which typically mask the analytical signal (noise). The cut-off frequency can be altered by changing the values of the capacitor and resistor in the circuit. For example, if the capacitor of the filter has a value of 1.0 μF and the resistor has a value of 1.0 kΩ, an input voltage with a frequency of 159 Hz would be attenuated to approximately 70% of its initial amplitude ($1/2\pi RC$).

An alternative to analog detection is to design the detection system in a photon counting configuration, which has been demonstrated to yield better sensitivity when compared to analog detection (see, for example, Nakamura and Schwarz, 1968; Jones *et al.*, 1971; Ingle and Crouch, 1972). In this approach, the output pulses from the photomultiplier are amplified and only those pulses whose magnitude exceeds a predetermined threshold are counted by a digital counter. The increased sensitivity of photon counting arises from the fact that thermionic noise generated in the dynode chain of the photomultiplier yields smaller amplitude pulses than those generated at the photocathode. Therefore, the thermionic noise from the detector can be effectively discriminated from the fluorescent photons. A disadvantage of photon counting is the limited dynamic range of the technique. High photon count rates result in nonlinearities in the response; therefore, photon counting is used only when the magnitude of the background is small.

In addition to photomultiplier tubes, another detector that shows great promise for fluorescence is single photon avalanche diodes. These devices have quantum efficiencies approaching 40–60% in the visible and near infrared region of the spectrum with dark count rates that are comparable to those of photomultiplier tubes. The primary disadvantage associated with these diodes is the small photoactive area, typically 150 μm in diameter. Good imaging optics are required to focus the fluorescence onto the small photosensitive area.

5.3. Applications in CE

A laser-induced fluorescence detector for CE is shown in Fig. 12. The excitation source is a He-Cd laser that can deliver 1.5 mW of power at 325 nm and 10 mW of power at 442 nm. The output of the laser is passed through an interference filter which removes plasma lines from the beam. In this system, part of the beam is split off and directed onto a photovoltaic cell, which reduces the low frequency noise and drift associated with the laser output by subtracting the signal of the photovoltaic cell from the photomultiplier tube output. The laser light is focused onto the capillary column and the fluorescence is collected at right angles to the excitation beam. In addition,

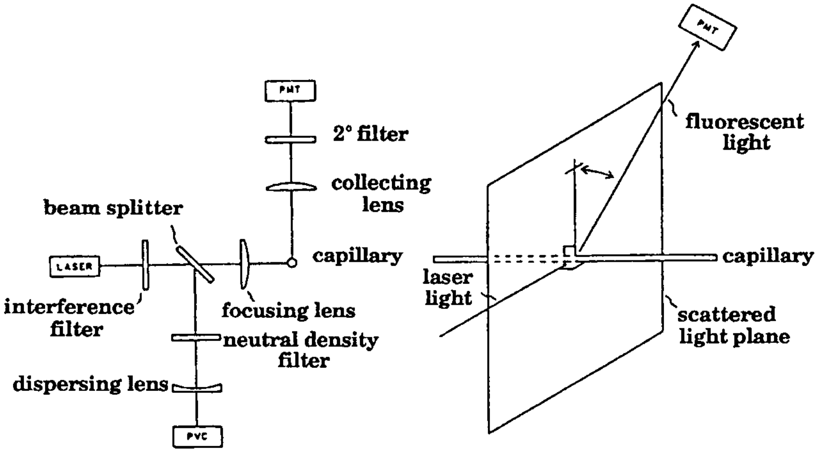


Fig. 12. A laser-induced fluorescence detector for CE using a He-Cd laser for excitation (Guthrie *et al.*, 1984). The detection optics are positioned 30° from the scattering plane to reduce the amount of background photons reaching the photodetector.

collection optics are situated 30° from the scattering plane in order to further reduce the background level. A low pass filter was used to eliminate the high frequency noise. The minimum detectable concentrations for 9-methylanthracene (325 nm excitation) and riboflavin (442 nm excitation) using this system were 25 and 0.36 ng/ml, respectively. For comparison purposes, the detection limit of 9-methylanthracene was determined with the same system, but using a mercury arc lamp as the excitation source (365 nm), and was found to be 3.7 ng/ml. The superior detection limit for the mercury arc lamp was a consequence of its higher energy output and the higher molar extinction coefficient of 9-methylanthracene at 365 nm. The authors have also used this laser fluorescence system to detect amino acids derivatized with OPA, NDA and fluorescein isothiocyanate (FITC) (Nickerson and Jorgenson, 1988a,b). The principal advantage of these derivatizing reagents is that the adducts have large absorption coefficients at 325 nm (OPA) and 442 nm (NDA, FITC). The minimum detectable quantities were found to be 80 amol for the OPA adducts, 12 amol for the NDA adducts and 1.1 amol for the FITC adducts. The poor detection limits for the OPA adducts are a consequence of UV excitation, giving rise to a larger background and the necessity for precolumn derivatization. The OPA adducts are chemically unstable (Chen *et al.*, 1979; Nakamura *et al.*, 1982; Stobaugh *et al.*, 1983) and show decreases in fluorescence intensities with time. Although the FITC adducts yielded the lowest detection limits, the native reagent is fluorescent, creating a large peak in the electropherogram from unreacted dye.

The minimum detectable quantities of fluorescein were determined using four different excitation sources: xenon arc lamp, deuterium lamp, tungsten lamp and an Ar ion laser operating at 488 nm (Albin *et al.*, 1991). The fluorescence was collected with dual optical fibers situated at an angle of 90° to the excitation beam and emission directed onto the photomultiplier tube. The concentration detection limits for fluorescein were found to be 4 ng/ml for the xenon lamp, 60 ng/ml for the deuterium lamp, 20 ng/ml for the tungsten lamp and 0.27 ng/ml for the Ar ion laser. The superior detection limits displayed for this laser result from higher output powers (40 mW) and a large absorption coefficient at the lasing wavelength.

Impressive mass detection limits using laser-induced fluorescence detection for CE have been obtained by Dovichi (1988, 1989). In this work, the detection is actually performed off-column in a sheath-flow cuvette. A schematic of the sheath-flow cuvette is shown in Fig. 13. The capillary is inserted into a 250 μm square bore glass cell. Surrounding the sample stream from the capillary is a sheath-flow composed of the buffer used in the electrophoresis.

The sheath-flow is driven through the cell with a stable syringe pump and connected to ground to complete the electrophoresis circuit. If the sheath stream is run at a flow rate exceeding that of the sample stream, the sample stream is reduced in diameter close to the output of the capillary. The diameter of the sample stream is determined by the ratio of the flow velocities between the sample and sheath streams. The advantage of using this arrangement results from the identical composition of the sheath and sample streams. Therefore, light scatter can be significantly reduced because virtually no refractive index change occurs at the sheath/sample stream interface. Figure 13 shows the electropherogram of 18 amino acids derivatized with FITC using the 488 nm output of the Ar ion laser for excitation. In this electropherogram, between 2 and 10 amol were introduced into the electrophoresis system. The detection limits for the FITC-labeled amino acids ranged between 0.9 and 15×10^{-20} moles, or approximately 6,000 to 90,000 molecules.

As previously mentioned, diode lasers are an attractive alternative to ion lasers due to their low cost and simplicity of operation. These lasers typically lase in the near infrared region of the spectrum, which can be an ideal region to observe the fluorescence due to the limited number of compounds that demonstrate intrinsic fluorescence

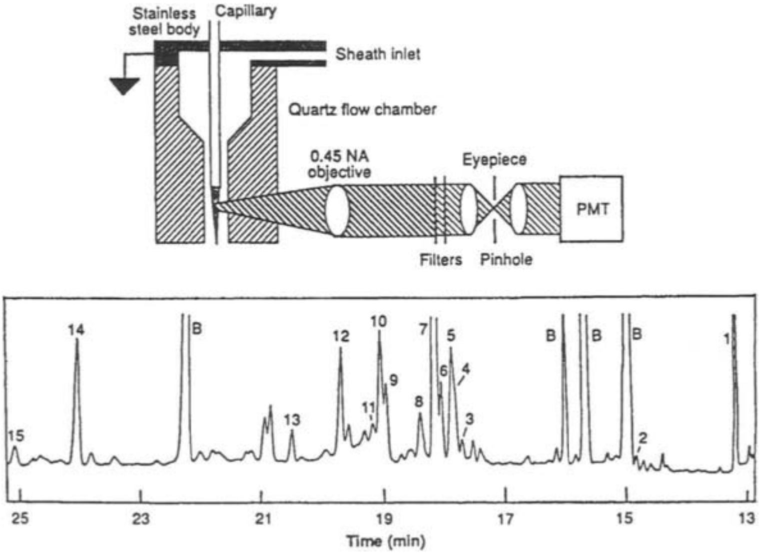
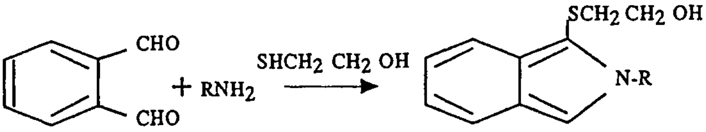


Fig. 13. Sheath flow cuvette used for laser-induced fluorescence detection in CE (see text for details) and electropherogram of amino acids (2–10 amol) derivatized with FITC and detected using laser excitation and the sheath flow cuvette (Dovichi and Cheng, 1988). Amino acid peaks are: 1, Arg; 2, Lys; 3, Leu; 4, Ile; 5, Trp; 6, Met; 7, Phe, Val, His and Pro; 8, Thr; 9, Ser; 10, Cys; 11, Ala; 12, Gly; 13, Tyr; 14, Glu; and 15, Asp. The peaks in the electropherogram marked with a B are reagent blank peaks.

in the near infrared. Higashijima and co-workers (1992) have recently demonstrated fluorescence detection of amino acids in the near infrared using a diode laser as the excitation source. The amino acids were labeled with a newly synthesized labeling reagent consisting of a thiazine chromophore (absorption maxima at 670 nm). The detection limits for the labeled amino acids using a diode laser (660 nm, 2 mW) for excitation were found to be in the range of 10 pmol. The rather poor detection limits were a consequence of the less than optimal conditions of the tagging reaction and the poor aqueous chemical stability of the fluoroprobe. The authors also used the frequency-doubled output of the diode laser to detect amino acids labeled with the visible dye 7-diethylaminocoumarin-3-carboxylic acid succinimidyl ester and reported detection limits of 100 amol. The use of diode lasers in the visible and near infrared will be an integral part of fluorescence detection for CE.

5.4. *Fluorescence Derivatization*

Although the selectivity of fluorescence can be an advantage, it may create problems due to the limited number of compounds that demonstrate intrinsic fluorescence, especially in the visible region of the spectrum. The visible region is particularly beneficial because of the limited number of interferences that may be present, permitting sensitive detection of analytes in rather complex matrices. In addition, since lasers are typically used as excitation sources for fluorescence, all of the components to be determined should display excitation maxima close to the fixed wavelengths of the laser. These concerns can be circumvented using derivatization to convert nonfluorescent components into fluorescent ones with similar excitation maxima. One common derivatizing agent is OPA (Roth, 1971), which reacts with primary amines in the presence of 2-mercaptoethanol at an appropriate pH to form isoindoles that have favorable quantum yields and excitation maxima at approximately 345 nm with an emission maxima at 450 nm. The reaction of OPA with a primary amine is shown below.



Unfortunately, these isoindoles are unstable, with the maximum in the fluorescence emission occurring within a time period of several minutes and then decreasing substantially with time. This requires labeling to be performed after the separation and immediately preceding detection in order to preserve the sensitivity of the analysis. Several approaches to postcolumn labeling of primary amines in CE have been described (Rose and Jorgenson, 1988; Tsuda *et al.*, 1988; Pentoney *et al.*, 1988; Rose, 1991). An example of a postcolumn labeling device using OPA as the derivatizing reagent for CE is shown in Fig. 14. The effluent from a 40 µm o.d. separation capillary is fed through a stainless steel tee and into a 50 µm i.d. reaction capillary. This capillary has an optical window produced by removing the polyimide coating and serves as the detection zone. The OPA reagent is supplied to the reaction capillary from the third arm of the tee. The reagents are mixed in the reaction capillary by diffusion, convection and migration effects. The use of the postcolumn reactor resulted in negligible losses in theoretical plate numbers, and the detection limit for the glycine derivative of OPA was 6.3 fg (83 amol).

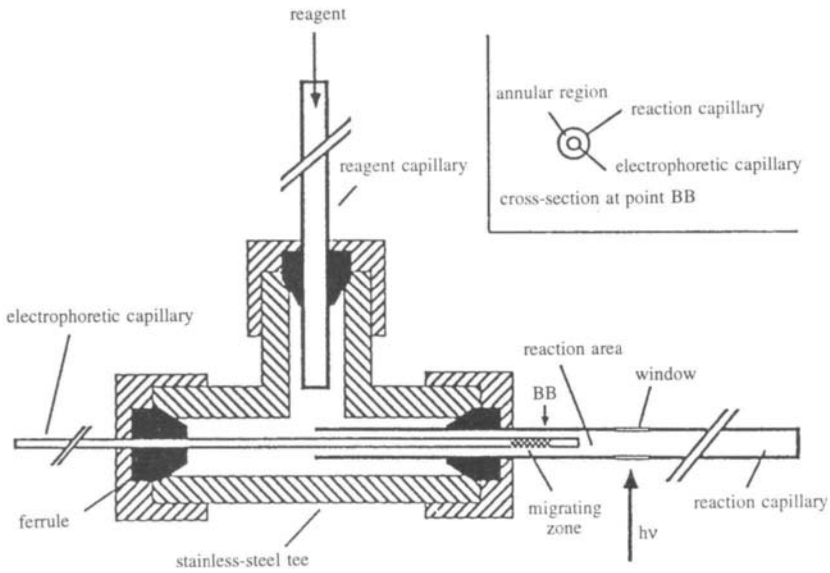
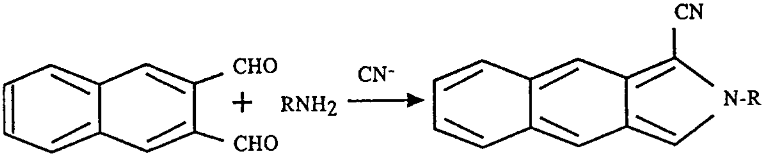


Fig. 14. Postcolumn reaction device for CE. (Reproduced with permission from Rose and Jorgenson, 1988).

An alternative reagent for the analysis of primary amines is naphthalenedialdehyde, NDA (Carlson *et al.*, 1986; Matuszewski *et al.*, 1987; de Montigny *et al.*, 1987; Roach and Harmony, 1987; Soper *et al.*, 1989; Soper and Kuwana, 1989). The reaction of NDA with a primary amine is shown below. The adducts (cyanobenz[f]isoindoles) demonstrate higher stability than do the OPA adducts and allow pre-column derivatization. In addition, the isoindoles have excitation and emission maxima in the visible region of the spectrum, which results in less impurity fluorescence in the electropherogram. Additionally, one of the absorption maxima of the CBI-amines is close to the 442 nm output of the He-Cd laser. Ueda and co-workers (1992), using the 442 nm line of the He-Cd laser for the excitation source, have reported detection limits of several amino acids derivatized with NDA in the range of 0.7 amol.



5.5. *Indirect Detection*

An approach for making fluorescence detection more universal is indirect fluorescence detection (Kuhr and Yeung, 1988a,b; Yeung, 1989; Gross and Yeung, 1990; Yeung and Kuhr, 1991). In this scheme, the running buffer is loaded with a fluorophore, and when the analyte zone migrates into the detection zone, the analyte effectively reduces the number of fluorogenic ions in the detection zone with a corresponding decrease in the fluorescence signal at the detector. Therefore, a constant signal is measured at the photodetector, with the analytical signal in the form of small changes in the background resulting in negative peaks arising from charge displacement. Figure 15 shows the detection of 200 and 20 fmol of underivatized amino acids using 200 μM solutions of salicylate in the running buffer excited by the 325 nm output of a He-Cd laser. The primary feature in this electropherogram is that the peaks are negative.

The major limitation on the detection sensitivity is the stability of the background. In many cases, active stabilization of the excitation source may be required in order to obtain favorable detection sensitivity. Experimental control of detection sensitivity is realized by reducing the concentration of the fluorogenic ion in the buffer. However, reductions in the ion concentration can create other problems, namely, lower conductivity in the buffer and reductions in the maximum concentration of material that may be detected. The ultimate detection sensitivity obtainable in indirect fluorescence detection is determined by shot-noise generated from the excitation source.

5.6. *Recent Innovations*

5.6.1. *Charge coupled device (CCD detection)*

The ability to obtain the entire emission spectrum of an eluting analyte would be beneficial in identifying unknown components in the electropherogram. Since the band resides within the effective detection zone for only a few seconds, the emission spectrum, which can be 50–200 nm wide, must be obtained in a relatively short period of time with sufficient signal-to-noise. Conventional photomultiplier tubes (one channel detectors) require the use of a monochromator to disperse the spectrum and a scan over the desired wavelength range.

Signal is lost due to the limited throughput of the monochromator and the small amount of time that each wavelength is allowed to illuminate the photodetector. A viable alternative is to remove the slits from the dispersing element and allow the entire spectrum to impinge upon many small detectors (multichannel detector). This has

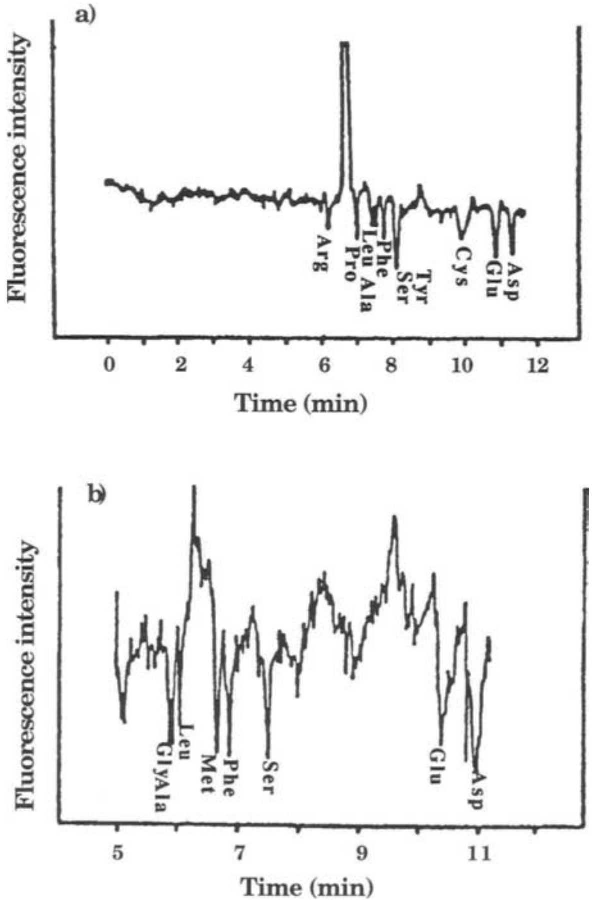


Fig. 15. Indirect fluorescence detection of native amino acids using salicylate as the fluorescing ion in the running buffer. The electropherogram represents the injection of (a) 200 fmol and (b) 20 fmol of the native amino acids into the capillary column. (Reproduced with permission from Kuhr and Yeung, 1988b)

been accomplished for CE using a charge coupled device (CCD). The CCD is a metal-oxide-semiconductor composed of several thousand to several million small optical detectors (pixels) arranged in a one- or two-dimensional format. When light strikes the device, charge is generated in the pixels with the amount of accumulated charge proportional to the product of the light intensity and exposure time of the affected pixel to the irradiating light. The charge is then read by moving the charge to an on-board amplifier. The advantages of CCD detectors are extremely low readout noise, low dark current, high quantum efficiency, operating wavelength range of 120 to 1000 nm, and the ability to bin together charge in adjacent pixels.

Fluorescence spectra of fluorescein injected into a CE system using 488 nm excitation from an Ar ion laser and CCD detection have been reported (Cheng *et al.*, 1990). Figure 16 shows the three-dimensional electropherogram of 2.0 fmol of fluorescein collected over the wavelength range of approximately 400 to 700 nm. The fluorescence was imaged onto a two-dimensional CCD with the integration time set at 0.2 s and a spectrum acquired every 6 s. Acquiring this type of data on a single channel photomultiplier system would result in severe losses in detection sensitivity and require longer data acquisition times to cover this spectral range.

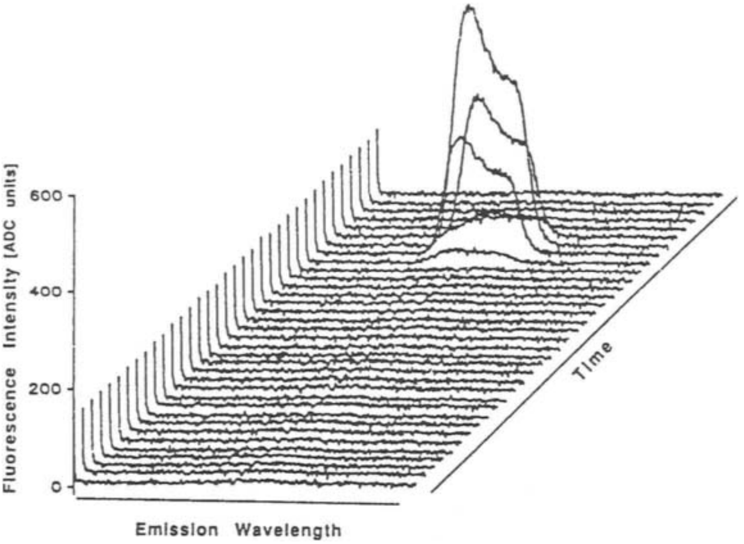


Fig. 16. Three-dimensional electropherogram of fluorescein using laser-induced fluorescence detection and a CCD detector. (Reproduced with permission from Cheng *et al.*, 1990).

In another use of CCD detectors in CE (Sweedler *et al.* 1992), a time-delayed integration mode of operation to reduce the readout time of the two-dimensional CCD detector and extend the integration time of the analytical signal was used. When a spectrum is acquired on the CCD, a shutter is closed and all of the pixels must be emptied of the accumulated charge before a subsequent spectrum can be taken. The effective readout time may be on the order of several seconds, depending on the number of pixels in the CCD array. This may result in the missing of bands during the readout time. In the time-delayed integration mode, the capillary tube is imaged onto the face of the CCD detector and the charge from the fluorescent species is moved from row to row toward the readout register at a rate corresponding to the migration rate of the band in the capillary column. This reduces the readout time and eliminates the need for a shutter. In addition, the column is irradiated in an axial fashion in order to increase the length of the detection zone. With this arrangement, detection sensitivity for amino acids labeled with FITC and excited by the 488 nm output of an Ar ion laser was 2–8 × 10^{-20} moles. In addition, complete fluorescence emission spectra of the compounds can be obtained without interrupting the electrophoretic flow.

5.6.2. Fluorescence-detected circular dichroism

Circular dichroism (CD) is a technique in which the intrinsic or induced chirality of a compound can be deduced from the degree to which it absorbs left and right circularly polarized light. Because the sensitivity of CD is dependent to a large degree on the optical pathlength, CD detection is not amenable to the small dimensions associated with capillary columns. An alternative to conventional CD spectroscopy is fluorescence-detected CD, in which the fluorescence signal is measured as a function of excitation with left and right circularly polarized light. Since the fluorescence signal depends upon the degree of light absorption (see Eq. 12), the amount of detected fluorescence will depend upon the chirality of the molecule when using left or right circularly polarized light. The advantage of fluorescence-detected CD is that fluorescence is much more sensitive than absorption measurements and is more amenable to the small dimensions associated with the CE column. The advantage of such an approach results from increased selectivity, because only components that display chirality and fluorescence are detected. In addition, the magnitude of the fluorescence-detected CD signal can give information regarding the optical activity of the particular component.

Christensen and Yeung (1989) have developed a fluorescence-detected CD detector for CE. The excitation source was the 488 nm linearly polarized line of the argon ion laser. In order to generate the left and right circularly polarized light, the laser beam was sent through a Pockels cell (electro-optical modulator). The excitation light impinged upon the capillary column at normal incidence in order to preserve the circular polarization purity of the beam. The fluorescence-detected CD signal is generated after demodulation from a lock-in amplifier. Figure 17 shows electropherograms of riboflavin and fluorescein using direct fluorescence and fluorescence-detected CD detection. Although fluorescein is fluorescent, it is achiral and thus does not produce a CD signal, while riboflavin is chiral and results in both a CD and a fluorescence signal in the electropherogram. The detection limit for riboflavin using fluorescence-detected CD was found to be 0.2 fmol, significantly higher than the mass detection limit for direct fluorescence detection. The poorer detection sensitivity for CD results from the fact that in the CD measurement the analytical signal is a difference signal between the fluorescence observed between left and right circularly polarized light.

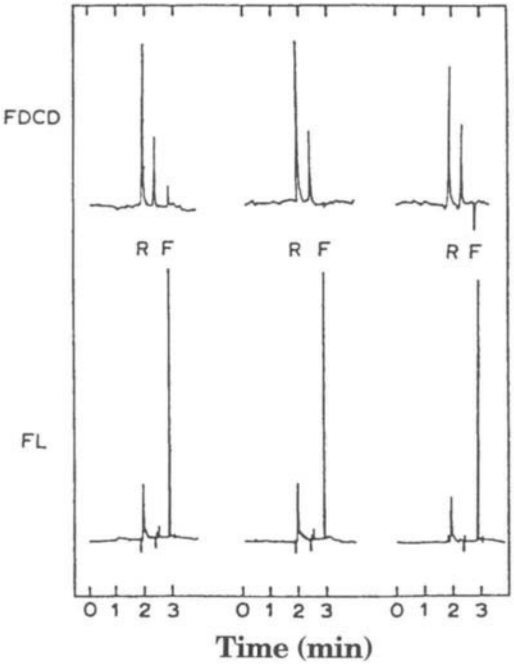


Fig. 17. Fluorescence-detected CD (FDCD) and fluorescence detection (FL) of riboflavin (R) and fluorescein (F) in CE using an argon ion laser as the excitation source (488 nm) (Christensen and Yeung, 1989). The second optically active component in the electropherogram is a decomposition product of riboflavin.

5.6.3. DNA sequencing and fluorescence detection

In another chapter, the use of capillary gel electrophoresis for the high speed sequencing of DNA is presented in detail. Here, we will offer the reader only a brief introduction to capillary gel electrophoresis and focus primarily on the fluorescence instrumentation used in capillary DNA analysis. DNA sequencing has evolved toward the use of capillary gel electrophoresis due to the fact that the high surface-to-volume ratio makes it possible to use larger applied voltages in the capillary column, significantly increasing the speed of the separation and resolution (Drossman *et al.*, 1990). For example, in slab gel electrophoresis, electropherograms typically require several hours to a day to obtain sufficient resolution to reliably detect all of the bands, whereas in capillary gel electrophoresis, oligonucleotide fragments may be separated in as little as 20 min. However, due to the small dimensions of the capillary column, small amounts of material must be identified in a rather complex matrix. In addition, identification of the chain terminator (four different terminators) must be accomplished if a single lane is used for the separation (requiring spectral differences in the tags). Tagging is a prerequisite for detection due to the poor intrinsic quantum yields of the nucleotide bases.

A diagram of a four spectral channel fluorescence detector used for capillary gel electrophoresis is shown in Fig. 18. Since the separation was performed in a single lane, four distinct tags, one for each nucleotide, were incorporated. The tagging dyes had distinct emission maxima and absorption maxima that required two different lasers for

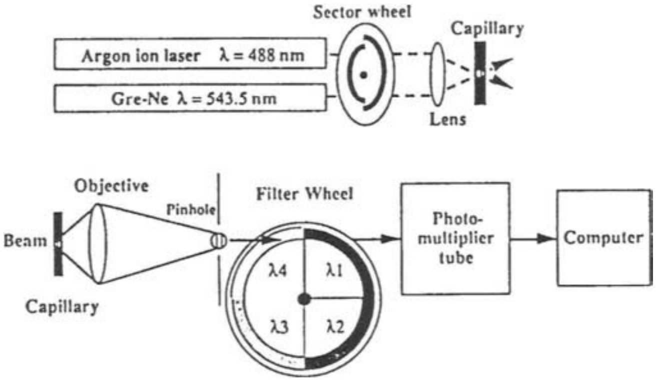


Fig. 18. Four-channel DNA sequencing device using capillary gel electrophoresis and fluorescence detection. (Reproduced with permission from Swerdlow *et al.*, 1991).

efficient excitation and minimization of Raman scattering. Two dyes (FAM, JOE) were excited with the 488 nm out-put of an argon ion laser and the other dyes (TAMRA, ROX) were excited with a green helium-neon laser (543.5 nm). The excitation beams were focused onto the column with the appropriate wavelength by a two-sector chopping wheel. The appropriate observation wavelengths were chosen by a filter wheel synchronized to the sector wheel, onto which four bandpass filters were affixed, each filter corresponding to the emission maxima of the respective fluorescent label. Detection limits for the dye-labeled oligonucleotides for this four-channel detector were on the order of 20×10^{-21} mol of material. In addition, resolution between adjacent peaks was typically 1.5 and sequencing rates of 300 bases/h were obtained at field strengths of 150 V/cm. It should be pointed out that in many cases spectral overlap of the four tagging dyes results in leakage of signal from one dye onto multiple channels, requiring the use of signal processing algorithms to correctly identify the chain terminator (Smith *et al.*, 1986).

There have been alternatives to the four-channel approach in capillary gel electrophoresis, for example, a two-channel scheme (Prober *et al.*, 1987) using four different succinylfluorescein dyes to label the chain terminators. The dyes have sufficient overlap in their excitation and emission spectra to warrant the use of a single laser to excite the fluorescence (488 nm output of the argon ion laser) and thus require only two detection channels. A system utilizing the two-channel protocol has recently been demonstrated (Swerdlow *et al.*, 1991) with a postcolumn sheath-flow detector. The emission is directed onto one of two detection channels using a dichroic beam splitter that transmits a particular wavelength region and reflects another. Detection limits for dye-labeled oligonucleotide fragments using two-channel fluorescence were on the order of 7×10^{-21} mol with sequencing rates of approximately 1000 bases/h at an electric field strength of 465 V/cm.

An additional approach, which uses a single detection channel, has been reported (Tabor and Richardson, 1987; Ansorge *et al.*, 1990), in which chain terminator identification is accomplished by varying the molar ratio of the four chain terminators in the polymerization reactions. The identification of the appropriate nucleotide is determined by observing differences in the amplitudes of the electrophoretic bands. In order to make this approach viable, uniform labeling between the four chain terminators is required during polymerization, which is performed using the manganese/T7 DNA polymerase enzyme system. The instrumentation for sequencing using this approach is essentially similar to the two-channel detector except that only a single detection channel is required.

6. Raman Detection

6.1. Introduction

Raman spectroscopy provides structural information not easily obtained from the spectroscopic techniques previously discussed. This information can assist in the identification of unknown components present in the electropherogram. In addition, quantitative information concerning the percent composition of a component in the sample can be deduced from the Raman spectrum, since the observed intensity of a Raman band is directly related to the concentration of the scattering component. However, the technique is not widely accepted as a detection mode in CE due to the lack of sensitivity and the complexity of the instrumentation needed to obtain a Raman spectrum. References are available which provide both classical and quantum mechanical descriptions of the Raman effect (Dollish *et al.*, 1974; Nakamoto and Strommen, 1984; Colthup *et al.*, 1990; Grasselli and Bulkin, 1991) as well as practical applications and instrumental components of a Raman spectrometer. Here we will present a brief introduction to the practice of Raman spectroscopy and its advantages and disadvantages for CE applications.

Raman spectroscopy yields a vibrational spectrum that may be similar to that observed in the infrared. For example, a ketone will display an IR band at 1710 cm^{-1}, while the Raman spectrum will also show a band at approximately 1710 cm^{-1} for the same ketone. The differences in IR and Raman result from the fact that IR spectroscopy is an absorption technique and, as such, uses infrared light as the excitation since these energies correspond to differences in the energy levels of vibrational states in the molecule. In Raman spectroscopy, practically any wavelength of light can be used for excitation. This is particularly beneficial experimentally, since sensitive photomultiplier tubes may be used as the photodetector in the Raman spectrometer. In addition, Raman is not an absorption technique. The Raman scattering photons are detected on a relatively dark background when sufficient filtering is used to reduce the amount of Rayleigh scattered photons reaching the detector. Another important advantage of Raman spectroscopy over IR is the fact that aqueous solvents present less interference. Since most CE separations are performed in aqueous buffers, the IR spectrum of components in the electropherogram would be masked by the broad and intense solvent bands. A major disadvantage associated with Raman spectroscopy is the very weak intensities in the Raman bands. Tuning the excitation laser to an electronic transition associated with the scattering components (resonance Raman) can enhance the intensity of the Raman bands, although in many cases interferences, namely fluorescence, obscure the weaker vibrational bands.

Energy level diagrams depicting the transitions observed in normal and resonance Raman spectroscopy are shown in Fig 19. In normal Raman spectroscopy, the energy of the photon lies below an excited electronic level (S_1) and collides inelastically with the scattering molecule with a concomitant loss in energy of the photon. This energy loss corresponds to a vibrational mode associated with the molecule. In resonance Raman spectroscopy, the incident photon possesses energy that permits the molecule to enter S_1 and relax into an excited vibrational level of S_0. The principal advantage associated with resonance Raman spectroscopy results from the larger observed signal intensities. Associated with resonance Raman spectroscopy is the problem that the Raman spectrum is dominated by bands from only the chromophoric region of the molecule and, as stated previously, the resonance Raman bands may be obscured by intense fluorescence from the molecule.

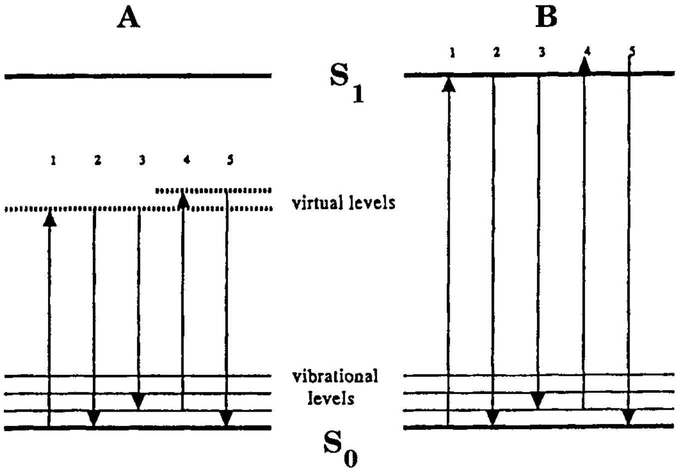


Fig. 19. Energy level diagrams depicting normal Raman (A) and resonance Raman transitions (B). The arrows represent: 1, excitation from ground vibrational level; 2, Rayleigh scattering; 3, Stokes scattering; 4, excitation from excited vibrational level; 5, anti-Stokes scattering.

Stokes and anti-Stokes shifts, two types of Raman shifts, can typically be observed. In the case of Stokes shifts, the initial vibrational state of the molecule lies below that of the final vibrational state, and the scattering photon has lower energy than the incident photon. In anti-Stokes shifts, the scattering photon is higher in energy than the incident photon. In a Raman spectrum, the reference or 0 energy shift band is called the Rayleigh band (the initial and final vibrational state of the molecule are the same, elastic scattering), which can be 1000 times more intense than the Raman bands, with the Stokes and anti-Stokes bands symmetrically distributed about the Rayleigh band. Since at room temperature most molecules are in the lowest vibrational level of S_0, the Stokes bands are usually more intense than the anti-Stokes bands. As with IR spectroscopy, the position of the bands is characteristic of the scattering molecule. The width of these bands is on the order of 1–100 cm^{-1}, while for fluorescence or absorption, bands widths of several thousand cm^{-1} are observed.

The observed intensity of a Raman band (I_R) for a scattering molecule can be calculated through the use of the general equation

$$I_R = \frac{N\,P\,\Omega\,d\sigma\,Q\,T_m T_o}{d\Omega} \tag{14}$$

where P is the incident photon density of the excitation source (photon cm^{-2} s^{-1}), N is the number of scattering molecules in the effective sampling area (related to the concentration), $d\sigma/d\Omega$ is the differential Raman cross section (cm^{-2}), Ω is the collection efficiency of the optics, Q is the quantum efficiency of the photodetector at the monitored wavelengths, and T_m and T_o are the throughput of the monochromator and collection optics, respectively, at the monitored wavelengths. If it is assumed that there are approximately 600,000 molecules in an electrophoretic band (1 amol), inserting typical values into the parameters of equation 14 ($T_m = 0.01$, $T_o = 0.9$, $Q = 0.1$, $P = 1.6 \times 10^{21}$ photons cm^{-2} s^{-1}, $\Omega = 1.0$ sr and $d\sigma/d\Omega = 1 \times 10^{-30}$ cm^2 sr^{-1} molecule^{-1}), indicates that the number of detected photoelectron per second from this group of molecules is approximately 10^{-6}. With these parameters it would require 1 pmol of material to generate a single photoelectron per second. The small number of detected photons is a direct consequence of the low scattering cross-sections associated with normal Raman processes. These calculations bring to light an important disadvantage of Raman spectroscopy, the inherently low signal intensities. These considerations become extremely important when one considers the small sample volume associated with CE.

6.2. *Instrumental Considerations*

The principal instrumental components of a Raman spectrometer include the excitation source, collection optics, a monochromator to disperse the spectrum and a photodetector. A laser is typically used in Raman spectrometers since the photon density is high and the spectral width is much narrower than a conventional light source with wavelength-selecting elements. A narrow linewidth is important in Raman spectroscopy in order to preserve the high spectral resolution. The monochromator can be a single grating device with relatively high throughputs of the scattered radiation, but often is not sufficient to filter out a significant fraction of the Rayleigh scattered photons. In order to reduce the intensity of the Rayleigh band, which can be several orders of magnitude more intense than the Raman intensities, double or triple grating spectrometers can be used, but suffers from poor throughput. An alternative is to employ notch filters in conjunction with single grating monochromators. Notch filters deeply attenuate the Rayleigh line and have a high transmission efficiency for the Raman scattered radiation. Cooled photomultiplier tubes operating in a single photon counting mode are most often used as the photodetector due to their low background levels. Since the photomultiplier is a single channel detector, the Raman spectrum is obtained by scanning the monochromator. This may require several minutes to obtain the entire spectrum with sufficient signal-to-noise. An alternative is to replace the photomultiplier with multichannel detectors, for example CCD detectors. In this approach, the grating is fixed and exit slits removed with the spectral bands imaged onto the individual pixels of the detector. The principal advantage of using the array detector is that much less time is required to acquire the spectrum.

6.3. Applications in CE

An application of resonance Raman spectroscopy in CE has been demonstrated for the electrophoretic separation and detection of the dyes methyl red and methyl orange (Chen and Morris, 1988). A He-Cd laser (442 nm) was used as the excitation source with the scattering photons collected using an array of ten 200 μm optical fibers and imaged onto a monochromator with a photomultiplier tube serving as the photodetector. The observation wavelength was fixed at 471.2 nm (1410 cm^{-1}) corresponding to the N=N stretching vibration of the dyes. Figure 20 shows the electropherogram for 0.1 mM injections of methyl red and orange. The detection limit for this system was found to be 2.5×10^{-6} M, comparable to optical absorption. Although only a single vibrational band was monitored, a vibrational Raman spectrum could be obtained over a range of approximately 1000 cm^{-1} by replacing the photomultiplier tube with an array detector. To obtain the spectrum with this configuration, the electrophoretic flow

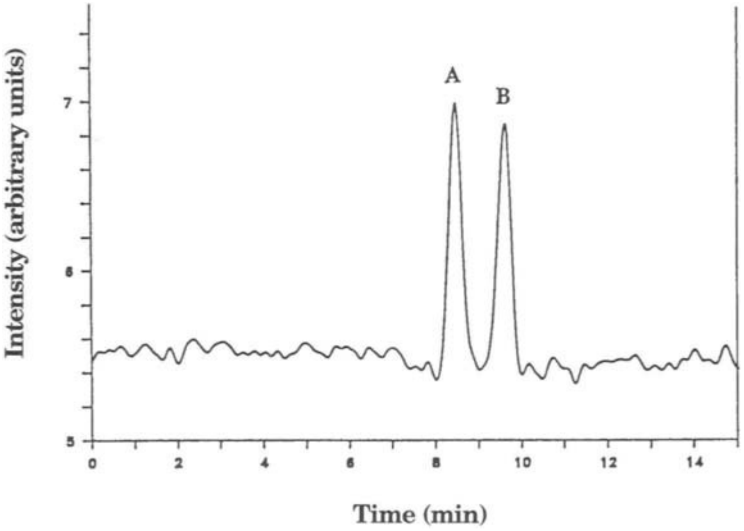


Fig. 20. CE electropherograms of (A) methyl red and (B) methyl orange using resonance Raman detection. The excitation source was the 442 nm output of a He-Cd laser (40 mW). (Reproduced with permission from Chen and Morris, 1988)

would have to be stopped to allow the grating to be scanned, with acquisition of the spectrum requiring several minutes for sufficient sensitivity. Utilizing an array detector, the spectrum could be obtained in a considerably shorter time, permitting uninterrupted flow through the capillary.

The lack of acceptance of Raman spectroscopy as a detection tool in CE analysis results from the poor sensitivity of the technique. As shown in Eq. 14, two methods can be used to enhance the sensitivity of Raman: (1) increase the excitation photon density or (2) increase the differential Raman cross section. Both of these can be accomplished using the technique of surface-enhanced Raman (SER) spectroscopy. A wealth of literature exists which describes this effect (Jeanmarie and Van Duyne, 1977; Albrecht and Creighton, 1977; Chang and Furtak, 1982; Weitz *et al.*, 1983; Moskovits, 1985), which arises when the scattering molecule is adsorbed on or near the surface of a roughened metal substrate, typically copper, gold or silver. Due to the optical properties of the metal, an intense electric field (high photon density) is observed near the surface. In addition, adsorption of the scattering molecule to the surface increases the cross-section by nearly twofold. The final result is enhancement in the Raman intensity of nearly 10^6. The metal substrate can be in the form of colloid suspensions or solid electrodes subjected to repeated oxidation-reduction cycles. Difficulties result from marked differences in the SER spectra when compared to the normal Raman spectrum of the same component. These differences can be in the form of shifts in the positions of bands, absence or presence of new bands in the SER spectra, and changes in the relative intensities of the bands. All of these differences occur from the perturbations of the metal substrate on the adsorbed molecule. Currently, no schemes have been reported using SER spectroscopy as a detection method for CE, although SER has been used in such techniques as thin-layer chromatography (Tran, 1984a; Tran, 1984b; Sequaris and Koglin, 1986) and liquid chromatography (Berthod *et al.*, 1987; Laserna *et al.*, 1987; Freeman *et al.*, 1988; Force, 1988; Ni *et al.*, 1989; Soper *et al.*, 1990).

Another prohibitive feature of Raman spectroscopy for CE detection results from the rather complex and expensive instrumentation needed. The upkeep of ion lasers and the associated optics makes the daily use of the detector difficult. These problems can be addressed through the use of diode lasers and CCD detectors as the principal components for the Raman spectrometer (Wang and McCreery, 1989). Diode lasers are inexpensive and do not require replacement of expensive ion tubes required of argon or krypton lasers. The use of the CCD array detector, whose quantum yields are nearly 0.80 at the lasing wavelengths of diode lasers, offer the sensitivity and speed to collect a Raman spectrum on a time scale appropriate for CE.

7. References

Albin, M., Weinberger, R., Sapp, E., Moring, S. (1991). *Anal. Chem.*, *63*, 417–422.

Albrecht, M. G. and J. A. Creighton (1977). *J. Amer. Chem. Soc.*, *99*, 5215–5217.

Ansorge, W., J. Zimmermann, C. Schwager, J. Stegemann, H. Erfle and H. Voss (1990). *Nucl. Acid Res.*, *18*, 3419–3420.

Berthod, A., J. J. Laserna and J. D. Winefordner (1987). *Appl. Spectrosc.*, *41*, 1137–1141.

Bornhop, D. J. and N. J. Dovichi (1986). *Anal. Chem.*, *58*, 504–505.

Bornhop, D. J. and N. J. Dovichi (1987). *Anal. Chem.*, *59*, 1632–1636.

Bruno, A. E., B. Krattiger, F. Maystre and M. Widmer (1991). *Anal. Chem.*, *63*, 2689–2697.

Carlson, R. G., K. Srinivasachar, R. S. Givens and B. K. Matuszewski (1986). *J. Org. Chem.*, *51*, 3978–3983.

Chang, R. and T. Furtak (1982). *Surface Enhanced Raman Scattering*. Plenum, New York.

Chen, C. Y., T. Demana, S. D. Huang and M. D. Morris (1989). *Anal. Chem.*, *61*, 1590–1593.

Chen, C. Y. and M. D. Morris (1988). *Appl. Spectrosc.*, *42*, 515–518.

Chen, R. F., C. Scott and E. Trepman (1979). *Biochim. Biophys. Acta*, *576*, 440–455.

Cheng, Y-F, R. D. Piccard and T. Vo-Dinh (1990). *Appl. Spectrosc.*, *44*, 755–765.

Christensen, P. L. and E. S. Yeung (1989). *Anal. Chem.*, *61*, 1344–1347.

Colthup, N.B., L. H. Daly and S. E. Wiberley (1990). *Introduction to Infrared and Raman Spectroscopy*. Academic Press, Boston.

de Montigny, P., J. F. Stobaugh, R. S. Givens, R. G. Carlson, K. Srinivasachar, L. A. Sternson and T. Higuchi (1987). *Anal. Chem.*, *59*, 1096–1101.

Dewey, T. G. (1991). *Biophysical and Biochemical Aspects of Fluorescence Spectroscopy*. Plenum Press, New York.

Dollish, F. R., W. G. Fateley and F. R. Bentley (1974). *Characteristic Raman Frequencies of Organic Compounds*. Wiley-Interscience,New York.

Dovichi, N. J., T. G. Nolan and W. A. Weimer (1984). *Anal. Chem.*, *56*, 1700–1704.

Dovichi, N. J. and Y. Cheng, (1988). *Science*, *242*, 562–564.

Dovichi, N. J., F. Zarrin, T. G. Nolan and D. J. Bornhop (1988). *Spectrochim. Acta*, *43B*, 639–649.

Dovichi, N. J. and S. Wu, (1989). *J. Chromatogr.*, *480*, 141–155.

Drossman, H., J. A. Luckey, A. J. Kostichka, J. D'Cunha and L. M. Smith (1990). *Anal. Chem.*, *62*, 900–903.

Flanagan, J. H., B. L. Legendre, R. P. Hammer and S. A. Soper (1995). *Anal. Chem.*, *67*, 341–347.

Force, R. K. (1987). *Anal. Chem.*, *60*, 1987–1989.

Freeman, R D., R. M. Hammaker, C. E. Meloan and W. G. Fateley (1988). *Appl. Spectrosc.*, *42*, 456–460.

Grasselli, J. G. and B. J. Bulkin (1991). *Analytical Raman Spectroscopy*. Wiley-Interscience, New York.

Gross, L. and E. S. Yeung (1990). *Anal. Chem.*, *62*, 427–431.

Guthrie, E. J., J. W. Jorgenson and P. R. Dluzneski (1984). *J. Chromatogr.* Sci., *22*, 171–176.

Haugen, G R. and F. E. Lytle (1981). *Anal. Chem.*, *53*, 1554–1559.

Higashijima, T., T. Fuchigami, T. Imasaka and N. Ishibashi (1992). *Anal. Chem.*, *64*, 711–714.

Ingle, J. D. and S. R. Crouch (1972). *Anal. Chem.*, *44*, 785–794.

Jeanmarie, D. L. and R. P. Van Duyne (1977). *J. Electroanal. Chem.*, *84*, 1–20.

Jones, R., C. J. Oliver and E. R. Pike (1971). *Appl. Opt.*, *10*, 1673–1680.

Kennedy, R. T. and J. W. Jorgenson (1989). *Anal. Chem.*, *61*, 436–441.

Kuhr, W. G. and E. S. Yeung (1988a). *Anal. Chem.*, *60*, 2642–2646.

Kuhr, W. G. and E. S. Yeung (1988b). *Anal. Chem.*, *60*, 1832–1834.

Lakowicz, J. R. (1983). *Principles of Fluorescence Spectroscopy*. New York: Plenum Press.

Laserna, J. J., A. Berthod and J. D. Winefordner (1987). *Talanta*, *34*, 745–747.

Matuszewski, B. K., R. S. Givens, K. Srinivasachar, R. G. Carlson and T. Higuchi (1987). *Anal. Chem.*, *59*, 1102–1105.

Moskovits, M. (1985). *Rev. Mod. Phys.*, *56*, 783–826.

Nakamoto, K. and D. P. Strommen (1984). *Laboratory Raman Spectroscopy*. Wiley-Interscience, New York.

Nakamura, H., A. Matsumoto and Z. Tamura (1982). *Anal. Lett.*, *15*, 1393–1410.

Nakamura, J. K. and S. E. (1968). *Appl. Opt.*, *7*, 1073–1078.

Ni, F., L. Thomas and T. M. Cotton (1989). *Anal. Chem.*, *61*, 888–894.

Nickerson, B. and J. W. Jorgenson (1988a). *J. High Resolut. Chromatogr. Chromatogr. Commun.*, *11*, 878–881.

Nickerson, B. and J. W. Jorgenson (1988b). *J. High Resolut. Chromatogr. Chromatogr. Commun.*, *11*, 533–534.

Nolan, T. G., W. A. Weimer and N. J. Dovichi (1984). *Anal. Chem.*, *56*, 1704–1707.

Oates, M. D., B. R. Cooper and J. W. Jorgenson (1990). *Anal. Chem.*, *62*, 1573–1577.

Pentoney, S. L., Jr., X. Huang, D. S. Burgi and R. N. Zare (1988). *Anal. Chem.*, *60*, 2625–2629.

Prober, J. M., G. L. Trainor, R. J. Dam, F. W. Hobbs, C. W. Robertson, R. J. Zagursky, A. J. Cocuzza, M. A. Jensen and K. Baumeister (1987). *Science*, *238*, 336–341.

Roach, M. C. and M. D. Harmony (1987). *Anal. Chem.*, *59*, 411–415.

Rose, D. L., Jr. and J. W. Jorgenson (1988). *J. Chromatogr.*, *447*, 117–131.

Rose, D. L., Jr. (1991). *J. Chromatogr.*, *540*, 343–353.

Roth, M. (1971). *Anal. Chem.*, *43*, 880–882.

Schulman, S. G. (1977). *Fluorescence and Phosphorescence Spectroscopy: Physicochemical Principles and Practice*. Pergamon, New York.

Scott, R. P. W. (1977). *Liquid Chromatography Detectors*. Chap. 2. Part I. Elsevier, Amsterdam.

Sequaris, J. L. and E. Koglin (1987). *Anal. Chem.*, *59*, 525–527.

Shear, J. B., R. Dadoo, H. A. Fishman, H. H. Scheller and R. N. Zare (1993). *Anal. Chem.*, *65*, 2977–2982.

Shera, E. B., N. K. Seitzinger, L. M. Davis, R. A. Keller and S. A. Soper (1990). *Chem. Phys. Lett.*, *174*, 553–557.

Smith, L. M., J. Z. Sanders, R. J. Kaiser, P. Hughes, C. Dodd, C. R. Connell, C. Heiner, S. B. H. Kent and L. E. Hood (1986). *Nature*, *321*, 674–679.

Smith, R. D., J. A. Olivares, N. T. Nguyen and H.R. Udseth (1988). *Anal. Chem.*, *60*, 436–441.

Soper, S. A., S. M. Lunte and T. Kuwana (1989). *Anal. Sci.*, *5*, 23–29.

Soper, S. A., T. Kuwana (1989). *Appl. Spectrosc.*, *43*, 883–886.

Soper, S. A., K. L. Ratzlaff and T. Kuwana (1990). *Anal. Chem.*, *62*, 1438–1444.

Soper, S. A., E. B. Shera, J. C. Martin, J. H. Jett, J. H. Hong, H. L. Nutter and R. A. Keller (1991). *Anal. Chem.*, *63*, 432–437.

Soper, S. A., L. M. Davis and E. B. Shera (1992). *J. Opt. Soc. Am. B.-Opt. Physics, 9*, 1761–1769.

Stobaugh, J. F., A. J. Repta, L. A. Sternson and K. W. Garren (1983). *Anal. Biochem.*, *135*, 495–504.

Swerdlow, H., J. Z. Zhang, D. Y. Chen, H.R. Harke, R. Grey, S. Wu and N. J. Dovichi (1991). *Anal. Chem.*, *63*, 2835–2841.

Sweedler, J. V., J. B. Shear, H. A. Fishman, R. N. Zare and R. H. Scheller (1991) . *Anal. Chem.*, *63*, 496–502.

Tabor, S. and C.C. Richardson (1987). Proc. Natl. Acad. Sci., *84*, 4767–4771.

Tran, C.D. (1984a). *J. Chromatogr.*, *292*, 432–438.

Tran, C.D. (1984b). *Anal. Chem.*, *56*, 824–826.

Tsuda, T., Kobayashi, Y., Hori, A., Matsumoto, T. and Suzuki, O. (1988). *J. Chromatogr.*, *456*, 375–381.

Tsuda, T., J. V. Sweedler and R. N. Zare (1990). *Anal. Chem.*, *62*, 2149–2152.

Ueda, T., R. Mitchell, F. Kitamura, T. Metcalf, T. Kuwana and A. Nakamoto (1992). *J. Chromatogr.*, *593*, 265–274.

Walbroehl, V. and J. W. Jorgenson (1984). *J. Chromatogr.*, *315*, 135–143.

Wallingford, R. A. and A. G. Ewing (1988). *Anal. Chem.*, *60*, 258–263.

Wang, Y. and R. L. McCreery (1989). *Anal. Chem.*, *61*, 2647–2651.

Wehry, E. L. (Eds., 1976). *Modern Fluorescence Spectroscopy*. Plenum, New York.

Weitz, D. A., S. Garoff, J. I. Gersten and A. Nitzan (1983). *J. Chem. Phys.*, *78*, 5324–5338.

Winefordner, J. D., S. G. Schulman and T. C. O'Haver (1972). *Luminescence Spectrometry in Analytical Chemistry*. Wiley-Interscience, New York.

Yeung, E. S. (1986). *Detectors for Liquid Chromatography*. Chap. I. Wiley-Interscience, New York.

Yeung, E. S. (1989). *Acc. Chem. Res.*, *22*, 125–130.

Yeung, E. S. and W. G. Kuhr (1991). *Anal. Chem.*, *63*, 275A–282A.

Yu, M. and N. J. Dovichi (1989). *Appl. Spectrosc.*, *43*, 196–201.

CHAPTER 5

Capillary Electrophoresis/Mass Spectrometry

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1. Introduction

The increasing application of capillary electrophoresis (CE) to pharmaceutical and biochemical analyses has inevitably led to a growing demand for detection methods which are more sensitive, selective, or structurally informative for the separated species. Mass spectrometry (MS) has long been recognized as perhaps the most selective and broadly applicable detector for analytical separations, but its combination with CE is relatively new, and the methodology continues to evolve rapidly. Recent and on-going developments in MS instrumentation are leading to continuing improvements in the sensitivity, speed, and level of structural detail obtainable from this powerful "detector." Concurrent developments involving tandem-MS methods (*i.e.*, MS/MS) and higher order-MS methods (*i.e.*, MS^n, where $n \geq 3$) promise that instrumentation offering even greater selectivity and structural information will be routinely achievable in the future. Although MS is still one of the most complex and expensive modes of CE detection, costs continue to decrease while user "friendliness" increases, and the density of information that can be gained from this combination increasingly mitigates its disadvantages. Indeed, when MS detection is desired to resolve an analytical issue, there is often no practical substitute.

The mass spectrometer provides the equivalent of up to several thousand discrete selective "detectors" functioning essentially in parallel; these are capable of providing molecular weight information from the intact "molecular ion" as well as structurally related information from its dissociation in the mass spectrometer. The CE-MS combination is, in one sense, nearly ideal: CE is based upon the differential migration of ions in solution, while MS analyzes ions by their mass-to-charge ratio (m/z) in the gas phase. On the other hand, these two highly orthogonal analytical methods exploit ion motion in two quite different environments—moderately conductive liquid buffers and high vacuum, respectively. A primary role of the CE-MS interface is to bridge these different environments. The combination of liquid chromatography (LC) with MS has long been of interest, and a variety of clever LC-MS interfacing schemes have been investigated over the last 25 years. However, the CE-MS combination places significantly different demands on an interface than does LC-MS. CE flow rates are quite low or negligible, the buffer is moderately conductive, electrical contact must be maintained with both ends of the capillary so as to define the CE field gradient, and (if the high separation efficiencies possible with CE are to be realized) any "dead volume" associated with detection or band-broadening due to laminar flow (arising from pressure differences between the capillary termini) must be avoided or minimized.

In this chapter, we review CE-MS interfacing methods based upon electrospray ionization (ESI) methods, experimental considerations, and selected applications. We emphasize CE separations by free-solution electrophoresis, *i.e.*, capillary zone electrophoresis (CZE). The bias of this chapter reflects the explosive growth in ESI applications into the field of mass spectrometry, the increasing availability of such instrumentation, the compatibility with MS methods capable of providing extensive structural information, and the authors' belief that the ESI interface is the method of choice for CE-MS due to its ease of implementation. (This emphasis is not meant to diminish the impressive results obtained with fast atom bombardment (FAB) interfaces, particularly for small molecule applications where results roughly comparable to ESI interfaces have been achieved.) Finally, we wish to describe clearly the current status of CE-MS, a highly promising combination still somewhat limited by imperfect understanding of the ESI interface, sensitivity, and the scan speed constraints of current MS technology. Since these MS limitations are almost sure to be overcome in the next few years (in the authors' optimistic assessment), the future for CE-MS holds exceptional promise.

2. CE-MS Interfacing Methods

In addition to a few reports of off-line CE-MS methods (Kenndler and Kanianski, 1981; Kenndler and Haidl, 1985; Takigiku *et al.*, 1990), two general on-line interfacing methods have been used for CE-MS based upon continuous flow fast atom bombardment (FAB) and electrospray ionization introduction methods for mass spectrometry. The two methods differ in the process used for ionization (or for transfer of ions from the liquid to the gas phase) so as to allow for the subsequent m/z analysis. We note that there are a number of references to FAB interfaces for CE-MS (DeWit *et al.*, 1988; Caprioli *et al.*, 1989; Minard *et al.*, 1989; Moseley *et al.*, 1989a,b, 1990, 1991; Reinhoud *et al.*, 1989, 1990; Caprioli, 1991; Deterding *et al.*, 1991; Moore and Caprioli, 1991; Suter *et al.*, 1991; Tehrani *et al.*, 1991; Verheij *et al.*, 1991; Wolf *et al.*, 1991; Suter and Caprioli, 1992) to which the interested reader is referred. We will not consider these methods further in this chapter.

CE-MS interfaces based upon ESI require a mass spectrometer incorporating an atmospheric pressure ionization/inlet. The availability of such MS instruments is rapidly increasing with numerous commercial implementations. Two variations on ESI interfaces based upon "liquid junction" and "sheath flow" (or "coaxial") approaches have been generally used and are shown schematically in Fig. 1. The ESI method is well-suited for CE-MS interfacing since it produces ions directly from liquid solutions at atmospheric pressure (Fenn *et al.*, 1990; Smith *et al.*, 1990b, 1991a). Considerations for interfacing generally derive from some limitations upon buffer composition for effective operation of the ESI source and the desire to position the ESI source (*i.e.*, the point of charged droplet formation) as close as possible to the analytical capillary terminus, so as to avoid lengthy transfer lines.

Figure 2 shows a general schematic illustration for CE-MS/MS instrumentation based upon the sheath flow interface, such as used in our laboratory. The ESI "source" is generally operated at an elevated voltage of 3-5 kV relative to the sampling orifice, where ions which are formed at atmospheric pressure enter the mass spectrometer entrained in a flow of gas (Smith *et al.*, 1990b). Many of the practical constraints and considerations for CE-MS interfacing derive from the voltage bias between these components. Some ESI sources allow the liquid effluent to be at ground potential, while others require the capillary terminus to be operated 3-5 kV relative to ground potential. The ESI source requires production of a high electric field that causes charge to accumulate on the liquid surface at the capillary terminus so as to disrupt the liquid flow and form droplets. The ESI liquid nebulization process can be mechanically or pneumati-

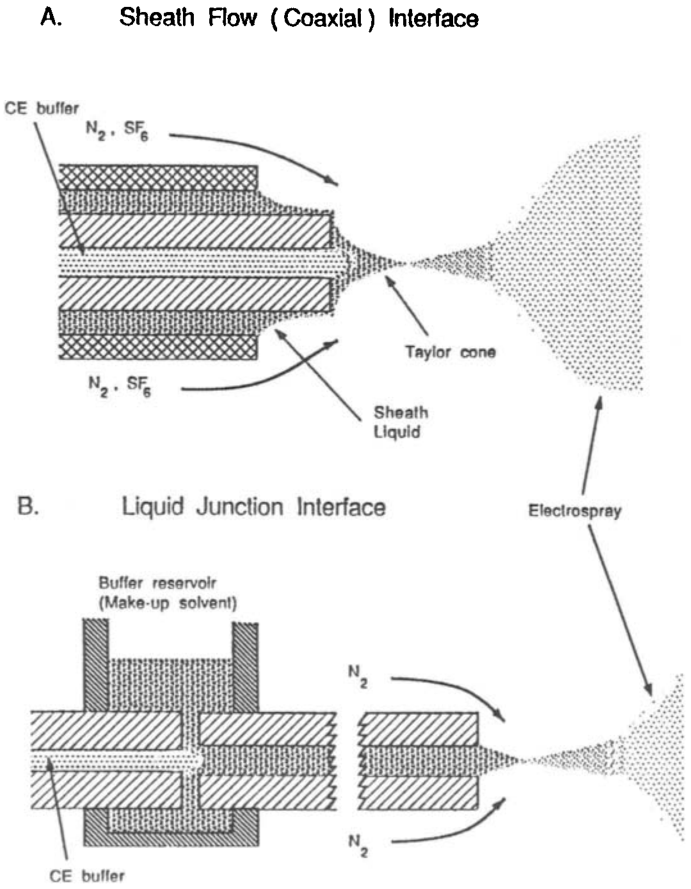


Fig. 1. Schematic electrospray ionization illustration of the (A) sheath flow (co-axial) and (B) liquid junction interfaces used for CE-MS. (Adapted with permission from Smith *et al.*, 1993.)

cally assisted (by vibration at high frequency or a high velocity annular gas flow at the capillary terminus, respectively). The pneumatically assisted electrospray is sometimes referred to as "ion spray" (Bruins *et al.*, 1987), an approach originally described for ESI by Dole and coworkers (Mack *et al.*, 1970).

The first CE-MS was based upon an electrospray ionization interface developed in our laboratory (Olivares *et al.*, 1987; Smith *et al.*, 1988a). This initial approach used a metalized capillary terminus to make contact with the CE eluent under conditions of high electro-

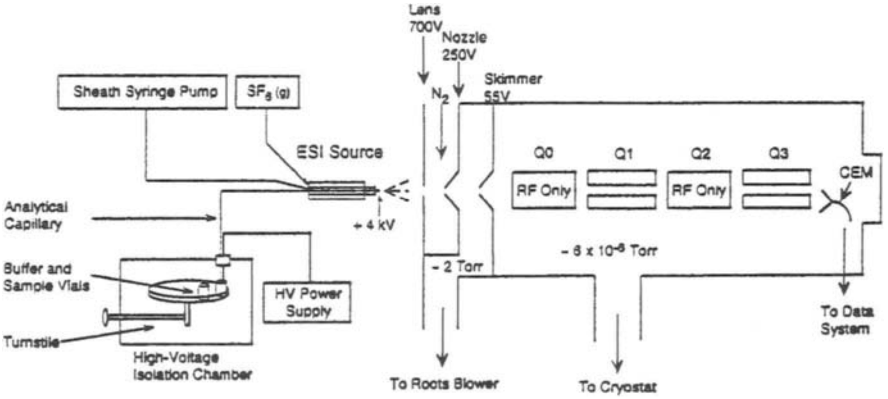


Fig. 2. Schematic illustration of the experimental arrangement for CE-MS. Details of one version of the sheath flow interface are given in Fig. 3. (Reprinted with permission from Smith *et al.*, 1991b.)

osmotic flow. We later reported an interface using a flowing sheath liquid (Smith *et al.*, 1988b), and also demonstrated the combination of capillary isotachophoresis (CITP) with MS (Udseth *et al.*, 1989; Smith *et al.*, 1990a) and CE-MS/MS (Smith *et al.*, 1990c; Edmonds *et al.*, 1990). More recently, other researchers have reported similar interfaces (Hail *et al.*, 1990; Hunt *et al.*, 1991; Shida *et al.*, 1989; Thibault *et al.*, 1991; Moseley *et al.*, 1992; Parker *et al.*, 1992; Pleasance *et al.*, 1992a,b.

The ESI interface:liquid junction variation was developed by Henion and coworkers (Lee *et al.*, 1988, 1989a,b) who have reported extensively upon its applications (Mück and Henion, 1989; Johansson *et al.*, 1991a,b; Wachs *et al.*, 1991; Garcia and Henion, 1992), and explored more recently by Pleasance *et al.* (1992b), who have compared its performance to the sheath flow approach. As indicated in Fig. 1B, electrical contact with the capillary terminus for the liquid junction interface is established through a liquid reservoir which surrounds the junction of the analytical capillary and a transfer capillary. The gap between the two capillaries is typically adjusted to 10–20 μm, a compromise resulting from the need for sufficient make-up liquid being drawn into the transfer capillary while avoiding analyte loss by diffusion into the reservoir. The flow of make-up liquid arises from a combination of gravity-driven flow due to the height of the make-up reservoir, and flow induced in the transfer capillary due to a mild vacuum generated by the venturi effect of the nebulizing gas used at the ESI source (Pleasance *et al.*, 1992b).

In the sheath flow (or coaxial) electrospray interface (Fig. 1A), a liquid (often methanol, methoxyethanol, or acetonitrile, but frequently augmented by as much as 10–20 percent formic acid, acetic acid, water, and/or other reagents) flows through the annular space between the ~200 µm o.d. CE capillary and a fused silica or stainless steel capillary (–250 µm i.d.). The fused silica separation capillary typically protrudes approximately 0.2 mm from the sheath capillary. A recent version of this interface developed at the authors' laboratory is shown in Fig. 3. In this design a 300 µm i.d. fused silica capillary surrounds the smaller diameter electrophoresis capillary. For the positive ion mode ESI operation, a voltage of +4 to +6 kV is applied indirectly to the sheath liquid from which a syringe pump delivers the flow of sheath liquid at 2–5 µl/min. Potential problems from the formation of bubbles in the connecting lines are minimized by the inclusion of a trapping volume. Enhanced stability can sometimes be obtained by degassing the organic solvents used in the sheath and by minimizing heating (*e.g.*, from the ion source or a "counter-current" gas flow). Cooling of the ESI source (Smith *et al.*, 1991b) or the use of

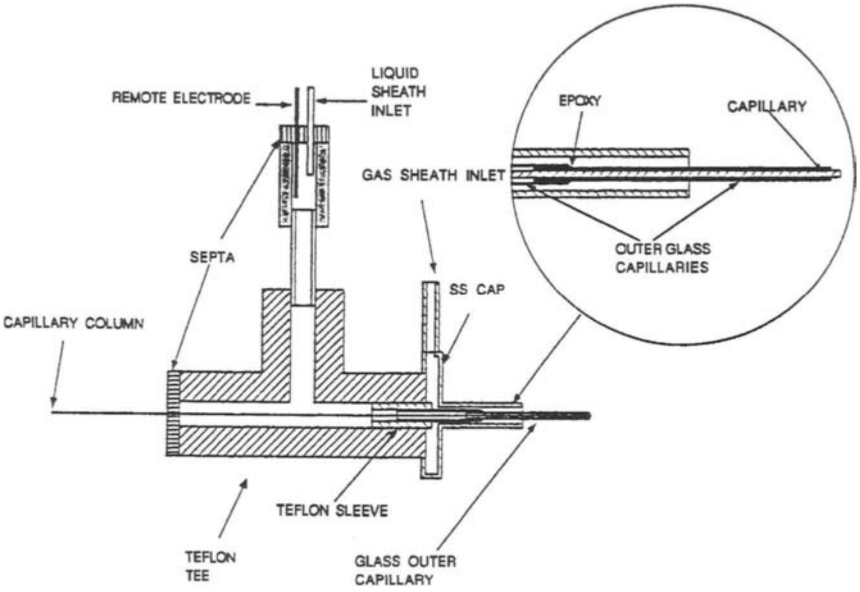


Fig. 3. One version of a sheath flow (or coaxial) electrospray ionization interface for CE-MS developed at the authors' laboratory. See also Figs. 1 and 2. (Reprinted with permission from Smith *et al.*, 1991b.)

less volatile sheath solvents such as methoxyethanol (Mylchreest and Hail, 1991) has also been useful in some situations. A number of reports from our laboratory have described work based upon the sheath flow approach (Edmonds *et al.*, 1989; Loo *et al.*, 1989, 1991; Smith *et al.*, 1989, 1990, 1991b).

It appears that most of the distinctions between the sheath flow and liquid junction ESI interfaces are relatively unimportant, and the performance in terms of spectral quality is similar. However, some differences have been noted. Pleasance *et al.* (1992b) have compared the liquid junction and sheath flow approaches using a pneumatically assisted ESI interface, and have noted that the latter provided a "more robust and reproducible interface with the added advantage of offering an independent means of calibration and quantitation . . . through the sheath" (Thibault *et al.*, 1991b). Improved signal-to-noise ratios and slightly improved separations were obtained with the sheath flow interface. Differences in elution times using the liquid junction interface were reported by these workers and attributed to modification of the liquid junction buffer due to flushing of the CE capillary between analyses.

The dependence of ESI ion current upon solution conductivity is relatively weak, generally 0.1–0.3 μA at atmospheric pressure, and only about 10–100 pA integrated ion current (*i.e.*, the sum of all ions) is actually transmitted through the MS and detected. An electron scavenger is often used to inhibit electrical discharge at the capillary terminus, particularly for ESI of aqueous solutions. Sulfur hexafluoride has also proven particularly useful for suppressing corona discharges for such applications and improving the stability of negative ion ESI. Introduction of this gas is most effectively accomplished using a gas flow (~50–200 mL/min) through an annular volume surrounding the sheath liquid capillary (Figs. 1A and 2).

The formation of ions suitable for MS requires conditions affecting solvent evaporation from the initial droplet population produced by ESI. Droplets must shrink to the point where repulsive coulombic forces approach the level of droplet cohesive forces (*e.g.*, surface tension). This evaporation can be accomplished at atmospheric pressure by a counter-current flow of dry gas at moderate temperatures (~80°C), by heating during ion transport through the sampling region, and (particularly in the case of ion trapping MS methods) by energetic collisions at relatively low pressure.

An important attribute of a CE-MS interface is the efficiency of ion sampling and transport through the mass spectrometer. The simplest commercially available instrumentation utilizes a 100–130 μm pinhole sampling orifice to a vacuum region maintained by a single stage of high-speed cryo-pumping (Sciex, Thornhill, Ontario, Canada). Charged droplets formed by ESI drift in the high electric field against a counter-current flow of dry N_2, which serves to speed desolvation and exclude high m/z residual particles and solvent vapor. Ions passing through the orifice into the vacuum region undergo further desolvation as the gas density decreases due to collisions and the ions are accelerated by the ion optics of the mass spectrometer.

Alternatively, many instruments have been developed based on differentially pumped interfaces. The ESI capillary inlet-skimmer interface developed by Fenn and coworkers (Fenn *et al.*, 1990) is widely used and is commercially available (Analytica, Branford, CT) in versions compatible with many mass spectrometers. A counter-current flow of N_2 bath gas is used to assist solvent evaporation, similar to the approach described above. Ions migrate towards the sampling orifice, where some small fraction is entrained in the gas flow entering a glass capillary (metalized at both ends to establish well-defined electric fields). Although some losses occur, ions are transmitted through capillaries with high efficiency. Ions emerge from the capillary in the first differentially pumped stage of the MS (~1 torr) as a component of a free jet expansion. A fraction of these ions are then transmitted through a "skimmer" and additional ion optics into the mass spectrometer. The electrically insulating nature of the glass capillary provides considerable flexibility because ion transmission does not depend strongly on the voltage gradient between the conducting ends of the capillary. This approach has the advantage that the CE capillary terminus can be at ground potential, simplifying CE current measurements and voltage manipulation during injection.

An alternative approach to droplet desolvation for ESI relies solely on heating during droplet transport through a heated metal (Chowdhury *et al.*, 1990; Rockwood *et al.*, 1991) or glass capillary (Fenn *et al.*, 1990; Ogorzalek Loo *et al.*, 1991). A counter-current gas flow with this approach is not essential and, in fact, significantly decreases obtainable ion currents. The electrospray source can be closely positioned to the sampling capillary orifice (typically 0.3–1.0 cm), since larger spacing is not needed for desolvation, resulting in more efficient charge transport into the capillary. The charged droplets from the ESI source are swept into the heated capillary, which heats the gas sufficiently to provide effective ion desolvation, particularly when augmented by a voltage gradient in the capillary-skimmer region. The disadvantage of this approach, and that of all sources not using a gas "curtain" or counter-current flow, is that much more solvent and residual material (*i.e.*, solute particles) enters the mass spectrometer, resulting in the need for more frequent disassembly and cleaning. Advantages of the heated capillary, however, are the ease with which it can be adapted to a variety of MS configurations, and the ability to "heat" ions for either desolvation or dissociation (Rockwood *et al.*, 1991) in a manner largely independent of m/z.

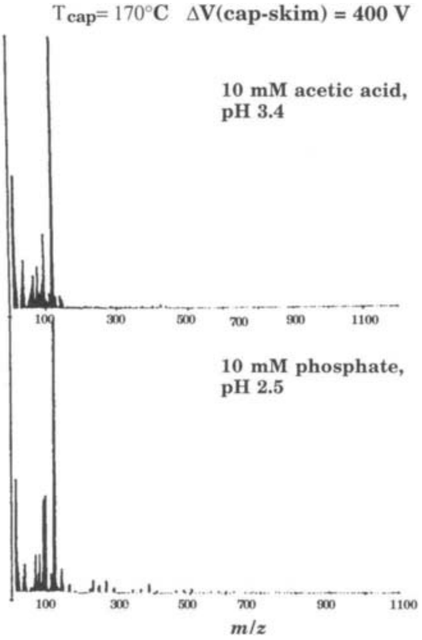
ESI is an extremely "soft" ionization technique and will, under appropriate conditions, yield intact molecular ions without contributions due to dissociation (unless induced during transport into the MS vacuum). Molecular weight measurements for large biopolymers which exceed the MS "mass range" can be obtained because their ESI mass spectra generally consist of a distribution of molecular ion charge states (Fenn *et al.*, 1990; Smith *et al.*, 1991a). The envelope of molecular ion charge states for proteins, arising generally from protonation for positive ion ESI, yields a distinctive pattern of peaks due to the discrete nature of the electronic charge; *i.e.*, adjacent peaks vary by addition or subtraction of one charge. In the case of noncovalently bonded species, such as multimeric proteins, mass spectra typically show only the individual subunits. With proper care, however, it is also possible to detect a range of noncovalently associated species by ESI-MS.

3. Experimental Methods and Considerations for CE-MS Analyses

The success of combined CE-MS methods depends upon a number of factors related to the interfacing techniques. In general, all of the concerns in CE relevant to sample injection, buffer composition, capillary surface interactions, and separation efficiency apply to CE-MS, and the reader is referred to other chapters in this volume. Most CE-MS has been conducted with capillaries of 50–100 μm i.d., although, as is discussed later, the best sensitivities for CE-MS are obtained with smaller diameter capillaries. However, most CE with other more conventional detectors is conducted with such capillaries, and selection represents a compromise between factors related to detector sensitivity, injection, capillary coating techniques, and the degradation of separation quality due to resistive heating in larger diameter capillaries. Both interfaces allow a wide range of CE buffers to be successfully electrosprayed (Smith *et al.*, 1990a,b). Since both the liquid junction and sheath flow interfaces utilize an effective dilution of the low CE effluent flow by a much larger volume of sheath liquid, considerable flexibility exists despite some constraints (very high salt and even moderate surfactant concentrations remain problematic). Buffer concentrations of at least 0.1 M can be used for CE-MS due to the dilution step. However, due to practical sensitivity constraints, CE buffer concentrations are generally minimized, and it has also been found that sensitivity can vary significantly with buffer composition. In general, the best sensitivity is obtained with volatile buffer components (such as acetic acid) at the lowest practical concentration, and by minimizing other nonvolatile and charge-carrying components. In addition, buffer components that interact strongly with the sample (*e.g.*, denaturants) substantially degrade sensitivity, largely due to association in the gas phase (*i.e.*, "clustering"). The use of surfactants, *e.g.*, sodium dodecyl sulfate (SDS), gives rise to intense signals in both positive and negative ion ESI and presents a major barrier to micellar electrokinetic capillary chromatography-MS.

Due primarily to their volatility, the most widely used buffers to date are acetic acid, ammonium acetate, and formic acid. It has sometimes been stated that involatile buffer constituents cannot be used for CE-MS. This is not the case, although different ESI interfaces may be required for their routine use. Figure 4 shows ESI mass spectra for comparison of a 10 mM acetic acid solution (pH ~3.4) and a 10 mM phosphate buffer (pH 2.5) with sheath liquids of the same composition. The major "background" ions due to the buffer are below *m/z* 150. However, as shown in Fig. 5, an inspection of the same mass spectra at higher gain (× 32.5) shows that the constraints due to "background" ions for these solutions are quite different. The phosphate buffer results in cluster ions extending to at least *m/z* 1000 with sufficient abundance to substantially limit CE-MS applicability. In contrast, acetic acid gives rise to broad "background" contributions presumably arising due to solution impurities; this illustrates the need to utilize the highest-purity buffers achievable for CE-MS. The MS sensitivity, which is ultimately limited by the ability of the mass spectrometer to efficiently analyze ions produced at atmospheric pressure by ESI, is probably the most important factor related to MS detection, and is discussed later at greater length. Sensitivity does not appear to depend significantly on the mechanical details of a well-designed and optimized electrospray source, although its design details can influence ESI stability and ease of operation.

Increasingly, CE-MS instrumentation benefits from automation of electrokinetic or hydrostatic injection, and capabilities for various on-column spectrophotometric detectors, as well as capillary temperature control. The advantages of computer-controlled injection are significant for both accuracy and precision, as well as freedom from artifacts arising from less reliable manual injection methods (Smith *et al.*, 1989b). Temperature control has also been recognized as important in obtaining good reproducibility. In the case of high CE currents, cooling the capillary can reduce difficulties with the interface. Gas generated in either the CE capillary or the ESI sheath capillary (which often contains more volatile organic solvent) can create a region of high resistance and effectively terminate a separation. The



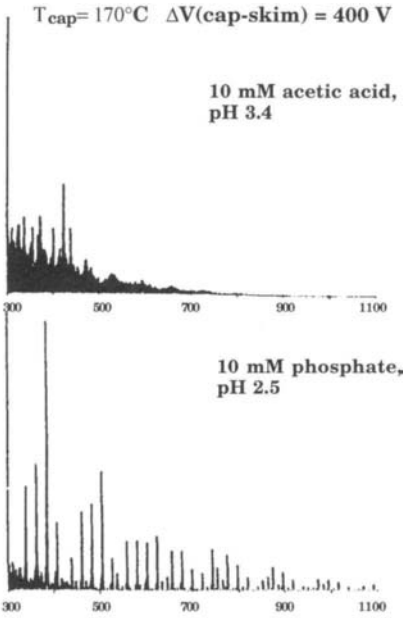


Fig. 4. (left) Comparison of "background" mass spectra from ESI of 10 mM acetic acid and phosphate buffer solutions obtained by direct infusion, and with a sheath liquid of the same composition.

Fig. 5. (right) Comparison of mass spectra at high gain (× 32.5 the scale in Fig. 4) for acetic acid and phosphate buffers.

use of a less volatile sheath liquid such as methoxyethanol has been reported to be useful for reducing such difficulties and may improve sensitivity, depending upon interface design (*i.e.*, due to the desolvation conditions). On the other hand, the ability to use higher temperatures can be essential to obtaining good separations of proteins, where higher order structural heterogeneity can be strongly temperature dependent. In addition, higher temperatures tend to denature proteins and increase the extent of charging during ESI (Loo *et al.*, 1991a). This can allow detection of some proteins (particularly glycoproteins) which might otherwise produce ions beyond the MS *m/z* range.

CE capillary surface coatings and conditioning often need to be optimized for particular applications. Protein separations are generally problematic, and our initial CE-MS of proteins in acidic buffers (pH 3–5) with uncoated capillaries produced relatively poor separations for proteins such as myoglobin and cytochrome *c* due to interactions with capillary surfaces (Loo *et al.*, 1989). However, excellent separations of myoglobin mixtures have been obtained with uncoated capillaries at higher pH (>8), an observation ascribed to the net negative charge of both the protein and the fused silica surface. In this case, a methanol-water sheath liquid containing 5 to 10% acetic acid was used to obtain the acidic conditions under which positive mode ESI provided the best sensitivity for these proteins. Under these conditions, reasonable detection limits (<10 fmol) could be obtained using multiple ion monitoring in buffers containing 10 mM Tris (Smith *et al.*, 1991b). However, the Tris buffer component, and perhaps limitations upon mixing of the CE and sheath flows, resulted in reduced sensitivity compared to that obtained for the same separations with volatile acidic buffers (Smith *et al.*, 1991b). Thibault *et al.* (1991a) demonstrated that protein separations conducted in acidic solution (*e.g.*, 10 mM acetic acid, pH 3.4) using reversible amino-based coated capillaries provided substantially improved sensitivity, and allowed useful full scan mass spectra to be obtained with hundred femtomole amounts of analyte. With these capillaries, the direction of electroosmotic flow is reversed, the inlet buffer is at a high negative voltage, and analytes with the smallest electrophoretic mobilities elute first.

4. Mass Spectrometric Considerations

Since the ESI interface is generally operated at atmospheric pressure, the pressure drop across the CE capillary can be made precisely zero, and ideally no degradation of separation efficiency should arise due to laminar flow. The ESI method is expected to be amenable to essentially any charged species in solution, but this in itself does not guarantee success of the combined CE-MS method. Gas phase ions must be substantially desolvated and relatively free of adducted buffer-related species, and must have an m/z within the capabilities of the MS. The flow rate necessary to sustain a stable ESI source from a capillary of conventional dimensions is generally 1–10 μL/min, but can ideally be higher or lower, depending on solution conductivity, source "tip" design, the use of nebulizing gas, etc. A crucial point is that CE flow rates are generally much lower, and the buffers used are often substantially more conductive than desired for existing ESI interfaces. Both the sheath flow and liquid junction interfaces introduce a "make up" flow of a less conductive (and generally more volatile) solution, which often serves to dilute the CE buffer and assist ESI. The result of this additional flow is that a wide range of CE buffer compositions and solvents can be effectively addressed, but with some constraints due to the volatility and any inherent ESI response (*i.e.*, "background") due to buffer components or their adduction with other solution species. Moseley *et al.* (1992) have reported an evaluation of sheath and buffer constituents upon ESI-MS, but it must be remembered that the variations in ESI response for different solvents could be dependent upon the details of the interface with regard to ESI droplet-evaporation and ion desolvation.

The major considerations relevant to MS detection are generally due to the nature and complexity of the particular sample, and pragmatic constraints due to MS detection sensitivity, resolution, and related scan-speed compromises. For quadrupole mass spectrometers, single or multiple ion monitoring, sometimes referred to as selected ion monitoring (SIM), leads to significantly enhanced detection limits compared to scanning operation, due to the greater "dwell time" at specific m/z values. For samples where analyte molecular weights are known and m/z values can be predicted, SIM detection is an obvious choice. If sufficient sample is available, a direct infusion experiment can be used to produce a mass spectrum of the unseparated mixture and the results used to select a suite of m/z values for a subsequent separation using SIM detection. This approach can be problematic, however, since relative ionization efficiencies can be different for direct infusion of the mixture compared to the separated species. In addition, noncovalent associations between mixture components may further complicate interpretation. Thus, important components may be overlooked during the direct introduction of mixtures. One of the advantages of CE-MS is that mixture components strongly discriminated against for such direct infusion experiments often show much more uniform response after CE separation.

The realization of high efficiency CE-MS separations, leading to peak widths of only a few seconds or less, presents challenges for most mass spectrometers. The on-line combination of capillary zone electrophoresis with mass spectrometry was first demonstrated in this laboratory for synthetic mixtures of quaternary ammonium salts (Olivares *et al.*, 1987; Smith *et al.*, 1988a). These initial separations used 100 μm i.d. by 100 cm-long uncoated fused silica capillaries and a "buffer" of 10^{-4} M KCl dissolved in water-methanol 1:1 (v/v) with a field gradient of 370 V/cm. As shown in Fig. 6, a mixture of five quaternary ammonium cations (m/z from 74 to 242) was detected at subfemtomole levels with separation efficiencies ranging from 35,000 to 140,000 theoretical plates. The use of "full scan" detection with quadrupole mass spectrometers often results in either limited signal intensities (due to the extremely short dwell time at each m/z) or too few scans obtained during peak elution. One option is to reduce MS resolution, which increases signal intensities and allows faster scan speeds. This is not an ideal solution, since mass measurement accuracy is reduced and chemical noise due to "background" signals at adjacent m/z values may mitigate any gain. For these reasons, obtaining the maximum number of theoretical plates possible with combined CE-MS has not been a major concern. Thus, CE conditions involving longer separations and larger injection volumes than optimum have often been used. Similar but magnified constraints arise with the use of tandem-MS methods for obtaining structural information, as in polypeptide sequencing, due to the low signal intensities with conventional MS/MS instruments. These limitations are a major driving force for the implementation of improved MS instrumentation using array detection or ion trapping methods. In the next section, we discuss the issue of sensitivity in more detail and describe two recently developed approaches to gaining improved sensitivity in CE-MS.

5. Practical Approaches to Improved CE-MS Sensitivity

5.1. Reduced Elution Speed Detection

The small quantities of solute used in CE require highly sensitive detection methods. The low signal intensities generally produced by ESI-MS, typically resulting in maximum analyte ion detection rates of no greater than 10^5 to 10^6 counts/s, effectively limit the maximum practical scan speeds with quadrupole mass spectrometers. Thus, depending on the desired m/z range, solute concentration, and other factors related to the nature of the solute and buffer species, maximum m/z scan speeds are often insufficient to exploit the high quality separations feasible with CE when coupled to quadrupole or other scanning mass spectrometers.

For fundamental reasons, the maximum electrospray ion current is only a relatively weak function of solution conductivity. When the delivery of charge-carrying solute entering the ESI source (*i.e.*, the product of concentration and mass flow rate) exceeds the capability of the electrospray process for complete ionization, the efficiency of solute ionization decreases. From an alternate viewpoint, charge separation due to ESI becomes less efficient at higher electrolyte concentrations, and a decreasing fraction of liquid-phase ions can be transferred to the gas phase at a given flow rate. Figure 7 qualitatively illustrates the nature of ESI ion formation vs. delivery rate to the ESI source. Two general regimes exist. At higher analyte concentrations or flow rates, ESI-MS signal strengths become relatively insensitive to flow rates. This explains the origin of the often-made

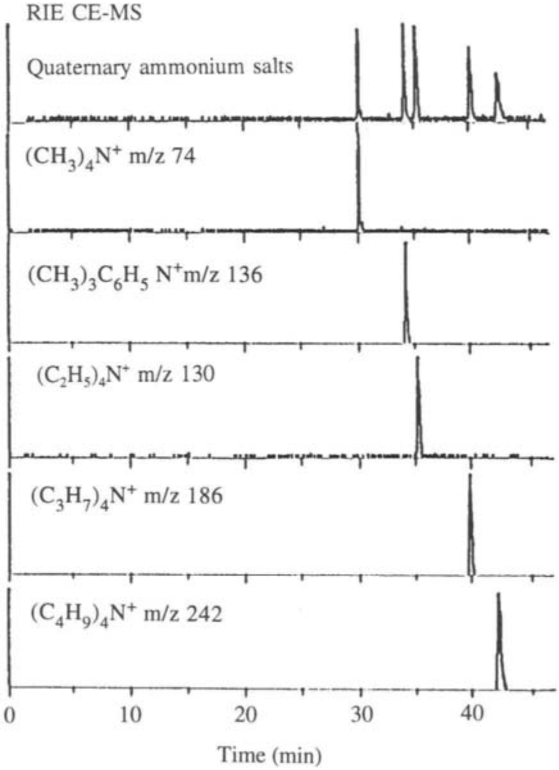


Fig. 6. Total and reconstructed single ion electropherograms for CE-MS of a mixture of five quaternary ammonium salts. The separation was conducted on a 2 m × 100 μm i.d. uncoated capillary in a 10 mM phosphate buffer at pH 4 with an electric field of 200 V/cm. (Reproduced with permission from Smith *et al.*, 1988a.)

comment that ESI-MS functions like a "concentration sensitive" detector. At low flow rates (or concentrations), the ESI signal strength becomes limited by the number of charge-carrying species in solution. In this regime, optimum sensitivity will be obtained.

In fact, most ESI work, and nearly all CE-MS, has been conducted in this region, where the efficiency of solute ionization is substantially limited by factors that include competition for available electrospray current between analyte and buffer components. CE separations generally incur higher currents (5–50 μA) than do typical ESI currents (0.1-0.5 μA), and therefore deliver charged species to the ESI source at a rate where ionization is necessarily inefficient. Any

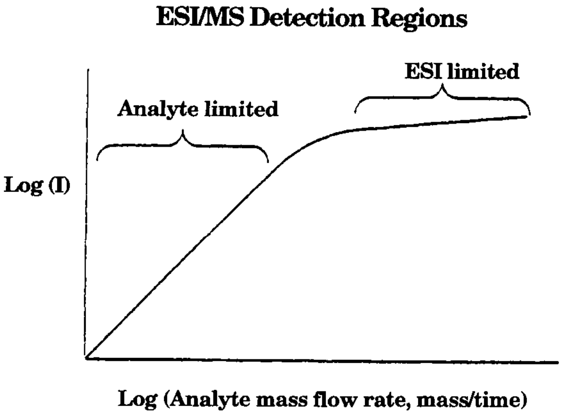


Fig. 7. Illustration of the two regimes for ionization efficiency in ESI. Analyte signal intensities are approximately proportional to analyte concentration in a direct manner at low CE flow rates where the delivery of charged species to the ESI source is less than the ESI current. At higher delivery rates, signal intensities are nearly independent of CE flow rates and related to analyte concentration.

contaminant present may also compete with the analyte for available charge, thus decreasing the solute ionization efficiency. Ideal buffer components have characteristics that allow CE separation of the analytes, are volatile, discriminated against during ESI, and form minimal gas phase contributions to the mass spectra arising from ionized clusters of buffer constituents.

An approach to obtaining enhanced sensitivity for CE-MS based upon reduced elution speed (RES) detection has recently been developed in our laboratory; it helps to alleviate both the sensitivity and scan speed limitations of CE-MS with scanning mass spectrometers (Goodlett *et al.*, in press). Involving only the ability to institute step changes in the CE electric field strength, the technique is simple and readily implemented. Figure 8 illustrates the basis of RES detection. A hypothetical CE-MS separation, where the electric field strength is held constant throughout the analysis, is shown in Fig. 8A. Alternatively, prior to elution of the first solute to the ESI source, the electrophoretic voltage is decreased in the RES CE-MS experiment, yielding the result shown in Fig. 8B for the same two solutes. Elution of solutes is slowed, allowing more scans to be recorded without significant loss in ion intensity. Under conditions where the quantity

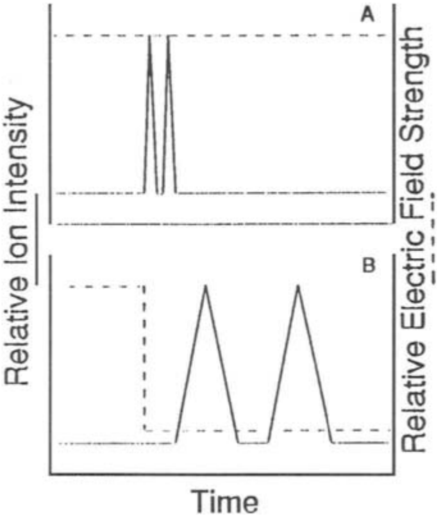


Fig. 8. Hypothetical CE/ESI-MS experiment showing the separation of a two-component mixture by (A) constant field strength CE/ESI-MS and (B) reduced elution speed CE/ESI-MS. Relative ion intensity is denoted by (—) and relative electric field strength by (– – –). (Reproduced with permission from Goodlett *et al.*, 1993.)

of solute entering the ESI source per unit of time exceeds the current available for complete ionization of the solute (*i.e.*, the solute "saturates) the electrospray process), we expect no substantial decrease in maximum ion intensity when the electric field strength is decreased. Thus, a larger fraction of the analyte ions will be transferred to the gas phase during RES CE-MS than during normal constant electric field strength CE-MS.

It has been shown previously (Jones *et al.*, 1990) that, in general, there is a negligible effect on separation quality (*i.e.*, due to diffusion) when the electric field strength is manipulated during an electrophoretic separation. Figure 9 illustrates the effect of both reversal of the CE field strength (A) and elimination of the field for extended periods (B). Jones *et al.* (1990) suggested that improved detection might be possible if the electric field strength were lowered to slow passage of the solute at the detector.

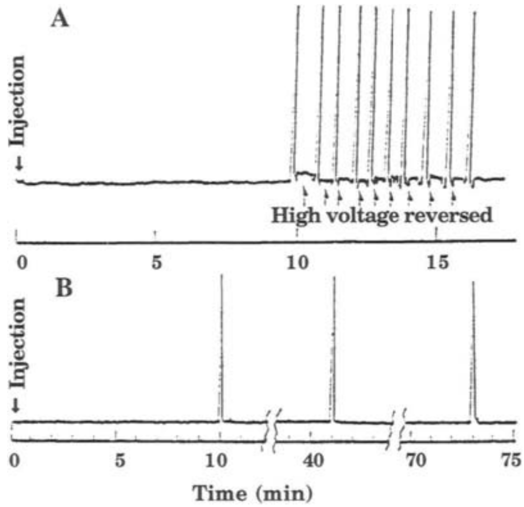


Fig. 9. Illustration of the effect of (A) multiple reversals of CE polarity and (B) for reversal of field strength with the field left "off" for 30-min periods. (Reproduced with permission from Jones *et al.*, 1990).

Figure 10 compares the RES method to normal CE-MS detection. Figure 10A shows the total ion electropherogram for the separation of 60 fmol (injected) each of carbonic anhydrase B, aprotinin, myoglobin, and cytochrome *c* using a constant electric field strength of 300 V/cm. In comparison, Fig. 10B shows the total ion electropherogram (TIE) for the same mixture, where the electric field strength was reduced to 60 V/cm one min prior to migration of the first protein into the ESI source. Figure 10B shows the same total ion electropherogram after the abscissa has been compressed by one-fifth (*i.e.*, by a factor equal to the inverse of the reduction in electric field strength). Only a 14% reduction in the ion intensity is observed for myoglobin, without an observable loss in separation quality. Each protein elutes five times slower in the RES CE-MS analysis than in the constant electric field strength CE-MS analysis, but the quality of separation is not affected; indeed, it actually appears better due to the increased number of scans recorded during elution. Mass spectra obtained during this RES CE-MS separation are shown in Fig. 11. The quality of data for m/z 600–1200 scans is sufficient to allow molecular weights to be calculated to 0.02 –0.1%, despite the modest separation quality (Smith *et al.*, 1990b).

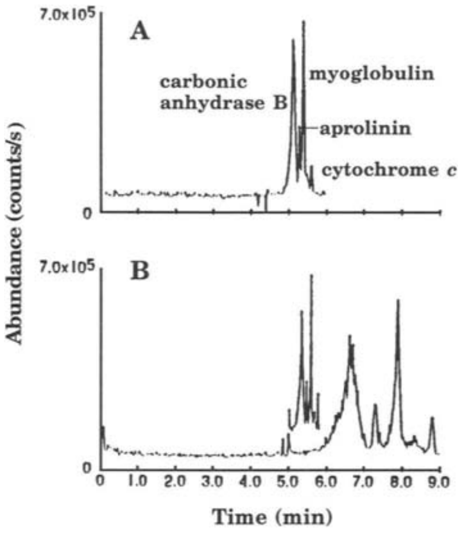


Fig. 10. Comparison of constant field strength (A) and reduced elution speed (B) CE/ESI-MS analysis of a mixture containing carbonic anhydrase, aprotinin, myoglobin and cytochrome *c*. The constant field strength CE/ ESI-MS analysis was conducted at 300 V/cm. The reduced elution speed CE/ESI-MS analysis was conducted at 300 V/cm until one min prior to elution of the first protein when the electric field strength was reduced to 60 V/cm. (Reproduced with permission from Goodlet *et al.*, 1993.)

Key advantages of RES CE-MS are the ability to scan an increased m/z range or to increase the number of scans collected during elution of a solute. If the electric field strength is reduced by a factor of five, then the solute elutes approximately five times slower, allowing five times as many scans to be recorded than at a constant electric field strength. Sixty femtomoles of carbonic anhydrase B injected and analyzed by constant electric field strength CE-MS allowed four scans to be averaged. In contrast, the 16 additional scans collected by RES CE-MS and not available during constant electric field strength CE-MS, provide better peak quality and more precise molecular weight measurements, as well as modest improvements in signal-to-noise ratios.

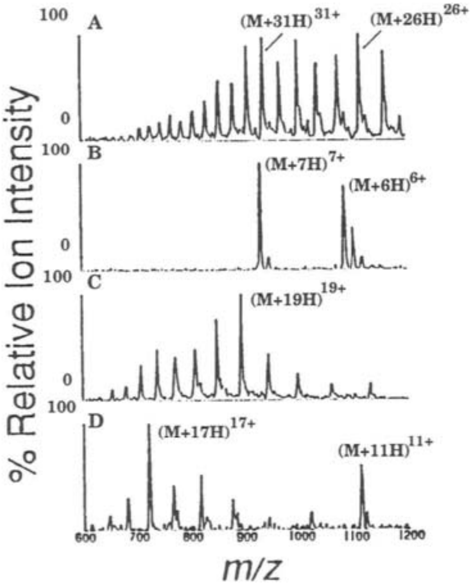


Fig. 11. Mass spectra recorded during reduced elution speed CE/ESI-MS analysis of a mixture of carbonic anhydrase (A), aprotinin (B), myoglobin (C) and cytochrome *c* (D) shown in Fig. 10. Each mass spectrum is the average of 20 scans recorded during the elution of each protein. (Reproduced with permission from Goodlet *et al.*, 1993.)

Some of the greatest challenges for CE-MS involve analysis of complex mixtures of biopolymers. An important goal is to decrease the quantity of protein required for sequencing using methods based upon an initial enzymatic digestion of a protein. Comparison of constant electric field strength and RES CE-MS for a 40 fmol injection of peptides produced by digestion of bovine serum albumin with trypsin (Figs. 12A,B) shows only a small decrease (20%) in ion intensity when the electrophoretic voltage was decreased to slow elution, similar to results for the protein mixtures. Single ion electropherograms extracted for the AWSVAR peptide, produced by cleavage of bovine serum albumin with trypsin at amide bonds 32 and 37, are shown in Fig. 13A for constant electric field strength CE-MS and in Fig. 13B for RES CE-MS. A total ion electropherogram for the bovine serum albumin tryptic peptide AWSVAR analyzed by RES CE-MS (Fig. 13B) showed only a minor increase in peak width at half height when com-

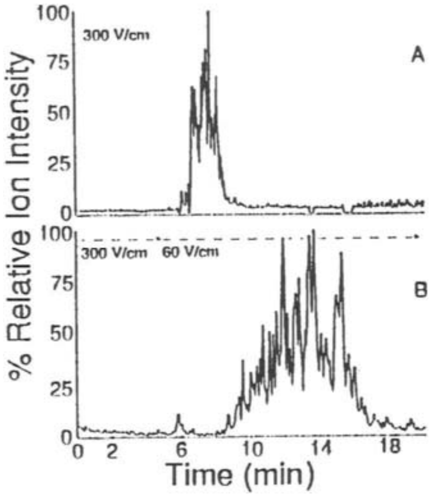


Fig. 12. Comparison of constant field strength (A) and reduced elution speed (B) CE/ESI-MS analysis of bovine serum albumin after digestion by trypsin. The constant field strength CE/ESI-MS analysis was conducted at 300 V/cm. The reduced elution speed CE/ESI-MS analysis was conducted at 300 V/cm until one min prior to elution of the first peptide when the electric field strength was reduced to 60 V/cm. (Reproduced with permission from Goodlet *et al.*, 1993.)

pared to the constant electric field strength CE-MS analysis (Fig. 13A), indicating that apparent peak width was largely dictated by the scan speed during normal elution.

Figure 14A shows a comparison of the mass spectra obtained during elution of this peptide. Five scans from the TIE shown in Fig. 12B, for the corresponding longer RES elution period, were averaged to produce the mass spectrum shown in Fig. 14B. The mass spectrum recorded during one scan under constant electric field strength CE-MS for peptide AWSVAR (Fig. 14A) shows the presence of ~15 additional significant peaks, due to a poorer overlap of the peak elution time with the MS scan period. A reduction in the complexity of individual scans can facilitate data interpretation of complex mixtures

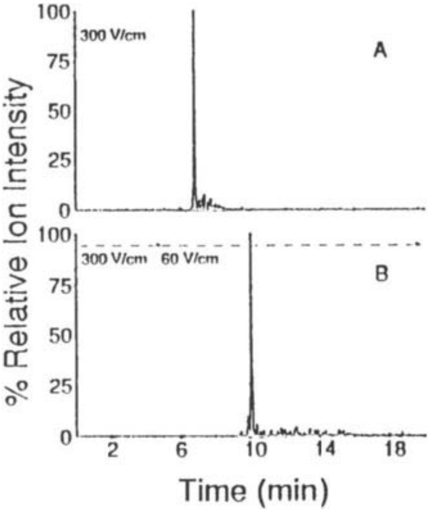


Fig. 13. Extracted single ion electropherograms for a single peptide (residues 32-37) from constant field strength (A) and reduced elution speed (B) CE/ESI-MS analysis of bovine serum albumin after digestion by trypsin (see Fig. 12). (Reproduced with permission from Goodlet *et al.*, 1993.)

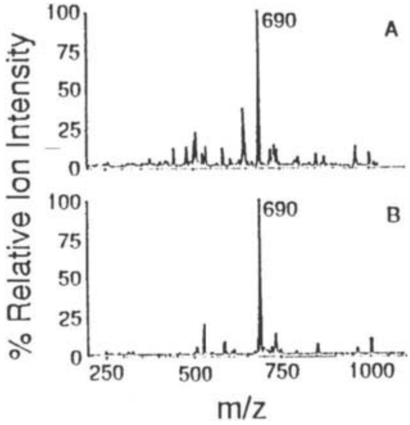


Fig. 14. Mass spectra of a peptide (residues 32-37) produced during digestion of bovine serum albumin with trypsin and analyzed by constant electric field strength (A) and reduced elution speed (B) conventional CE/ESI-MS (see Fig. 12). (Reproduced with permission from Goodlet *et al.*, 1993.)

due to the greater effective scan speed and the reduced likelihood of other components eluting during the same scan.

Reduced elution speed CE-MS provides an increase in the efficiency of mass spectrometric scanning compared to conventional CE-MS methods. The prolonged residence time of solute in the electrospray can be exploited in several ways, including: 1) increasing the m/z range scanned, 2) increasing the number of scans recorded during elution of a given solute, and 3) enhancement of sensitivity (integrated ion intensity) for a given solute. Additionally, a reduction of migration rate into the ESI source may help offset any negative effects (*e.g.*, signal "drop out") and other fluctuations in the electrospray signal. The method does not increase solute consumption, provides improved sensitivities for peptide and protein analyses extending into the low femtomole region, and incurs very little loss in ion intensity, particularly important for MS/MS methods and their potential application to peptide sequencing.

5.2. *Effect of Capillary Diameter upon CE-MS Sensitivity*

As indicated by Fig. 7, optimum ESI-MS sensitivity will generally be obtained at the lowest CE currents, where the rate of delivery of charged species to the source is minimized. This expectation is in contrast to the expectations of several early workers who utilized larger diameter capillaries in order to increase CE sample loading. In contrast, an optimum capillary diameter is that which meets several criteria: it should (1) be available commercially (or readily prepared), (2) be amenable to alternative detection methods, and (3) allow optimum detector sensitivity. For the last criterion, we would expect that optimum sensitivity would be obtained at CE currents approximately equal to or less than the ESI current. Figure 15 shows the ESI and CE current for a 10 mM acetic acid buffer (pH 3.4) with a 75/24/1 v/v/v methanol/water/acetic acid sheath at a flow rate of 2 μL min^{-1}. It can be seen that, even for this relatively low conductivity buffer, large CE capillary diameters (>40 μm) will have currents that exceed that of the ESI source.

Wahl *et al.* (1993) have examined ESI-MS detection sensitivity as a function of capillary diameter using standard analyte mixtures. Figure 16 shows total ion current profiles for the separation of a three-component mixture in capillaries with diameters of 100, 50, 25, and 10 μm. Because the same electromigration injection techniques were used for each capillary, the injection volume is (to a good approximation) proportional to the square of the diameter of the capillary. As shown in Fig. 16, only relatively small decreases in ESI-MS ion currents were observed for the smaller capillary diameters.

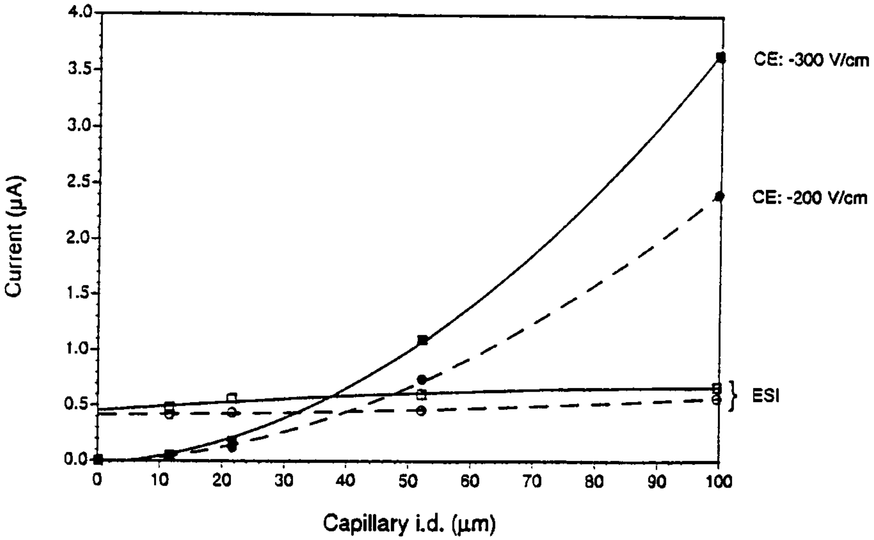


Fig. 15. Comparison of the CE and ESI currents for a 10 mM acetic and buffer as a function of capillary diameter for two different CE field strengths. The CE current has the expected quadratic dependences upon capillary inner diameter. (Reproduced with permission from Wahl *et al.*, 1993.)

For the 10 μm i.d. capillary, where a factor of ~100 less sample was injected, analyte signal intensities are observed to decline by only factors of 2–4, compared to the 100 μm diameter capillary.

Figure 17 shows the actual ESI-MS response observed for one analyte (leucine enkephalin) as a function of injection size for the four capillary diameters (Wahl *et al.*, 1993). Consistent with the above results and qualitatively with the behavior predicted by Fig. 7, sensitivity increases as capillary diameter decreases. Also in accord with this is the observation that roughly equivalent sensitivity is obtained using capillaries of 10 and 20 μm diameter. Figure 18 shows the exceptional sensitivity that can be obtained using a 20 μm i.d. capillary for a simple mixture of polypeptides with selected ion monitoring (of seven m/z values).

Although there are increased difficulties when using small i.d. capillaries, *e.g.*, plugging of the capillary, the MS interfacing methods are substantially unchanged. One concern relevant to the use of small i.d. capillaries was that incomplete mixing between the CE buffer system and the sheath liquid would cause a decrease in analyte ion current (because the analyte would then be present in only a fraction of the electrospray droplets). Observation of the terminus of an

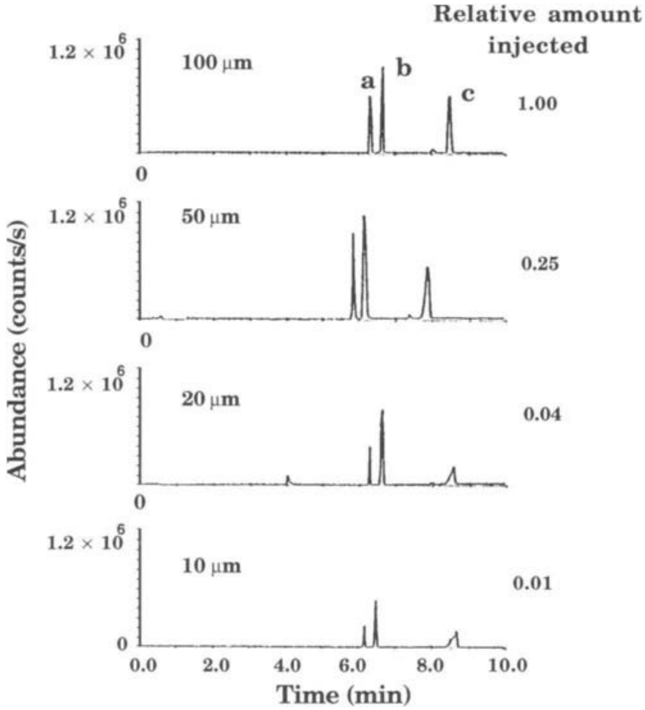


Fig. 16. Comparison of the CE-MS total ion electropherograms for separation of (a) tryptophan, (b) leu-enkephalin, and (c) melittin for four different capillary diameters. Electromigration injection and separation conditions were identical for all experiments and signal intensities are shown on the same scale. The relative amount of analyte injected is a factor of 100 lower for the 10 µm capillary compared to the 100 µm capillary. (Reproduced with permission from Wahl *et al.*, 1993.)

uncoated fused-silica capillary of small i.d., squarely cut with the polyimide coating intact, showed that the electrospray could emanate from both the outer diameter and near the inner diameter of the capillary. Points of electrospray emanation were sometimes observed to move rapidly, and apparently in a random manner, between various sites on the capillary terminus. We believe that such a mode of operation may degrade signal stability and sensitivity because electrosprays emanating from the capillary outer diameter may contain less analyte (due to ineffective mixing). For this reason, the capillary terminus was etched to provide a tapered capillary terminus, allowing the liquid to flow to the conical apex and promote more effective mixing.

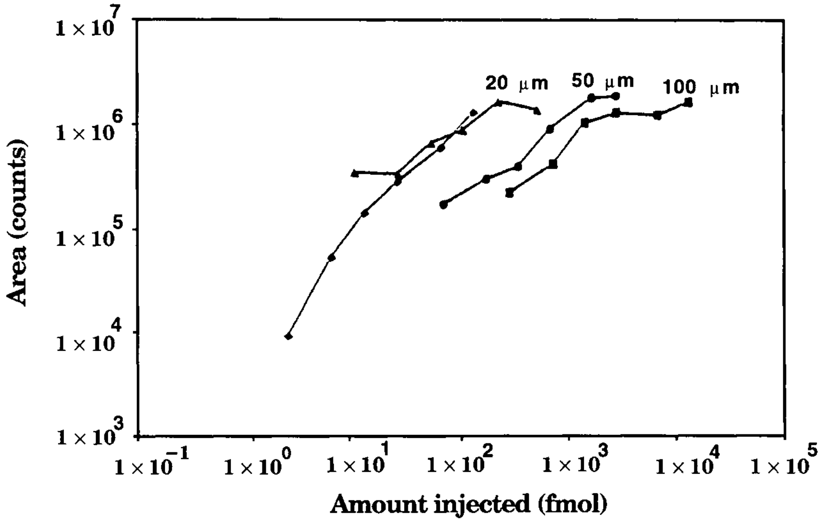


Fig. 17. CE-MS signal intensities for leucine-enkephalin observed as a function of sample size injected using four different capillary diameters. Injections utilized similar electrokinetic conditions and a range of analyte concentrations (Reproduced with permission from Wahl *et al.*, 1993).

The extension to even smaller capillaries for protein analysis is shown in Fig. 19, where the total ion current electropherograms obtained for the protein mixture using 50 μm (top) and 5 μm (bottom) i.d. capillaries are illustrated on the same absolute scale. In both experiments, the mass spectrometer is scanning from m/z 600–1200 at 1.5 s scan^{-1}. In general, the surface treatment for the 5 μm i.d. capillary appears to be less homogeneous, based upon electroosmotic flow rates which are observed to be slower presumably due to less effective surface coverage for the smaller capillaries. Injected amounts for the separation shown in Fig. 19 are 60 fmol/ protein for the 50 μm i.d. capillary and 600 amol/protein for the 5 μm i.d. capillary. This corresponds to a 100-fold difference in the injected sample amount between the two capillaries; however, comparison of the two electropherograms shown in Fig. 19, as for Fig. 16, shows only a two- to fourfold difference in signal intensity.

The mass spectra obtained for the 600 amol/protein injection into the 5 μm i.d. capillary are shown in Fig. 20, and are of sufficient quality to determine the molecular weight of the proteins to better than 0.03%. These results clearly indicate that CE-MS of proteins is feasible at subfemtomole levels, and that sensitivity for the selected

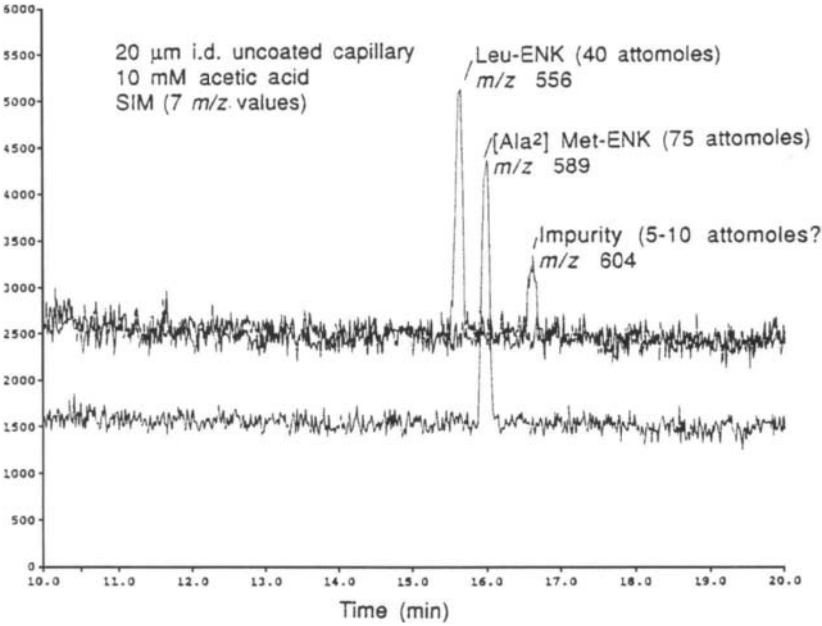


Fig. 18. CE-MS obtained with a 20 µm i.d. capillary for two pentapeptides showing the high sensitivity with SIM detection. The amount of the unknown impurity is estimated. (Reproduced with permission from Smith *et al.*, 1993.)

ion monitoring (SIM) mode of operation should extend to the low attomole range for the proteins studied because a sensitivity improvement of generally 10–100 is noted for SIM relative to "full spectrum" detection.

In the regime of ultra-sensitive analysis, particularly for biopolymer characterization, mass spectrometry (MS) has lagged far behind techniques such as laser-induced fluorescence and electrochemical detection. These detection methods have provided the most impressive sensitivities yet demonstrated with CE, but application is restricted and information for compound identification is generally limited due to a reliance on electrophoretic mobilities. The attraction of MS detection is that accurate molecular weight information and component identification can be performed, and techniques for obtaining structural information based upon tandem methods are currently being extended to larger molecules. These results and on-going developments in mass spectrometric instrumentation suggest that CE-MS has the potential to move into the regime of ultrasensitive detection.

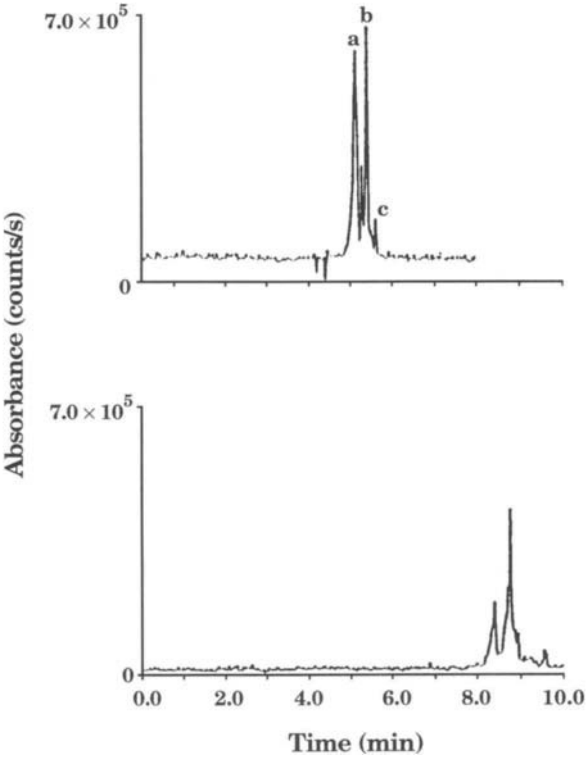


Fig. 19. Total ion current electropherograms obtained for CE-MS analysis of a 30 mM/protein mixture containing (a) carbonic anhydrase, (b) aprotinin, myoglobin and (c) cytochrome *c* using 50 μm (top) and a 5 μm (bottom) i.d. capillaries. Injected amounts were approximately 60 fmol/ protein for the 50 μm i.d. capillary and 600 amol/protein for the 5 μm capillary by electrokinetic injection. Comparison of the two electropherograms, shows only a two- to fourfold difference in signal intensity. Experimental conditions: 100 cm capillary lengths; 0.01 M acetic acid buffer solution, pH = 3.4; electric field, -300 V cm^{-1}; MS scanning conditions from m/z 600 to 1200 at 1.5 s/scan. (Reproduced with permission from Wahl *et al.*, 1993.)

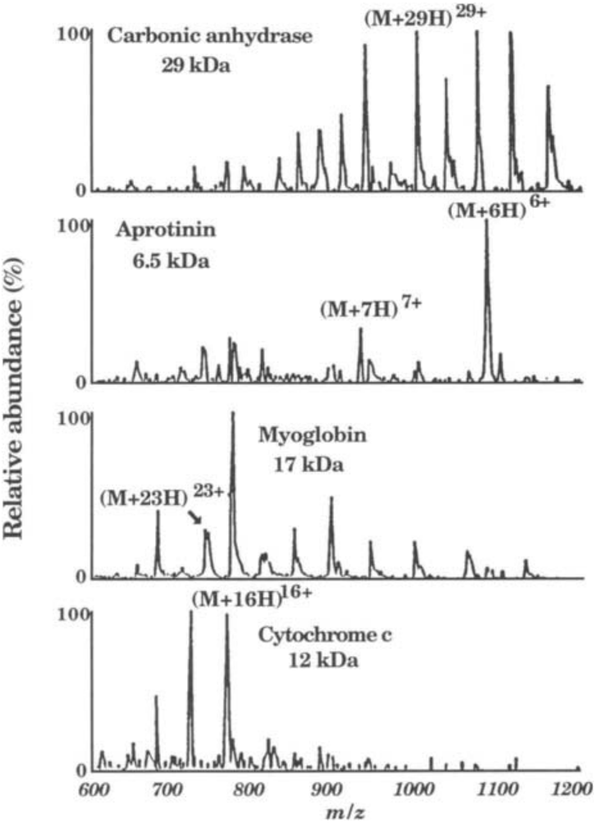


Fig. 20. Mass spectra obtained from the individual solute zones for 600 amol/protein injected using the 5 μm i.d. capillary shown in Fig. 19 (bottom). (Reproduced with permission from Wahl *et al.*, 1993.)

6. CE-MS Applications

To this point, much of the reported CE-MS has been concerned with investigating and demonstrating the methodology and comparing it to other techniques (particularly micro-column LC-MS). Since CE-MS is a less familiar technique to the analytical chemist, the "first wave" of reports generally are those arising from laboratories often more interested in new methodologies and instrumental approaches than in routine applications or specific analytical problems. Gradually, however, more "real problems" are being examined (the "second wave"), and such reports should increase greatly as CE becomes more widespread.

Tomer and co-workers have recently applied CE-MS to the analysis of macrolide antibiotics (Parker *et al.*, 1992). These workers used 75 μm i.d. capillaries that were either uncoated or treated with 3-aminopropyl trimethoxysilane (APS). The APS coating reverses the charge at the column-buffer interface and, thus, the direction of the osmotic flow compared to uncoated capillaries. This has been found useful for acidic buffers, where ESI is often most sensitive for polypeptides and where many ions of interest are positively charged and may interact strongly with the negatively charged silanols on untreated fused silica. A field strength of 300 V/cm was used, and the buffers employed were 0.01 M acetic acid or ammonium acetate. The pH was adjusted by the addition of ammonium hydroxide or acetic acid to pH 3.4 and by the addition of formic or trifluoroacetic acid for pH below 3.4. Using a Vestec ESI-MS instrument with a sheath flow (coaxial) interface, they examined mixtures of erythromycin A, spiramycin, tylosin, midecamycin, miokamycin, and rokitamycin and the second contained the four macrolides oleandomycin, josamycin, troleandomycin, and leucomycin-A5. These workers noted that the addition of trifluoroacetic acid decreased the sensitivity at the lowest pH. They also experimented with the addition of organic modifiers in the buffer (up to 10% 2-propanol). They found no improvement, but observed the expected decrease in electroosmotic flow and correspondingly longer analysis times.

Thibault and coworkers have recently shown the application of CE-MS to compounds of interest to the aquaculture industry such as antibiotics and marine toxins (Pleasance *et al.*, 1992a). They demonstrated separations of saxitoxin, neosaxitoxin, C_{11}-sulfated gonyautoxins (GTX-2 and GTX-3), erythromycin, ometoprim, trimethoprim, sulfaisomidine, sulfamethazine and okadaic acid in 50 μm i.d. capillaries. These samples are difficult to analyze by more conventional means. They compared both the liquid junction and sheath flow interfaces using a 0.2% formic acid solution as either the sheath or junction solution. Both CE-MS and CE-MS/MS experiments were performed with detection limits down to the 1 ppm range. They reported the expected discrimination effects during electrokinetic injection, finding hydrostatic injections to be much more useful than electrokinetic injections for quantitation.

Application to more complex polypeptide mixtures (*i.e.*, tryptic digests of proteins), potentially an extremely important area of application due to the significance of peptide mapping and sequencing using ever smaller sample sizes, has also been investigated by a number of researchers. With these mixtures, high efficiency separations have been demonstrated, generating over 250,000 theoretical plates. Complex mixtures of peptides generated from tryptic digestion of large proteins present a difficult analytical challenge, since the fragments cover a large range of both pI and hydrophobicity. Since trypsin specifically cleaves peptide bonds C-terminally at lysine and arginine, the resulting peptides generally form doubly as well as singly charged molecular ions by positive ion ESI. Such doubly charged tryptic peptides generally fall within the m/z range of modern quadrupole mass spectrometers. The resolving power of these methods is illustrated in Fig. 21, which shows a UV electropherogram (top), obtained in conjunction with a CE-MS analysis (Smith *et al.*, 1991b), for a separation of a tryptic digest of tuna cytochrome *c* in a 50 mM acetate buffer (at pH 6.1) mixed with an equal volume of acetonitrile. Shown are single ion electropherograms for two charge states of two tryptic fragments. In this work, a commercial CE instrument (Beckman P/ACE 2000) was modified to allow UV detection of the separation at one-half the MS detection time, providing a "preview" of ESI-MS detection at twice the separation time. The individual peaks indicate a separation efficiency corresponding to approximately 4×10^5 theoretical plates. It was noted that the apparent efficiency for MS detection is significantly greater than that for UV detection (1.2×10^5 theoretical plates for the same peak), a fact that can be largely attributed to the longer separation time and the very small effective "dead volume" or residence time in the ESI interface (Smith *et al.*, 1991).

Lee *et al.* (1989) showed the detection and identification of 11 components of a tryptic digest of recombinant bovine somatotropin using SIM detection. Their separations were conducted in a 100 μm i.d. capillary using a 50:50 acetonitrile-5 mM ammonium acetate buffer, and obtained efficiencies as high as 300,000 theoretical plates. In separate experiments, CE-MS/MS was demonstrated for one of the components, providing information on the amino acid sequence. Subsequently, Johansson *et al.* (1991) reported the analysis of a hemoglobin tryptic digest. They use a 100 μm i.d. capillary, a field strength of 260 V/cm and a 50:50 acetonitrile-15 mM ammonium acetate buffer adjusted to pH 5.0 with acetic acid. Approximately 10 pmol was injected electrokinetically (25 s at 10 kV from a 154 pmol μL^{-1} digest). The digest was expected to have 28 components and a complex total ion electropherogram with several co-eluting peaks was observed from a scanning experiment covering a 600 m/z range. The authors reported that all of the expected enzymatic products were detected. In separate experiments, a 10 pmol injection of the digest was used to obtain a MS/MS spectrum of the T-3 fragment. The product daughter spectrum showed fragment ions which provided information on the peptide. In another experiment these workers used a 75 μm i.d. capillary with 15 mM ammonium acetate buffer adjusted to pH 2.5 by the addition of TFA and 15% methanol. They monitored 8 ions (SIM) characteristic of the expected fragments identified from their previous

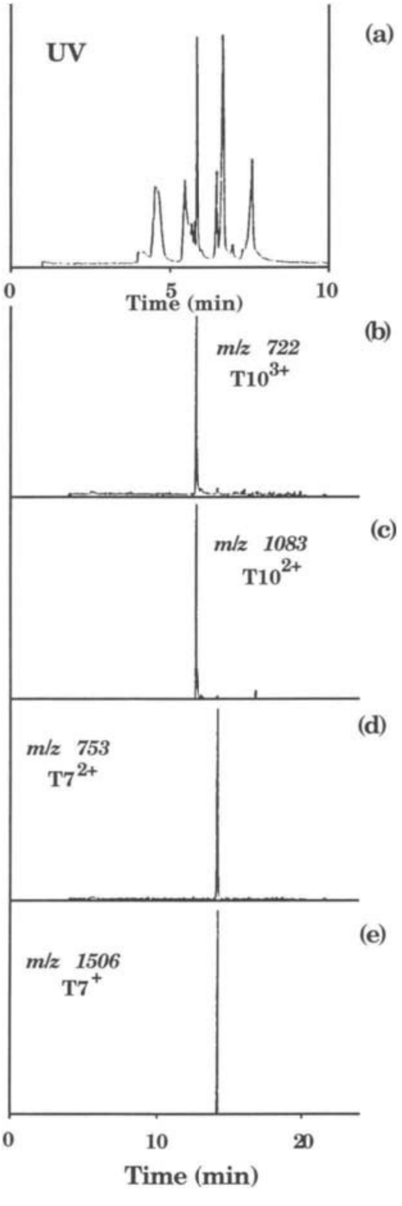


Fig. 21. Comparison of the UV (214 nm) detection (top) and MS reconstructed ion electropherograms (bottom), with time axes adjusted for the corresponding peaks, for a CE-MS separation of a tryptic digest of tuna cytochrome *c*. (Reproduced with permission from Smith *et al.*, 1993.)

experiments and demonstrated efficiencies from 85,000 to 135,000 theoretical plates for an 8 pmol injection.

Thibault *et al.* (1991a) examined a separation of the tryptic fragments of a glucagon digest. The separation was done in a 50 μm i.d. capillary at 390 V cm^{-1}, using either a 10 mM (pH 3.4) or 2.5 M (pH 2.1) acetic acid buffer. They used a commercial (Microcoat™) polymeric coating reagent. Two pmol of glucagon (digested) were injected. The four expected fragments (yielding both singly and doubly charged ions) were observed using MS scanning (a 950 m/z wide range). In addition, evidence for the partial oxidation of the sample was obtained along with a small amount of CID fragmentation that occurred in the ESI-MS interface. Higher efficiency (~34,000 theoretical plates) but longer analysis times were obtained with the lower pH buffer.

Experience to date suggests that nearly all tryptic fragments can be effectively detected by ESI-MS methods if first separated; infusion of the unseparated digest often results in dramatic discrimination against some components. Fragments observed by the UV detector are almost always detected by ESI-MS. However, detection has sometimes been problematic for very small fragments (*i.e.*, amino acids and dipeptides) due to difficulties in obtaining optimum ESI-MS conditions over a wide m/z range. Excessive internal excitation or large solvent related background peaks at low m/z appear to be the origin of some difficulties. There is also evidence that some interface designs discriminate strongly against low m/z ions. However, available evidence indicates that if tryptic fragments are not detected by CE-MS, they were generally "lost" prior to the separation, a problem particularly evident with "nanoscale" sample handling. As observed in the past, full realization of more sensitive analytical methods will depend upon concomitant improvements in the earlier stages of sample handling and preparation.

The ESI-MS interface provides the basis for detection of molecules having molecular weights exceeding 100,000. The analysis of proteins by CE-MS is challenging due to the well-established difficulties associated with protein interactions with capillary surfaces. Often, capillaries must be coated to minimize or prevent wall interactions (Thibault *et al.*, 1991a; Moseley *et al.*, 1992). It is unlikely that one capillary or buffer system will be ideal for all proteins but, rather, as for LC separations, procedures optimized for specific classes of proteins will be developed. Most CE separations require the ionic strength of the buffer to be about 10^2 greater than that of the sample to prevent degradation of the separation due to perturbation of the local electric field in the capillary. Detection sensitivity for proteins is generally lower than for small peptides due to the greater number of charges per molecule and the greater number of charge states. An exception to this is where much greater concentrations of volatile buffer components (such as acetic acid) are acceptable.

An important attribute of the ESI-MS interface is the capability to obtain structurally related information on large molecules by dissociation in the interface by a "collisional heating" process (induced by a simple electric field gradient). This capability was first demonstrated for large molecules by our laboratory in 1988 (Loo *et al.*, 1988). It has the advantage compared to CID MS/MS methods of much greater selectivity, but the disadvantage of greater ambiguity since only one MS step is utilized and a good separation is generally required. The complexity of large molecule collisional dissociation spectra precludes obtaining sequence information with current MS quadrupole technology, but its potential for "fingerprinting" has been established (Smith *et al.*, 1990d, 1991a). As shown in Fig. 22, extensive sequence-related fragmentation can be obtained for molecules as large as serum albumins (~66 kDa), yielding spectra suitable for fingerprinting purposes. We have also shown that direct infusion experiments using low pmol sample quantities provide spectra for which a limited amount of sequence information can be obtained for materials of substantially known sequence. This suggests the potential utility of direct CE-MS analysis of proteins for evaluation of suspected primary structural or post-translational modifications.

Figure 23 shows a separation of three myoglobins obtained on an uncoated 50 μm capillary of 8 cm length (Smith *et al.*, 1991b). The separation was obtained in 10 mM TRIS buffer at pH 8.3 with an electric field strength of 120 V/cm. Approximately 100 fmol per component was injected onto the capillary column. The top of the figure shows the UV trace for the separation and the bottom shows two single ion electropherograms from MS detection for the same separation. Whale myoglobin (M_r 17,199) was detected at m/z 861 (due to loss of the heme) and carried 20+ charges. The resolution of the mass spectrometer was too low to separate the 19+ molecular ion of horse myoglobin (M_r 16,950) from sheep myoglobin (M_r 16,923), and both contribute to the m/z 893 ion in the single ion electropherogram. A signal ion at m/z 617 due to the heme group of the myoglobins is also observed and clearly remains associated with the protein during CE separation (Smith *et al.*, 1991b). Since the heme is not covalently bound to these proteins, it is labile under the ESI conditions used. Peak widths in the UV trace are 3.5 s wide (half-height) at half the MS detection time, compared to 4.5 s for MS detection. This suggests that under optimum conditions, more efficient injection methods designed to concentrate the analyte at the head of the column in conjunction with smaller sample loading could potentially reduce the peak width in these separations to approximately 1 s.

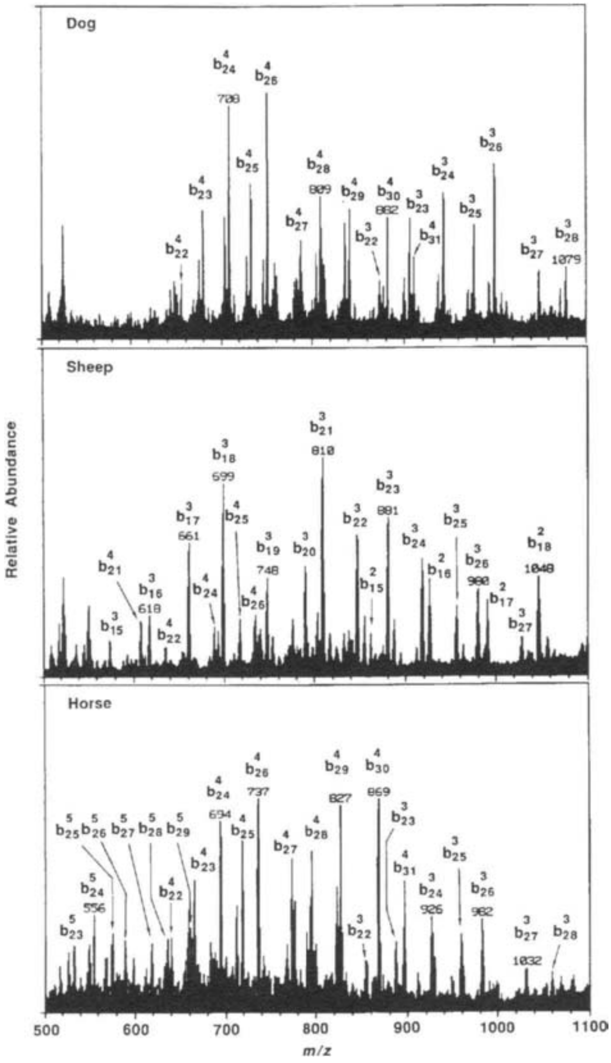


Fig. 22. Electrospray ionization mass spectra of three serum albumins (M_r ~66,000) obtained with a nozzle-skimmer voltage (DNS) of 335 V using a modified Sciex TAGA 6000E triple quadrupole mass spectrometer. The high DNS spectra provide structurally related information, with much greater sensitivity than possible with triple quadrupole (MS/MS) methods, that is useful for fingerprinting. The labels (b_y^x) refer to ions arising due to products incorporating the amino terminus at the x peptide bond and having a charge state of y+. (Reproduced with permission from Luo *et al.*, 1991b.)

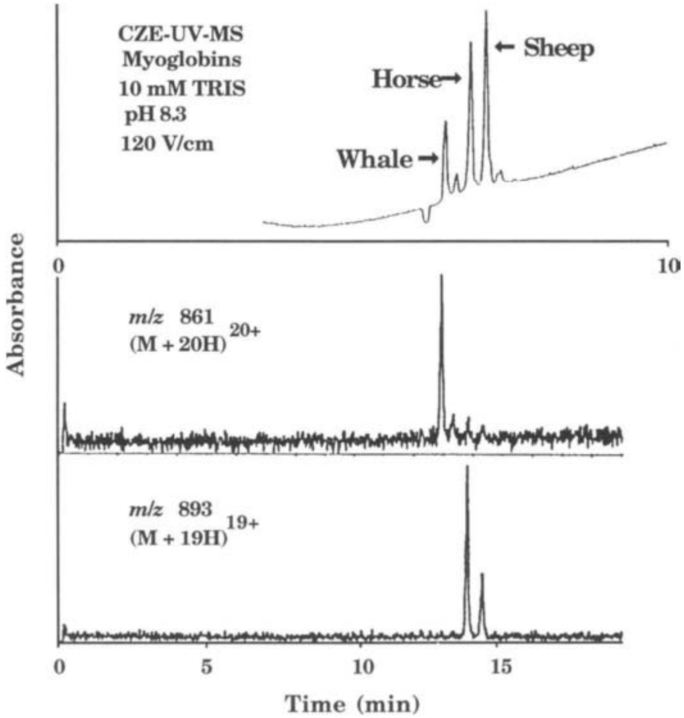


Fig. 23. CE-MS separation of a mixture of whale (M_r 17,199), horse (M_r 17,951), and sheep (M_r 16,923) myoglobins. Approximately 100 fmol per component was injected. The on-line UV response and later MS response (at approximately twice the separation time) are shown with adjusted time scales. The separation was conducted in 10 mM Tris (pH 8.3) at 120 V/m with a 1 m × 50 μm i.d. uncoated capillary. A sheath liquid incorporating acetic acid was used to assist positive ion ESI. (Reproduced with permission from Smith *et al.*, 1991b.)

The peak widths obtained in these studies are already sufficiently narrow to exceed current capabilities for obtaining high quality full-scan spectra when signal intensities are low and the mass range is large. For targeted compound analysis, where a monitoring of only a small number of *m*/*z* values is necessary, detecting peaks of <1 s in width is quite practical. The reduced elution speed techniques discussed earlier provide one approach to allowing full scan spectra to be obtained under such conditions. Obtaining full scan spectra for fast or high resolution separations will also benefit from future advances in ion trap-MS or array-based instrumentation.

In addition to our laboratory, others have also demonstrated CE-MS protein separations. Particularly successful have been separations in acetic acid buffer solutions using capillary coatings designed to reverse (make positive) capillary surface charge. Thibault *et al.* (1991a) have obtained mass spectra while scanning (m/z 1400–2350 range) using 0.2–0.8 pmol injections of the proteins lysozyme, α-lactalbumin, chymotrypsinogen A, and bovine apotransferrin. Moseley *et al.* (1992) used an APS-treated capillary (75 μm i.d.) with a 10 mM, (pH 3.4) acetic acid buffer, and a field strength of 300 V/cm to evaluate a separation of the proteins myoglobin and cytochrome *c*. Scanning over an m/z 1200 range allowed proteins to be detected using only a 130 fmol injection.

The CE-MS method can also provide information on impurities, or possibly multimeric species, specifically non-covalently associated dimers or larger complexes stable in solution (Smith *et al.*, 1991a). For example, the CE-MS analysis of a sample of B-chain insulin in 10 mM TRIS (at pH 8.3), at an electric field strength of 150 V cm^{-1}, using 0.7 pmol injection of a sample showed a second smaller, slightly lower peak eluting by both MS and UV detection. The second peak was attributed to either an impurity of very similar M_r or a dimer (and perhaps larger multimers) that would dissociate to multiply charged monomers due to collisional heating in the ESI interface (Smith *et al.*, 1991a). Such species can generally be separated due to differences in electrophoretic mobility and, depending upon interface conditions and multimer stability, can be detected as either the monomer or intact multimer. As molecular weight increases, ESI sensitivity for similar compounds is expected to decrease (Smith *et al.*, 1990c). Figure 24 shows a separation obtained for a serum albumin sample using conventional scanning detection with an APS coated capillary. The separation, obtained with a 140-fmol injection, provided CE-MS results of sufficient quality for accurate molecular weight determination, as well as detection of a significant albumin dimer impurity. The use of smaller i.d. capillaries is expected to result in similar results from much smaller injections.

7. Capillary Isotachophoresis-MS

Isotachophoresis in a capillary is an alternative electrophoretic method which provides an attractive complement to CE. The instrumentation used for capillary isotachophoresis (CITP) can be nearly identical to that of CE. In CE, analyte bands are separated on the basis of the differences in their electrophoretic mobilities in an electric field gradient, which ideally is unperturbed by their presence (owing to the much lower effective concentrations). In contrast, in isotacho-

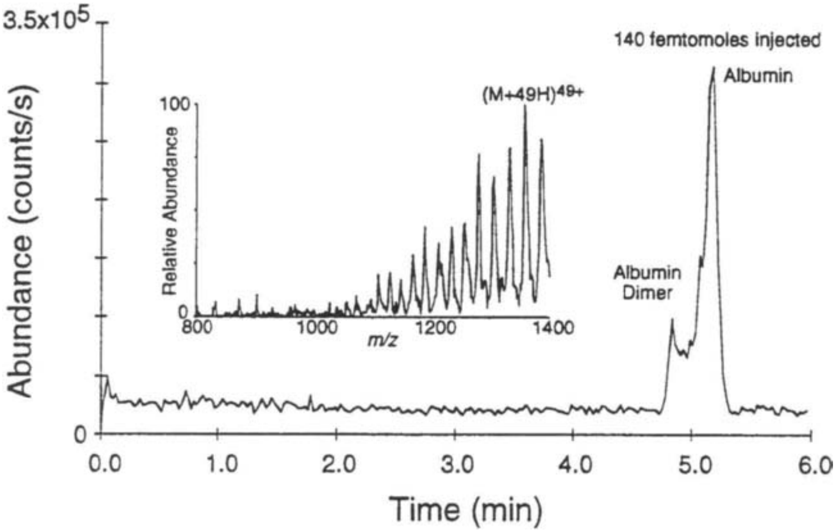


Fig. 24. CE-MS of a bovine albumin sample showing the separation and mass spectra obtained for a 140 fmol injection. The poorly resolved smaller peak at shorter time corresponds to an albumin dimer impurity.

phoresis, the analyte mobilities define the electric field strength. Two different electrolyte solutions are chosen as the leading and terminating electrolyte solutions, which have sufficiently high and low electrophoretic mobilities, respectively, to bracket the electrophoretic mobilities of the sample components of interest. The sample is inserted in the capillary between the two electrolyte solutions, and the sample components are separated into distinct bands on the basis of their electrophoretic mobilities. The electric current in the capillary is largely determined by the leading electrolyte, which then defines the ion concentration in each band. The electric field strength varies in each band, the highest field strength being found in the bands with the lowest mobility (*i.e.*, greatest resistance). The length of a band is then proportional to the amount of its ions in the sample. As with CE, resolution is ultimately limited by band broadening due to molecular diffusion, Joule heating, and particularly electroosmosis (due to differences between bands).

A direct CITP-MS combination allows sample sizes to be increased by several orders of magnitude compared to CE, and is limited primarily by the volume of the capillary and the separation time allowed (which is inversely related to the voltage). In addition, CITP often results in concentration of analyte bands (depending on their concentration relative to the leading electrolyte), which is in contrast to the inherent dilution obtained with CE. These two features can provide a substantial improvement in concentration detection limits compared to CE. Although detection limits better than 10 attomole are feasible for CE-MS (see Fig. 18), the 10 nL injection volume typical of CE corresponds to concentration detection limits generally in the range of 10^{-7} to 10^{-9} M with SIM detection. If the ionization efficiency is lower, or full-scan mass spectra are required, the CITP-MS detection limits will be substantially better than those obtainable by CE-MS. All CITP bands have similar ion concentrations, so there should ideally be no large differences in signal intensities between bands.

Figure 25 demonstrates an early CITP-MS separation of dopamine and epinephrine, showing the characteristic band-like structure of CITP for the two components. The separation was conducted at 175 V cm^{-1}, with a 2 m × 100 μm i.d. untreated fused-silica capillary. The leading electrolyte was 10^{-3} M ammonium acetate and the trailing electrolyte was 10^{-3} M tetraoctylammonium bromide in a 1:1 v/v water-methanol mixture as solvent. Figure 26 shows the CITP-MS single and total ion isotachopherograms for a mixture of phosphonium salts. The sample was loaded by electromigration for 60 s at 30 kV. The separation is well developed, with minimum band overlap and sharp edges. The widths of the four bands are nearly equivalent, consistent with the similar sample concentrations and the minor discrimination expected from use of electromigration for introduction of of the mixture. The most striking feature of the separation shown in Fig. 26 is the excellent resolution of the four phosphonium salts, particularly for the vinyl and ethyl triphenylphosphonium salts which were not resolved in the shorter CE separations. The fact that the vinyltriphenyl- and ethyltriphenylphosphonium ions are resolved illustrates the potential of CITP-MS for high-resolution separations.

CITP-MS and CITP-MS/MS have been demonstrated for peptide sequencing (Smith *et al.*, 1990a). A potentially important capability of CE-MS (and CE-MS/MS) is a method that will yield primary structural information (*i.e.*, sequence) for polypeptides and small proteins. The fact that ESI mass spectra generally consist of only intact multiply charged molecular ions is sometimes cited as a disadvantage of this method, since it is claimed that structural information cannot be obtained. However, effective dissociation of molecular ions can be induced in the nozzle-skimmer, capillary-skimmer, or equivalent regions of the ESI interface (Loo *et al.*, 1988; Smith *et al.*, 1990b, 1991a). A more powerful approach, but one often limited by available signal strengths, is tandem MS of molecular ions from several of the major charge states.

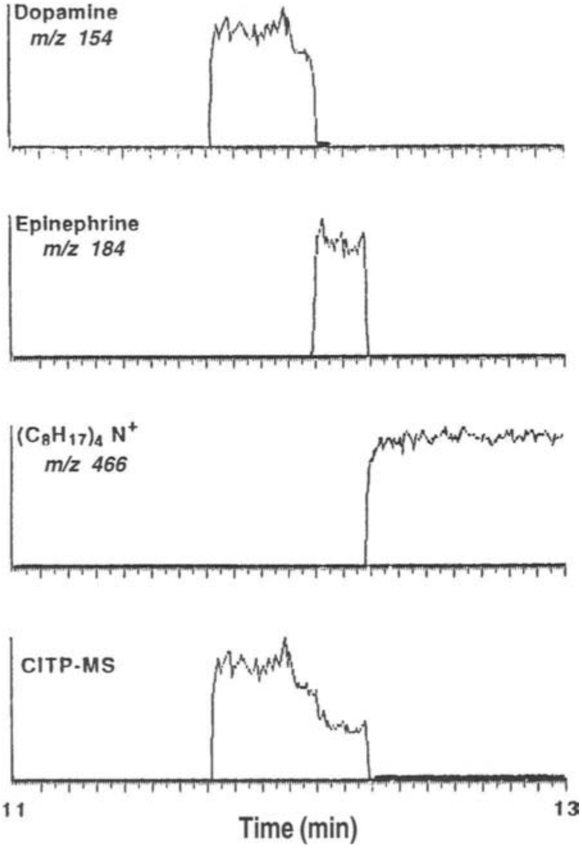


Fig. 25. CITP/MS separation of a dopamine and epinephrine in a 0.8-m capillary at 30 kV. Tetraoctylammonium bromide was used for the terminating electrolyte. (Reproduced with permission from Udseth *et al.*, 1989.)

The tandem mass spectra for the various tryptic fragments (currently obtained in separate experiments due to limitations of our data acquisition) can be assembled as demonstrated previously for the smaller polypeptide, melittin. The fragments observed from MS/MS spectra often provide large amounts of redundant information regarding the peptide sequence. For smaller peptides such as glucagon, major fragment ions are observed, which provide information on the entire glucagon sequence (Smith *et al.*, 1990a). CITP provides a relatively pure analyte band to the ESI source, without the large concentration of a supporting electrolyte demanded by CE. This latter char-

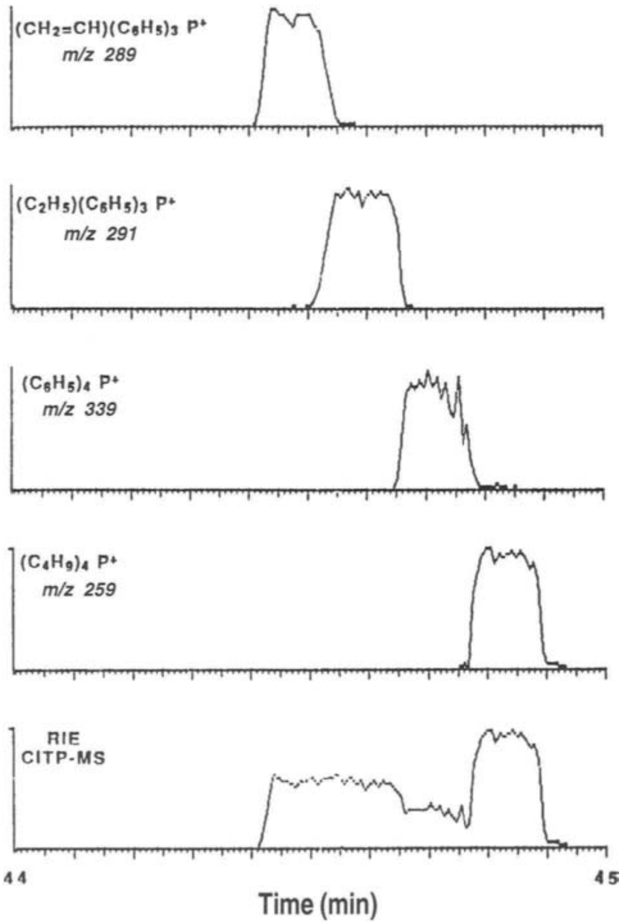


Fig. 26. CITP/MS separation of a mixture of four quaternary phosphonium salts showing the single ion plots for the four cations and reconstructed ion electropherogram (RIE). A 2-m capillary was used at 35 kV and the sample loaded by electromigration at 35 kV for 60 s. (Reproduced with permission from Udseth *et al.*, 1989.)

acteristic circumvents a disadvantage of CE-ESI-MS related to the reduced maximum analyte ion current feasible due to ionization of supporting electrolyte. Thus, CITP-MS has the potential of allowing greater analyte ion currents than feasible with CE-MS, facilitating MS/MS experiments. Although CITP-MS has thus far attracted relatively little attention, these characteristics make CITP-MS/MS potentially well-suited for characterization of enzymatic digests of proteins.

8. Limitations to Current CE-MS Methods

Most ESI-MS instruments to date have been based upon quadrupole mass spectrometers. The limitations upon obtaining high quality full scan mass spectra for CE-ESI-MS experiments with quadrupole mass spectrometers, and even more demanding tandem MS/MS experiments (such as for peptide sequencing), require further discussion since the constraints arise directly from the present levels of ESI-MS performance. Consideration and understanding of the nature of these constraints is important for understanding the trade-offs between scan speed, resolution, *m/z* range and sensitivity, as well as for the development of improved methods and instrumentation which may lead to their resolution or circumvention.

Perhaps the greatest limitation of present ESI-MS instrumentation arises due to the small fraction of the total ESI ion current actually introduced and analyzed by the mass spectrometer. At present, a typical ESI-MS quadrupole instrument will provide an ion current of approximately 10^{-11} A to the detector, corresponding to ~6×10^7 singly charged ions s^{-1}. Although not always the case, the buffer ionic strength will generally exceed the analyte by about 10^2 to realize the high efficiency possible with CE. Thus, if the ESI process does not discriminate greatly between buffer ions and sample, only about 6×10^5 sample ions s^{-1} will be detected (at most). In practice, ESI generally discriminates in favor of higher molecular weight species, and somewhat higher sample ion currents can be obtainable using "volatile buffers" such as acetic acid. Examination of full scan spectra from, say, *m/z* 10–2000, under conditions which minimize discrimination, generally shows that even the most intense peaks correspond to «10% of the **total** ion current, consistent with such expectations. The best resolved and fastest eluting peaks for capillaries of conventional dimensions have widths at half height of about 1–3 s. If ten scans are required during peak elution, and the region *m/z* 100–2000 is scanned in 0.2 *m/z* steps, then the most intense peak that can be detected would correspond to only ~20 ions. Even with pulse-counting detection >3-5 ions are required to provide minimal confidence of detection, suggesting a dynamic range of ~5. Clearly, this quality of MS performance is impractical with present quadrupole instruments. For scans at a reduced width of 1000 *m/z* units and 1 *m/z* steps, ~200 ions can result for major peaks, giving a more reasonable dynamic range of ~50. Slower scans are also useful in practice. For a 1.4 scan second over a 500 *m/z* unit window, with 1 *m/z* step size, a dynamic range of ~400 would be estimated, qualitatively consistent with the present results. Clearly, while results obtained at slower scan speeds, lower resolution, and a smaller *m/z* scan range are far less than ideal, simultaneous optimization of both CE and MS components is generally impractical given existing instrument performance. The reduced elution speed method described earlier provides one approach to circumvent some of these compromises. The situation is more severe for tandem MS/MS using triple quadrupole instrumentation, due to the much lower signal intensities after collisional dissociation (generally at least one order of magnitude smaller, often a factor of 100).

There are several possible approaches to resolving these limitations. One is to increase the efficiency of ion transport from the ESI source into the mass spectrometer, now generally only ~0.01% or less in overall efficiency (Smith *et al.*, 1990b). A second approach is to analyze all the ions which enter the mass spectrometer, using either ion-trapping methods or array detection. Indeed, a number of workers are already beginning to examine the quadrupole ion trap mass spectrometer (ITMS) for this purpose (Van Berkel *et al.*, 1990; Cooks *et al.*, 1991). Since the ITMS can trap and accumulate ions over a wide *m/z* range, and then rapidly conduct a swept ejection/detection step, the fraction of total ions detected (compared to that entering the ITMS) is much greater, allowing the utilization of a much larger fraction of the total ESI current (assuming efficient ion injection and trapping). Much faster scan speeds over extended *m/z* ranges are thus possible. The best MS performance in principle would be obtained with full range array detection, but practical instrumentation for this purpose is not yet available, and would likely be much more expensive than either the ITMS or existing instrumentation. It should be noted that projections regarding ITMS performance must be made with caution, since the trapping efficiencies upon injection from external ESI sources are not yet well established, and may be on the order of only one percent. In addition, the *m/z* measurement accuracy currently obtainable has been reported to be significantly lower than for quadrupole instruments, a particular concern for multiply charged species and sequencing applications. However, preliminary results indicate the sensitivity obtainable with ESI is indeed better than for quadrupole instrumentation (Van Berkel *et al.*, 1990), although concerns regarding mass measurement accuracy have not been completely addressed, and capabilities for both high resolution and high *m/z* detection have been demonstrated (Cooks *et al.*, 1991).

Other developments are promising, but less certain. McLafferty and coworkers have pioneered the combination of ESI with Fourier transform-ion cyclotron resonance mass spectrometry (FT-ICR) (Henry *et al.*, 1991) and have shown that very high resolution is obtainable. Laude and coworkers have demonstrated that substantial gains in sensitivity are obtainable by having the ESI interface in the high magnetic field of an ICR using a superconducting magnet (Hofstadler and Laude, 1992). The potential for higher order mass spectrometry, MS^n ($n \geq 3$), using ICR or ITMS instruments also appears promising. High resolving power at moderate m/z, a capability of ion-trapping devices, has been demonstrated for proteins and appears to have considerable potential, although technical difficulties remain to be solved, particularly in conjunction with CE methods.

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9. References

Bruins, A. P., T. R. Covey and J. D. Henion (1987). *Anal. Chem.*, *59*, 2642–2646.

Caprioli, R. M. (1991). *Proceedings of the Japanese Soc. Biomed. Mass Spectrom.*, 3–13.

Caprioli, R. M., W. T. Moore, M. Martin and B. B. DaGue (1989). *J. Chromatogr.*, *480*, 247–257.

Chowdhury, S. K., V. Katta and B. T. Chait (1990). *Rapid Commun. Mass Spectrom.*, *4*, 81–87.

Cooks, R. G., G. L. Glish, S. A. McLuckey and R. E. Kaiser (1991). *Chem. Eng. News*, March 25, 26–41.

de Wit, J. S. M., L. J. Deterding, M. A. Moseley and K. B. Tomer (1988). *Rapid Commun. Mass Spectrom.*, *2*, 100–104.

Deterding, L. J., M. A. Moseley, K. B. Tomer and J. W. Jorgenson (1991a). *J. Chromatogr.*, *554*, 73–82.

Deterding, L. J., C. E. Parker and J. R. Perkins (1991b). *J. Chromatogr.*, *554*, 329–338.

Edmonds, C. G., J. A. Loo, C. J. Barinaga, H. R. Udseth, and R. D. Smith (1989). *J. Chromatogr.*, *474*, 21–37.

Edmonds, C. G., J. A. Loo, S. M. Fields, C. J. Barinaga, H. R. Udseth and R. D. Smith (1990). In: *Biological Mass Spectrometry* (A. L. Burlingame and J. A. McCloskey; eds.) pp. 77–100, Elsevier Science Publishers B.V., Amsterdam.

Fenn, J. B., M. Mann C. K. Meng and S. F. Wong (1990). *Mass Spectrom. Rev.*, *9*, 37–70.

Garcia, F. and J. D. Henion (1992). *Anal. Chem.*, *64*, 985–990.

Goodlett, D. R., J. H. Wahl, H. R. Udseth and R. D. Smith (1993) *J. Microcol. Sep.*, *5*, 57–62.

Hail, M., J. Schwartz, I. Mylchreest, K. Seta, S. Lewis, J. Zhou, I. Jardine, J. Liu and M. Novotny (1990). In: *Proceedings of the 38th ASMS Conference on Mass Spectrometry and Allied Topics*, Tucson, AZ, pp. 353–354.

Henry, K. D., J. P. Quin and F. W. McLafferty (1991). *J. Am Chem. Soc.*, *113*, 5447–5449.

Hunt, D. F., J. E. Alexander, A. L. McCormack, P. A. Martino, H. Michel, J. Shabanowitz, M. A. Moseley, J. W. Jorgenson and K. B. Tomer (1991). In: *Techniques in Protein Chemistry II,* (J. J. Villafranca, ed.) pp. 441–454, Academic Press, San Diego, CA.

Johansson, I. M., E. C. Huang, J. D. Henion and J. Zweigenbaum (1991). *J. Chromatogr.*, *554*, 311–327.

Johansson, I. M., R. Pavelka and J. D. Henion (1991). *J. Chromatogr.*, *559*, 515–528.

Jones, H. K., N. T. Nguyen and R. D. Smith (1990). *J. Chromatogr.*, *504*, 1–19.

Kenndler, E. and E. Haidl (1985). *Fresenius J. Anal. Chem.*, *322*, 391–396.

Kenndler, E. and D. Kaniansky (1981) *J. Chromatogr.*, *209*, 306–309.

Lee, E. D., Mück, W., J. D. Henion, and Covey, T. R. (1988). *J. Chromatogr.*, *458*, 313–321.

Lee, E. D., Mück, W., J. D. Henion, and T. R. Covey (1989a). *Biomed. Environ. Mass Spectrom.*, *18*, 253–257.

Lee, E. D., Mück, W., J. D. Henion, and T. R. Covey (1989b). *Biomed. Environ. Mass Spectrom.*, *18*, 844–850.

Loo, J. A., H. K. Jones, H. R. Udseth and R. D. Smith (1989). *J. Microcolumn Separations*, *1*, 223.

Loo, J. A., R. R. Ogorzalek Loo, H. R. Udseth, C. G. Edmonds and R. D. Smith (1991a). *Rapid Commun. Mass Spectrom.*, *5*, 101–105.

Loo, J. A., C. G. Edmonds and R. D. Smith (1991b). *Anal. Chem.*, *63*, 2488–2499.

Mack, L. L., P. Kralik, A. Rheude and M. Dole (1970). *J. Chem. Phys.*, *52*, 4977–4986.

Minard, R. D., P. D. Curry, Jr. and A. G. Ewing (1989). In: *Proceedings of the 39th ASMS Conference on Mass Spectrometry and Allied Topics*, May 21–26, Miami Beach, FL., pp. 30–33.

Moore, W. T. and R. M. Caprioli (1991). In: *Techniques in Protein Chemistry II*, p. 511–528, Academic Press, New York.

Moseley, M. A., L. J. Deterding, K. B. Tomer and J. W. Jorgenson (1989a). *Rapid Commun. Mass Spectrom.*, *3*, 87–93.

Moseley, M. A., L. J. Deterding, K. B. Tomer and J. W. Jorgenson (1989b). *J. Chromatogr.*, *480*, 197–209.

Moseley, M. A., L. J. Deterding, K. B. Tomer and J. W. Jorgenson (1990). *J. Chromatogr.*, *516*, 167–173.

Moseley, M. A., L. J. Deterding, K. B. Tomer and J. W. Jorgenson (1991). *Anal. Chem.*, *63*, 109–114.

Moseley, M. A., J. Shabanowitz, D. Hunt, K. B. Tomer and J. W. Jorgenson (1992). *J. Am. Soc. Mass Spectrom.*, *3*, 289–300.

Mück, W. M. and J. D. Henion (1989). *J. Chromatogr.*, *495*, 41–59.

Mylchreest, I. and M. Hail (1989). In: *Proceedings of the 39th ASMS Conference on Mass Spectrometry and Allied Topics*, May 21–26, Miami Beach, FL., p. 316.

Olivares, J. A., N. T. Nguyen, C. R. Yonker and R. D. Smith (1987). *Anal. Chem.*, *59*, 1230–1232.

Parker, C. E., J. R. Perkins and K. B. Tomer (1992). *J. Am Soc. Mass Spectrom.*, *3*, 563–574.

Perkins, J. R., C. E. Parker and K. B. Toner (1992). *J. Am Soc. Mass Spectrom.*, *3*, 139–149.

Pleasance, S., S. W. Ager, M. V. Laycock and P. Thibault (1992a). *Rapid Commun. Mass Spectrom.*, *6*, 14–24.

Pleasance, S., P. Thibault and J. Kelly (1992b). *J. Chromatogr. 591*, 325–339.

Reinhoud, N. J., W. M. A. Niessen and U. R. Tjaden (1989). *Rapid Commun. Mass Spectrom.*, *3*, 348–357.

Reinhoud, N. J., E. Schroder, U. R. Tjaden, W. M. A. Niessen, M. C. Ten Noever de Brauw and J. Van der Greef (1990). *J. Chromatogr.*, *516*, 147–155.

Rockwood, A. L., M. Busman, H. R. Udseth and R. D. Smith (1991). *Rapid Commun. Mass Spectrom.*, *5*, 582–585.

Shida, Y., C. E. Parker, J. R. Perkins, K. O'Hara, M. Kono and K. B. Tomer (1989). In: *Proceedings of the 39th ASMS Conference on Mass Spectrometry and Allied Topics*, May 21–26, Miami Beach, FL., pp. 587–588.

Smith, R. D., J. A. Olivares, N. T. Nguyen and H. R. Udseth (1988a). *Anal. Chem.*, *60*, 436–441.

Smith, R. D., C. J. Barinaga and H. R. Udseth (1988b). *Anal. Chem.*, *60*, 1948–1952.

Smith, R. D., J. A. Loo, C. J. Barinaga, C. G. Edmonds and H. R. Udseth (1989a). *J. Chromatogr.*, *480*, 211–232.

Smith, R. D., H. R. Udseth, J. A. Loo, B. W. Wright and G. A. Ross (1989b). *Talanta*, *36*, 161.

Smith, R. D., S. M. Fields, J. A. Loo, C. J. Barinaga, H. R. Udseth, and C. G. Edmonds (1990a). *Electrophoresis*, *11*, 709–717.

Smith, R. D., J. A. Loo, C. G. Edmonds, C. J. Barinaga, and H. R. Udseth (1990b). *Anal. Chem.*, *62*, 882–899.

Smith, R. D., J. A. Loo, C. G. Edmonds, C. J. Barinaga, and H. R. Udseth (1990c). *J. Chromatogr.*, *516*, 157–165.

Smith, R. D., J. A. Loo, C. J. Barinaga, C. G. Edmonds, and H. R. Udseth (1990d). *J. Am. Soc. Mass Spectrom.*, *1*, 53–65.

Smith, R. D., J. A. Loo, R. R. Ogorzalek Loo, M. Busman and H. R. Udseth (1991a). *Mass Spectrom. Rev.*, *10*, 359–451.

Smith, R. D., H. R. Udseth, C. J. Barinaga, and C. G. Edmonds (1991b). *J. Chromatogr.*, *559*, 197–208.

Smith, R. D., J. H. Wahl, D. R. Goodlett and S. A. Hofstadler (1993) *Anal. Chem.*, *65*, 574A–584A.

Suter, M. J.-F., B. B. DaGue, W. T. Moore, S.-N. Lin and R. M. Caprioli (1991). *J. Chromatogr.*, *553*, 101–116.

Suter, M. J.-F. and R. M. Caprioli (1992). *J. Am. Soc. Mass Spectrom.*, *3*, 198–206.

Takigiku, R., T. Keough, M. P. Lacey and R. E. Schneider (1990). *Rapid Commun. Mass Spectrom.*, *4*, 24–29.

Tehrani, M., R. Macomber and L. Day (1991). *J. High Resolut. Chromatogr.*, *14*, 10.

Thibault, P., C. Paris and S. Pleasance, S. (1991a). *Rapid Commun. Mass Spectrom.*, *5*, 484–490.

Thibault, P., S. Pleasance and M. V. Laycock (1991b). Analysis of paralytic shellfish poisons by capillary electrophoresis, *J. Chromatogr.*, *542*, 483–501.

Thibault, P., S. Pleasance and M. V. Laycock (1991c). In: *Proceedings of the 39th ASMS Conference on Mass Spectrometry and Allied Topics*, May 21–26, Miami Beach, FL., pp. 593–594.

Udseth, H. R., J. A. Loo, and R. D. Smith (1989). *Anal. Chem.*, *61*, 228–232.

Van Berkel, G. J., G. L. Glish and S. A. McLuckey (1990). *Anal. Chem.*, *62*, 1284–1295.

Verheij, E. R., U. R. Tjaden, W. M. A. Niessen and J. Van Der Greef (1991). *J. Chromatogr.*, *554*, 339–349.

Wachs, T., J. C. Conboy, F. Garcia and J. D. Henion (1991). *J. Chromatogr. Sci.*, *59*, 357–366.

Wahl, J. H., D. R. Goodlett, H. R. Udseth and R. D. Smith (1992) *Anal. Chem.*, *64*, 3194–3196.

Wahl, J. H., D. R. Goodlett, H. R. Udseth, and R. D. Smith (1993) *Electrophoresis*, *14*, 448–457.

Wolf, S. M., C. Norwood, E. Jackim and P. Vouros (1992). *J. Amer. Soc. Mass Spectrom.*, *3*, 757–761.

CHAPTER 6

Electrochemical Detection for Capillary Electrophoresis

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1. Introduction

Electrochemical (EC) detection is widely accepted as a technique that can provide sensitive and selective measurements. Many articles concerning its theory, design, performance, and application have appeared in the scientific literature (Weber, 1986; Ryan *et al.*, 1994; Lunte and Lunte, 1996). These deal principally with its use in conjunction with liquid chromatography (LC), where it has been demonstrated to be one of the most sensitive detection techniques available.

Electrochemical measurements offer significant advantages over the classical spectroscopic techniques, particularly with respect to capillary electrophoresis (CE). Because electrochemistry is a surface technique, the limits of detection are not compromised by the small dimensions inherent in CE. This is in contrast to pathlength-dependent spectrophotometric detectors. In addition, electrochemical detectors do not require an optical carrier and, as a result, are much less costly than absorption and fluorescence detectors. This is especially significant when one considers that electrochemical detectors are easily tunable without such parts as monochromators and filters. While laser-induced fluorescence detection does offer comparable sensitivity, the number and wavelength of lasers is limited (see Soper chapter). Derivatization (either pre- or postcolumn) is often required, which limits its usefulness. The main drawbacks associated with EC detection for CE are problems with reliability and its lack of acceptance by many chemists more familiar with Beer's law.

In this chapter, a brief introduction on the basic principles of electrochemistry that are necessary for understanding electrochemical detection will be given. For those wishing to delve deeper, numerous detailed texts on this subject exist (Bard and Faulkner, 1980; Kissinger and Heineman, 1984, 1996). The focus of this chapter is primarily on amperometric detection; however, potentiometry and conductivity detection will also be addressed. The emphasis will be on developments in the field, describing the designs of the various systems and summarizing specific applications of the technique.

1.1 Principles of Electrochemical Detection

The application of electrochemical detection to an analysis problem depends ultimately upon the voltammetric characteristics of the analyte(s) of interest under a particular set of experimental conditions. Therefore, before beginning an analysis, it is important to evaluate the voltammetric behavior of the analyte(s) under conditions befitting the separation experiment. In typical circumstances, this is initially achieved by cyclic voltammetry (CV), a technique which can be used to rapidly assess the thermodynamic and kinetic parameters of the redox reaction. Hydrodynamic voltammetry (HDV), a steady-state technique from which amperometry is derived, is also used to determine the detection potential; it is more closely related to the actual situation encountered in an CEEC experiment.

Amperometry is the most widely applied technique in electroanalytical flow-through detection systems. In these systems a fixed operating potential is applied and the resulting current is measured as the compound undergoes a redox reaction at the electrode surface. To determine the optimum operating or detection potential for a particular compound, the relationship between the electrolysis current and the applied potential under the experimental conditions of the separation should be determined. Illustrated in Fig. 1 is the relationship of current to applied potential for the oxidation of an electroactive compound. The HDV begins at a potential at which the analyte is not electroactive and, therefore, no faradaic response is generated. The curve then proceeds into a region of increasing response characterized by the half-wave potential $E_{1/2}$, defined as the potential at which the current is half its limiting value. The slope of this region is determined by the Nernst equation and the kinetics of electron transfer at the electrode. The response finally attains a limiting current or mass transport-limited plateau. It is clear that the maximum current response is obtained at the mass transport-limited region; however,

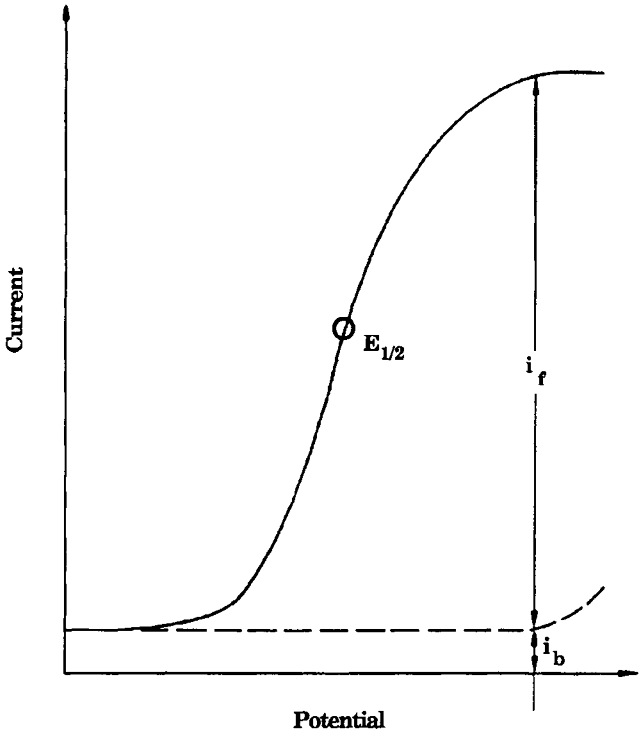

Fig. 1. Hydrodynamic voltammograms of redox species. $E_{1/2}$, half-wave potential; i_b, background current; i_f, faradaic current.

the influence of the background current (electrolysis of the carrier solution constituents) should also be taken into consideration in order to obtain the optimum signal-to-noise (S/N) ratio.

Selectivity is another important parameter influenced by the operating potential. Because each class of analytes exhibits different potential behavior (Fig. 2), the detection potential can be exploited for selective detection. In general, for oxidation reactions, the lower the detection potential, the greater the selectivity. Electropherograms are obtained by plotting current as a function of time at a selected (constant) potential. Faraday's law is crucial to amperometric determinations. It is described by:

$$Q = nFN \qquad (1)$$

where the number of coulombs (Q) passed is directly proportional to the number of moles (N) converted to product in the electrophoresis

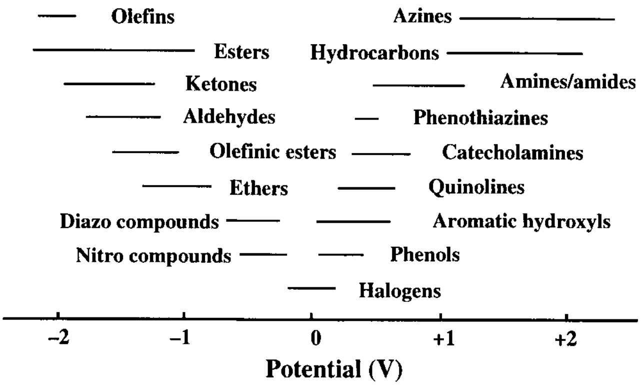

Fig. 2. Electrochemical behavior of several electroactive organic functional groups.

zone, number of electrons (n) involved in the reaction, and the Faradaic constant (F = 96,500 coulombs/mole of electrons). Differentiation of this equation with respect to time yields the instantaneous current, which is a measure of the rate at which the analyte is converted and is given by:

$$i_t = dQ/dt = nF\ dN/dt \qquad (2)$$

In the CEEC experiment, the current response is defined by Eq. 2, where the response is due to the change in concentration as a function of time. The peak area is defined by equation (1).

In practice, amperometric detectors exhibit conversion efficiencies of up to 5%; the term coulometric detector is employed if yields surpass 95%. Between these limits the detector may be termed quasi-amperometric, which is the design generally employed in CEEC systems. The amperometric rather than the coulometric mode of detection is more desirable in flow-through detectors. This is principally due to the fact that as the size of the electrode surface is increased (to increase efficiency), the added surface area contributes proportionally less to the analyte converted but is directly proportional to the background current. This results in poorer signal-to-noise ratios (S/N) at larger electrodes. Sources of noise and other parameters affecting detection limits in electrochemical detection have been investigated by Weber and Long (1988).

Redox reactions that obey the Nernst equation are termed reversible. Irreversible reactions occur when the current from electrolysis of the analyte is limited by the rate of electrochemical or chemical steps at the electrode surface. This limitation may be lessened by the application of an operating potential that exceeds the value according to the Nernst equation. In effect, this increases the thermodynamic driving force, causing the electrode reaction to occur more favorably.

1.2. Microelectrodes

The small dimensions inherent in CE necessitate the use of microelectrodes as working electrodes for electrochemical detection. The electrochemical responses at these electrodes can differ significantly from those observed at conventional-sized electrodes. This is due to the fact that the dimensions of the electrode are smaller than the distance the molecules diffuse in solution during the experiment, which leads to a steady-state response. These electrodes exhibit several unique properties which make their use as detectors very favorable, including reduced ohmic drop and capacitive charging currents and greatly increased rates of mass transfer. For comprehensive discussions on the theory, design and application of these electrodes, several excellent reviews are available (Wightman and Wipf, 1989; Wang, 1990).

1.3. The Electrochemical Cell

Electrochemical cells are usually based on a three-electrode configuration comprising a working, auxiliary and reference electrode controlled by a potentiostat. Typically, the working electrode is held at ground, and the auxiliary electrode is used to apply the potential difference between the cell solution and the working electrode. Another option is to set either the reference or auxiliary to ground potential. In this way, the potential difference between the electrode and the solution and not the potential of the working electrode material itself is the applied potential. The role of the reference electrode is to provide a stable known half-wave potential; it serves as the electrochemical zero. The purpose of the potentiostat is to control the potential difference while converting the Faradaic current into a measurable voltage. As a result of the reduced effects of solution resistance at microelectrodes, three-electrode potentiostats are not always necessary, particularly if the currents measured are in the low to sub-nanoampere range. Less sophisticated home-built two-electrode systems will suffice as long as the reference electrode is of low impedance.

1.4. Electrode Materials

Because of their excellent mechanical and electrical properties, cylindrical carbon fibers ranging in diameter from 5–33 μm have been the most widely employed electrode material for CEEC. This material can be used over a potential window of −1.0 V to 1.3 V vs. Ag/AgCl, making it possible to carry out both oxidation and reduction reactions. Recently, metal electrodes made from copper (Engstrom-Silverman and Ewing, 1991; Colón *et al.*, 1993; Ye and Baldwin, 1994), gold (O'Shea *et al.*, 1993) and amalgamated gold (O'Shea and Lunte, 1993) have found increasing use in CEEC. Chemically modified microelectrodes have also been used for CEEC, and tailoring of electrode surfaces for specific applications should provide the key to improved amperometric detectors in the future (O'Shea and Lunte, 1994; Zhou *et al.*, 1994).

2. Capillary Electrophoresis with Electrochemical Detection (CEEC)

2.1. System Design

Although electrochemical detection offers both sensitivity and selectivity for CE, it is not routinely employed. This is due primarily to the difficulty in performing electrochemistry in the presence of the high voltage power supply associated with CE. Therefore, the main design feature in interfacing an electrochemical detector to a CE system is the electrical isolation of the electrophoretic current from the electrochemical cell. Typically, the current generated by the application of the high voltage separation current is several orders of magnitude greater than that measured in the electrochemical cell. Wallingford and Ewing (1987) were the first to report the design and use of a CEEC system. They employed a porous glass sleeve into which two sections of capillary column were joined near the cathodic end of the capillary. This conductive joint (rather than the end of the column) is immersed in the cathodic buffer reservoir where it permits the flow of ions but not bulk electrolyte. This allows grounding of the separation voltage, and enables one to hold the detection end of the column at ground. Buffer and solute zones are then pumped by the electroosmotic flow generated in the separation capillary past the joint, through the detection end of the column and into the electrochemical cell, where detection can be performed without the adverse effects of the applied electrical field. Figure 3 shows a schematic diagram of this system. An initial concern was that the second capillary segment, the detection capillary, would lead to band broadening;

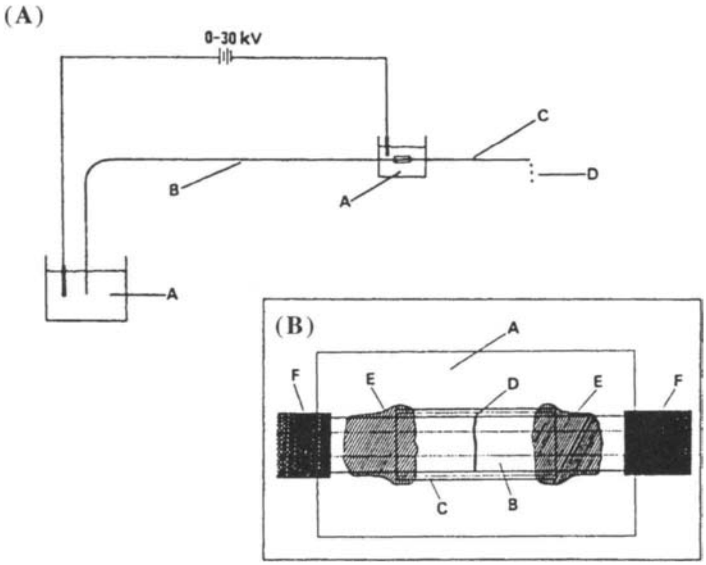

Fig. 3. (A) Schematic diagram of coupled CZE system. A, buffer reservoirs; B, separation capillary; C, detection capillary; D, eluent. (B) Detailed schematic of porous joint. A, microscope slide; B, fused silica capillary; C, porous glass capillary; D, joint; E, epoxy; F, polymer coating. (Reproduced with permission from Wallingford and Ewing, 1987.)

however, it was shown that this was minimal if the capillary length were less than 2.5 cm. In a recent report on the use of a 13 cm detection capillary, Kok (1993) demonstrated that zone broadening could be greatly decreased by the application of compensating pressure on the front end of the separation capillary during electrophoresis.

A more rugged and easily constructed improvement of this type of design was reported by O'Shea *et al.* (1992a), who employed Nafion, an ion-exchange membrane, as the conductive material. The use of this flexible material avoided the fragile nature of porous glass and did not require the capillary sections to be realigned after being fractured, as this is carried out within the Nafion tube. In this design, a short length of Nafion is threaded over a previously prepared score mark on the capillary column and sealed in place. Gentle pressure is applied, causing the score mark to fracture; this allows conduction through the fracture and grounding is thereby accomplished. A schematic representation of this joint is illustrated in Figure 4. Other

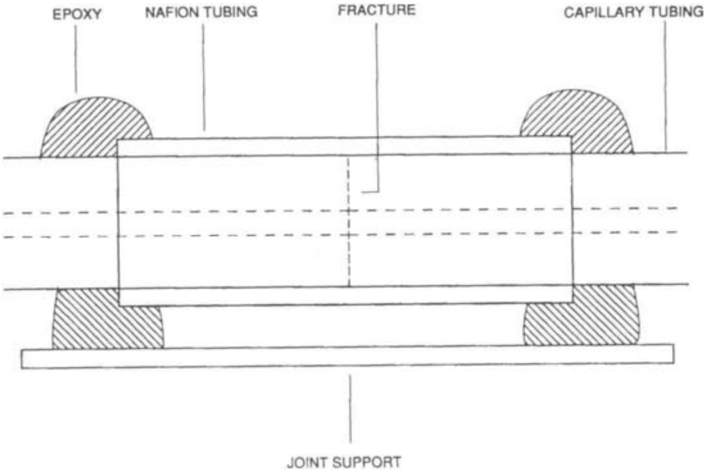

Fig. 4. Schematic diagram of Nafion joint. (Reproduced with permission from O'Shea *et al.*, 1992a).

materials which have been successfully implemented in this fashion include porous graphite (Yik *et al.*, 1991), cellulose acetate (Wang and Chen, 1992) and palladium (Kok and Sahin, 1993). Another decoupling device design was reported by Huang and Zare (1991), who used a CO_2 laser to drill a hole (ca. 40 µm in diameter) in the side of a capillary, which was subsequently filled and sintered in place by a mixture of solder glass and powdered fused silica. The glass frit allowed electrical contact to be established, facilitating its use either in the application of sample collection or for electrochemical detection.

An alternative design for an end-column amperometric detector that eliminated the need for a decoupling device for CE was described by Huang *et al.* (1991) and is illustrated in Fig. 5. In this design the working electrode is placed at the end of the capillary column (but not inside). By employing capillary columns of very small internal diameter (<20 µm) with zwitterionic buffers, virtually all of the voltage associated with the separation is dropped across the capillary. Therefore, the amperometric detector can be operated in the millivolt range provided that the working electrode is outside the end of the capillary column. However, because the detector is not isolated from the separation voltage, the actual detection potential is influenced by the electrical field present at the end of the separation capillary. The magnitude of this electrical field is proportional to the diameter of the capillary, and thus this design works best with capillaries of less than

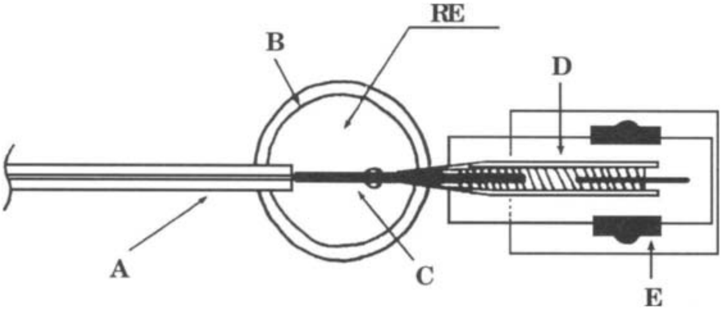

Fig. 5. Schematic drawing of CZE with end-column amperometric detection. (A) capillary; (B) cathodic buffer reservoir and electrochemical cell; (C) carbon fiber electrode; (D) electrode assembly; (E) micromanipulator; (RE) reference electrode. Reproduced with permission from Huang *et al.*, 1991).

20 μm i.d. Limitations of this design are that small fluctuations in the voltage of the power supply used for the separation can translate into noise at the electrochemical detector, and that the electrode must be equilibrated each time the separation voltage is switched on at the start of a run (Kok, 1993). Due primarily to these factors, higher concentration detection limits are consistently achieved when using this design rather than decoupling devices.

An additional drawback of end-column detection is the difficulty in placing the electrode at the end of the capillary. A solution to this problem was provided by chemically etching the end of the capillary with hydrofluoric acid to enlarge the internal diameter at the detector end (Sloss and Ewing, 1993). The electrode can then be positioned more precisely and easily, and is less susceptible to changes in position due to vibrations from external sources. The noise associated with the separation voltage was examined in detail by Lu and Cassidy (1993b). They concluded that in order to achieve optimal results a decoupling device should be employed when the internal diameter of the capillary is greater than 25 μm.

The electrochemical cells employed for CEEC are based on either a two- or three-electrode configuration, depending on the range of faradaic current being measured. Typically, a cylindrical-shaped microelectrode is inserted into the detection end of the capillary column using micromanipulators while viewing under an optical microscope. The coulometric efficiency of this type of detection scheme is dependent on the i.d. of the capillary column and the dimensions of the microelectrode. Insertion of a carbon fiber 200 μm in length with 33 μm i.d. resulted in coulometric efficiencies of approximately 50% for flow velocities typically utilized in CE separations (O'Shea *et al.*, 1992a).

2.2. *Applications*

2.2.1. *Direct amperometry*

2.2.1.1. Carbon electrodes. Early research using CEEC was focused primarily on the analysis of single cells. The research highlighted the power of the technique for analysis of small sample volumes (picoliter range). Sampling of the cytoplasm was accomplished by insertion of a capillary glass microinjector fitted onto the anodic end of the electrophoresis capillary and injected directly into a nerve cell of an invertebrate (Wallingford and Ewing, 1988c). A refinement of this procedure where the anodic end of the capillary was used as the injector has also been reported (Olefirowicz and Ewing, 1990a). This was accomplished by etching the capillary with hydrofluoric acid to form a microinjector with a tip diameter of 8–10 μm. In that work, 5 μm i.d. columns were used, necessitating electrochemical etching of the 5 μm commercially available carbon fibers to ca. 2 μm to facilitate insertion into the column. An electropherogram of a cytoplasm sample obtained from a snail neuron is shown in Fig. 6. This particular study represented the first direct detection of neurotransmitters in a single nerve cell. Detection limits in the range of 10^{-7}–10^{-9} M were obtained for a range of indoles and catecholamines in a series of studies carried out by Wallingford and Ewing (1988b,c; 1989).

Another application of CEEC that has exploited the small sample volume requirement is the analysis of *in vivo* microdialysis samples (O'Shea *et al.*, 1992b,c). In this technique, a liquid perfusate is pumped through a probe consisting of a short length of dialysis tubing implanted in the tissue of interest. Through precisely controlled flow rates, chemicals can be predictably introduced or removed from the extracellular space by establishment of a diffusional steady-state across the membrane wall. The major limitation of microdialysis sampling is the small volumes obtained, as the best recovery is obtained at flow rates of less than 1 μl/min. Thus, in order to obtain good temporal resolution, a technique having the capability to analyze submicroliter samples is highly desirable. Since CE consumes sample volumes of nanoliters or lower, enhanced temporal resolution is possible as well as the ability to perform multiple analyses on a single sample. For further discussion of this technique, see Chapter 12.

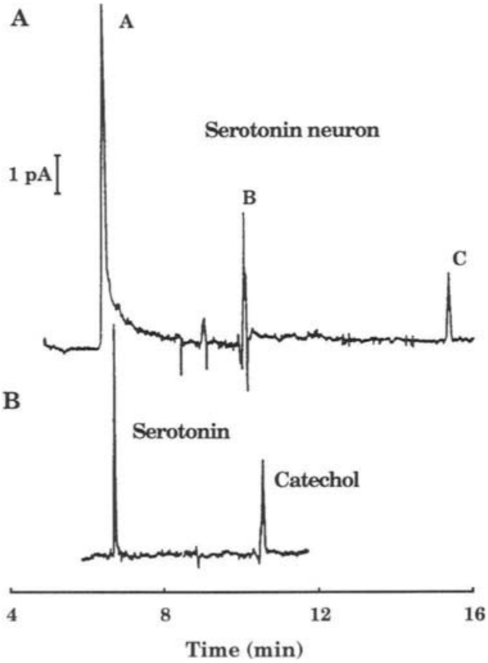

Fig. 6. Comparison of electropherograms of a cytoplasmic sample obtained from a large serotonin neuron of *Planorbis corneus* (Part A) to an electropherogram of serotonin and catechol obtained from a standard solution (Part B); separation capillary length, 77 cm; capillary i.d., 5 μm; buffer, 25 mM 2-[N-morpholino]-ethanesulfonic acid (MES) (pH 5.65); injection 5 s at 10 kV (i = 9 nA); separation potential 25 kV (i = 21 nA). The electrophoretic mobilities of peaks A and C in part A correspond to the calculated electrophoretic mobilities of serotonin (basis for identification) and an unidentified anion, respectively. (Reproduced with permission from Olefirowicz and Ewing, 1990a.)

In one report in which CEEC was employed for microdialysis studies, a dose of 25 mg/kg of the drug L-dopa was administered to a rat through an implanted cannula and was continuously monitored in blood over a 2–h period (O'Shea *et al.*, 1992b). The authors compared their results to those in a previously reported work of the same nature using LC, which suffered from poorer resolution and in which it was necessary to dilute samples in order to obtain usable injection quantities. Another advantage of the small sample requirements of CE is that multiple analyses can be carried out on a single microdialysis sample. This makes it possible to perform voltammetric characterization of analytes in a single microdialysis sample to confirm peak identity and purity. As with any separation technique, migration time is not always a reliable indicator of peak identity. This is perhaps more important in the case of CE, where the sample matrix may have a considerable effect on the migration of sample components. Therefore, it is always desirable to obtain further information for confirmation. Identification by this approach is based upon the $E_{1/2}$ value and the shape of the current voltage curve, which may be used in conjunction with migration time to characterize the analyte. The combination of these two parameters substantially increases the probability that the identification is correct.

Voltammetric characterization is accomplished by comparison of current responses in the region where most change occurs, the current-limiting plateau. This produces characteristic ratio values and is analogous to wavelength ratioing with UV absorbance detection. An example of this is illustrated in Fig. 7, where the voltammetric ratios for peaks identified as α-methyldopa and L-dopa were obtained. The ratios are shown in Table 1 for the sample and standards and confirm the purity and identity of the L-dopa peak. On the other hand, the ratios were significantly different for α-methyldopa and the sample peak, indicative of misidentification or the presence of a co-eluting peak.

Table 1. Voltammetric Characterization of L-dopa and its Metabolites

	+500/+800 mV	+650/+800 mV
Peak 1	0.26	0.63
α-methyldopa	0.19	0.52
Peak 2	0.23	0.44
L-dopa	0.24	0.43

(Reproduced with permission from O'Shea *et al.*, 1992b.)

The effect of pretreatment on the response of the carbon fiber microelectrode was also demonstrated in this study. It is well known that the surface of carbon electrodes can be gradually altered over time as a result of fouling. In conventional-sized electrodes this can be overcome with polishing, but this approach is obviously not feasible

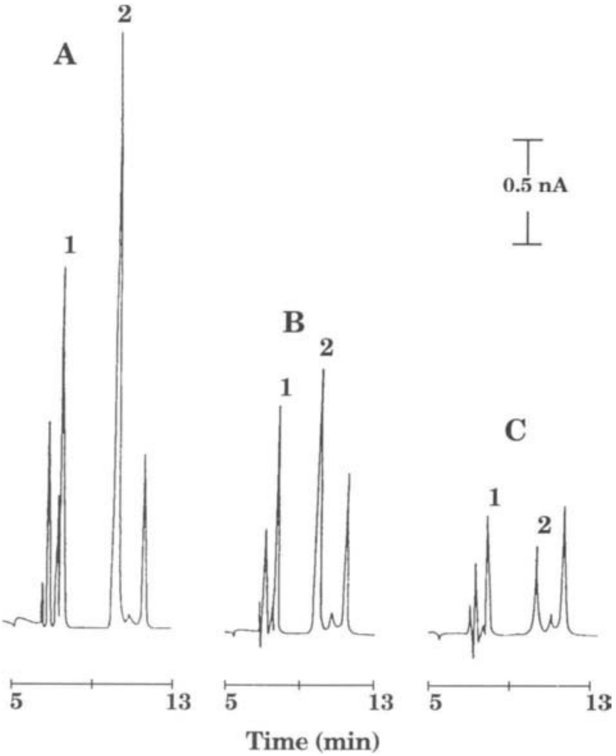

Fig. 7. Electropherograms of the dialysate, obtained 40 min after administration of L-dopa, used for voltammetric characterization study. Separation conditions: 0.01 M borate (pH 8.3); voltage, 30 kV; column length, 75 cm; detection potential, (A) +800 mV, (B) +650 mV and (C) +500 mV vs. Ag/AgCl. (Reproduced with permission from O'Shea *et al.*, 1992b.)

for cylindrical microelectrodes. An effective type of electrochemical pretreatment, which was first used by Gonon *et al.* (1981) for carbon fiber microelectrodes, is based upon the application of a high frequency waveform to the electrode surface. An example of the effectiveness of a 2 V square wave of 30 s duration on the current response of a carbon fiber for the oxidation of L-dopa is shown in Fig. 8. It was observed that the response rapidly decreased between successive injections of the drug (Fig. 8A); however, if the pretreatment were applied between each run, a reproducible response was obtained (Fig. 8B).

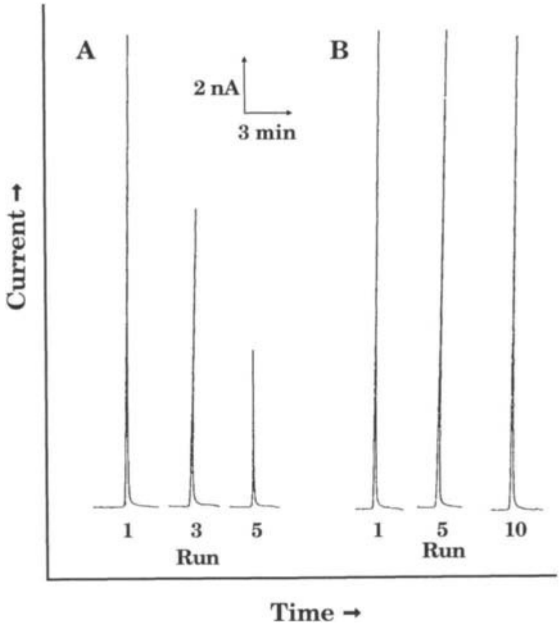

Fig. 8. Electropherograms of 25 μg/ml L-dopa. Separation conditions same as in Fig. 7 except that the detection potential is +800 mV vs. Ag/AgCl. (A) Successive injections (1,3,5) of L-dopa; (B) successive injections (1,3,5) with intermediate pretreatment. (Reproduced with permission from O'Shea *et al.*, 1992b.)

CEEC and microdialysis were also combined for the monitoring of excitatory amino acid release in the brain of a rat (O'Shea *et al.*, 1992c). Extracellular levels of aspartate, glutamate and alanine in the frontoparietal cortex of the brain were determined. The effect of an influx of high concentrations of potassium ion on the overflow of the amino acids was also monitored. As these and the majority of amino acids lack electrochemically active moieties necessary for direct detection, derivatization with naphthalene-2,3-dicarboxaldehyde (NDA), which reacts with primary amines in the presence of cyanide to produce electroactive cyano[*f*]benzisoindole (CBI) derivatives, was employed (Lunte and Wong, 1989). Detection limits reported for the derivatized amino acids were in the 10^{-7} to 10^{-8} M range.

Deacon and co-workers (1993) employed a Cu(II)-coated capillary for the determination of peptides by CEEC; this permitted direct detection without the need for precolumn derivatization. The Cu(II)-

coated capillaries were prepared by flushing fused-silica capillaries with the biuret reagent. The Cu(II) in the reagent then associated with the negatively charged silanol groups on the walls of the capillary. Under alkaline conditions, injected peptides complexed with the Cu(II) and were oxidatively detected at a carbon fiber microelectrode. Di-, tri-, tetra and pentaglycine were determined with detection limits ranging from 7.0×10^{-7} to 5.5×10^{-6} M. The sensitivity decreased with increasing peptide size.

Amperometric detection has also been used with MECC to enhance the separation of nonionic and cationic analytes. This technique was first introduced by Terabe and coworkers (1984) and is based primarily on partitioning phenomena rather than electrophoretic effects (see Chapter 3). Wallingford and Ewing (1988b) first demonstrated the use of micellar solutions with CEEC using sodium dodecyl sulfate (SDS) for the separation of neutral and cationic catecholamines. In this case, the presence of micelles in the buffer solution was observed to affect the detection. To further evaluate this effect, voltammetric experiments were performed in various concentrations of SDS. It was found that higher concentrations of SDS cathodically shifted the half-wave potentials of the catechols, with the effect more pronounced for cationic catechols. In addition, as the SDS concentration was increased, the limiting current was observed to decrease to a constant value. In contrast, an increase in response was observed for neutral catechols at low concentrations of SDS. Sensitivity was therefore severely affected, resulting in detection limits in the femtomole rather than the attomole range commonly obtained with MECC. It was surmised that this reduction in sensitivity was caused by adsorption of SDS to the electrode, which resulted in fouling of the surface. In a further study, it was found that surface modification of the electrode surface with Nafion alleviated this problem somewhat; however, conclusive quantitative data were not reported (Wallingford and Ewing, 1989).

2.2.1.2. Metal electrodes. Other electrode materials in addition to carbon have been employed for direct amperometric detection in CE. The use of an amalgamated gold wire microelectrode was reported by O'Shea and Lunte, 1993. This type of electrode was prepared by dip-coating a 50 µm diameter cylindrical gold microelectrode in mercury. Alternatively, the mercury can be electrodeposited from a mercurous solution onto the microelectrode. The resulting electrode was used for the detection of biological thiols, including cysteine and glutathione, down to the nanomolar range. The detection of the thiols is indirect and is based upon the catalytic oxidation of mercury in the presence of thiols as described by:

$$\mathrm{Hg} + 2\,\mathrm{RSH} \rightarrow \mathrm{Hg(SR)_2} + 2e^- + 2\mathrm{H}^+ \qquad (3)$$

This reaction occurs at +0.1 V vs. Ag/AgCl, and is therefore very selective. This system was employed for the determination of glutathione in a rat brain homogenate. Only one other peak was detected in this complex sample (Fig. 9). The applicability of the system was extended to the detection of thiol-containing drugs, including D-penicillamine, captopril, and 6-mercaptopurine, at the submicromolar level. The average lifetime of the electrode was reported to be ca. one week; however, it was emphasized that this was a direct function of sample throughput and thiol concentration.

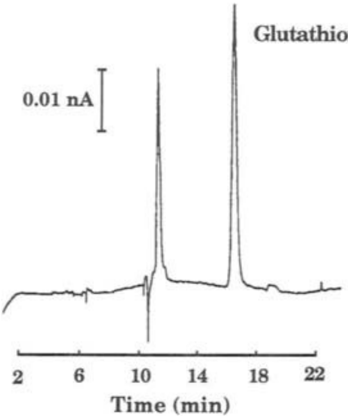

Fig. 9. Electropherogram of rat brain tissue homogenate. Peak corresponds to 58 fmol of glutathione. Operating buffer, 10 mM MES (pH 5.5); separation voltage, 30 kV; detection potential, +150 mV vs. Ag/AgCl. (Reproduced with permission from O'Shea and Lunte, 1993.)

Recently, the application of a mercury liquid film on a gold microdisk electrode for CEEC was reported (Lu and Cassidy, 1993a). This electrode was used for the determination of 14 metal ions detected as their complexes with the complexation reagent α-hydroxyisobutyric acid, which was present in the buffer system. A limitation associated with the Hg-film electrode was its lack of long-term stability. This was demonstrated by a decrease of up to 50% for certain complexes over a 5–h period. It was speculated that this was caused by alterations in the physical nature of the electrode such as increased formation of the Hg/gold amalgam and/or oxidation of the mercury surface.

A copper wire microelectrode detector has been described by Engstrom-Silverman and Ewing (1991). The detection principle was based upon the complexation of certain analytes, including amino acids, with a copper oxide film on the surface of the electrode. This complexation and subsequent dissolution of the copper oxide resulted in an oxidation current that is dependent on the analyte concentration. The detection of nonelectroactive amino acids and dipeptides as well as electroactive catechols using a 10 μm copper wire electrode was demonstrated. The separation and detection of amino acids and peptides using a copper electrode has been further investigated by Ye and Baldwin (1994) and Zhou and Lunte, (1995).

Copper wire microelectrodes were also used for the detection of carbohydrates that had been separated in strongly alkaline solutions (pH 13) without prior derivatization or complexation (Colón *et al.*, 1993). The technique used was termed amperometric detection at constant potential (ADCP). The separation conditions employed were suitable for the separation of 15 different sugars (Fig. 10), and reportedly did not result in any apparent deterioration of the microelectrode. The linear response of the detector was found to be over three orders of magnitude (μM–mM), and limits of detection for the carbohydrates studied were in the fmol range.

2.2.1.3. Chemically modified electrodes. Direct amperometry of many important analytes is often compromised by slow electron transfer kinetics at the electrode surface, which causes oxidation (or reduction) to occur at a potential greater than the expected thermodynamic potential. This can result in poor selectivity and higher detection limits. In some cases, detection of an analyte may require an overpotential that is beyond that of the solvent electrolysis, thus making detection infeasible. Chemically modified electrodes (CME) can overcome this problem through the use of surface-bound mediators that can catalyze the redox reaction of specific solution species at substantially reduced potentials. The capabilities of such CMEs for CEEC were first demonstrated by O'Shea and Lunte (1994) with electrodes based on the immobilization of two different types of modifiers into a carbon paste matrix. Electrodes were composed of a length of 150 μm i.d fused silica capillary packed at one end with carbon paste containing the modifier. The disc electrode was then aligned with the end of the CE capillary in a wall-jet configuration. The first design of the electrode incorporated the electrocatalyst cobalt phthalocyanine, which was used to catalyze the oxidation of several analytes including cysteine, glutathione and thioguanine with detection limits in the 10^{-8} M range. The enhanced selectivity achieved with this electrode was demonstrated by the analysis of cysteine in urine.

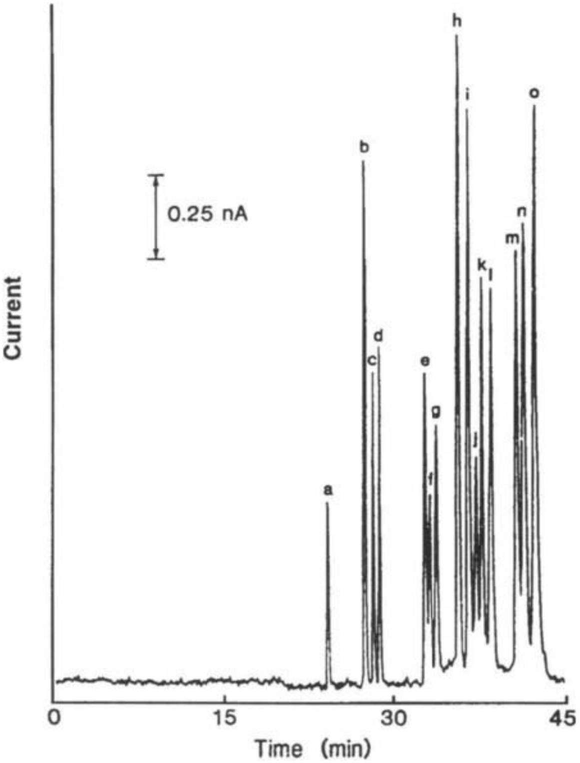

Fig. 10. CZE/ADCP electropherogram of a mixture containing 15 different carbohydrates (80–150 μM). Conditions: separation electrolyte, 100 mM NaOH; fused-silica capillary, 50 μm i.d. × 73 cm; injection 10 s by gravity (10 cm height); separation voltage, 11 kV; carbohydrates (a) trehalose, (b) stachyose, (c) raffinose, (d) sucrose, (e) lactose, (f) lactulose, (g) cellobiose, (h) galactose, (i) glucose, (j) rhamnose, (k) mannose, (l) fructose, (m) xylose, (n) talose, (o) ribose. (Reproduced with permission from Colón *et al.*, 1993.)

The use of a RuCN-based modified electrode for the simultaneous determination of thiols and disulfides has also been reported in conjunction with CE separation (Zhou *et al.*, 1994). The electrode used in this case requires solutions of low pH and high ionic strength for maximum sensitivity.

An additional design is based on an immobilized enzyme. In one case, glucose oxidase was employed for the selective detection of glucose (O'Shea and Lunte, 1994). The electrodes were reported to be very stable and reproducible when used as detectors for CE. Based on the diversity of chemical and biological modifiers available, CMEs should expand the utility of CEEC to include several classes of analytes that are otherwise poorly suited to electrochemical detection. Other types of CMEs have found application in LCEC and should be exploitable for CE. These include permselective coatings, which can enhance selectivity or inhibit electrode fouling, and electroactive polymer films for the electrochemical detection of nonelectroactive ionic analytes.

2.2.2. Pulsed amperometry

Aldehydes and terminal alcohol moieties in carbohydrates can be detected by their electrocatalytic oxidation at gold electrodes under alkaline conditions. However, as a consequence of the adsorption of free-radical intermediate product as well as solution impurities, the anodic response of carbohydrates is rapidly attenuated. Johnson and LaCourse (1990) were the first to develop a commercially exploitable pulsed waveform for LC to overcome this limitation. In this technique, termed pulsed amperometric detection (PAD), three sequential potential steps manage the process of sampling the faradaic signal from the analyte, followed by oxidative cleaning and reductive reactivation of the electrode surface. Examples of the potential-time waveforms used for PAD are illustrated in Fig. 11. By judicious selection of the PAD waveform, several other classes of compounds, including aliphatic amines and amino acids as well as numerous sulfur compounds, are amenable to detection.

O'Shea *et al.* (1993b) were the first to report results from CE-PAD. They applied the waveform illustrated in Fig. 11a to a 50 μm diameter gold wire microelectrode. Several carbohydrates of physiological interest were detected, including glucose, with detection limits of 0.9 μM or 22.5 fmol. The method was employed for the detection of glucose in blood; an example of such an electropherogram is shown in Figure 12. It has also been expanded to the detection of glycopeptides and carbohydrates isolated from recombinant coagulation factor VIIa (Weber *et al.*, 1995). Lu and Cassidy (1993a) recently described a two-step pulse waveform for the detection of Tl^+, Pb^{2+} and Cu^{2+}. The procedure was based upon the application of a negative potential to reduce the analyte ions, followed by a positive potential in which the current was sampled from the oxidation of the reduced metal.

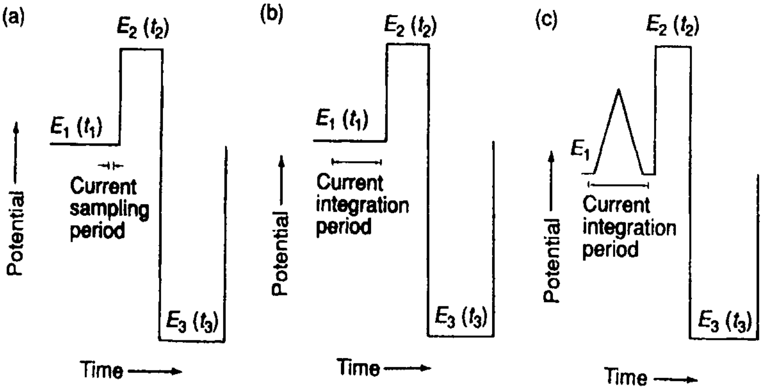

Fig. 11. Potential–time (E–t) waveforms. Processes: E1, anodic detection; E2, oxidative cleaning; E3, cathodic reactivation. Waveforms: (a) pulsed amperometric detection (PAD) with a short current sampling period (*e.g.*, 16.7 ms); (b) PAD with a long current integration period (*e.g.*, 200 ms); (c) integrated PAD with a long integration period (*e.g.*., 200 ms).

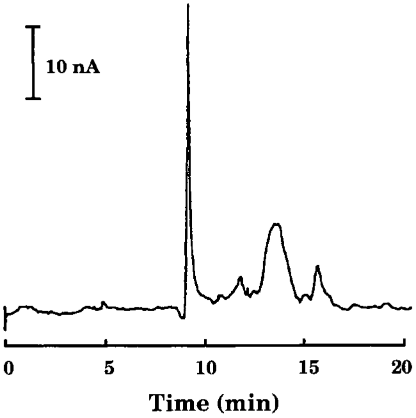

Fig. 12. Electropherogram of human blood. Peak corresponds to 85 μM glucose. Operating buffer, 10 mM NaOH–8 mM Na_2CO_3; capillary column 75 μm i.d.; separation voltage, 25 kV. (Reproduced with permission from O'Shea *et al.*, 1993.)

2.2.3. Indirect amperometry

Although direct and pulse amperometry can provide very sensitive measurements, many analytes are not electroactive and therefore cannot be detected by this approach. Indirect detection can circumvent this limitation; it is based upon the analyte displacing a mobile-phase additive in the eluted band. The analytical signal is derived from the additive itself rather than the analyte. The displacement of the additive by the analyte causes a decrease in the signal because the concentration of the additive in the analyte zone is lower than its steady-state concentration. Olefirowicz and Ewing (1990b) were the first to demonstrate the feasibility of indirect amperometric detection for CE. Dihydroxybenzylamine (DHBA), an electroactive compound, was used as the cationic electrophore, providing a stable background by its continuous oxidation at +700 mV vs. SCE. Zones of nonelectroactive cations displaced the DHBA during the electrophoretic run and were detected as negative peaks. Several amino acids and peptides were detected in this manner with detection limits in the subfmol range. In addition, direct amperometric detection of catechols could be performed simultaneously, allowing both electroactive and non-electroactive analytes to be detected in the same run, as illustrated in Fig. 13.

2.2.4. Conductivity detection

Conductivity detection is based upon the measurement of the potential difference between two indicator electrodes when a constant current is applied to them. Analytes possessing conductivities different than that of the electrophoretic buffer are detected as they pass by the indicator electrodes. Conductivity is a bulk property and therefore provides universal detection; however, this also severely limits its selectivity and yields detection limits comparable to those of UV detection. Nonetheless, it can be useful for the detection of inorganic ions.

The first experiments using a conductivity detector in CE were performed by Mikkers *et al.* (1979), who employed a potential drop between two electrodes separated along the flow direction in a polytetrafluoroethylene (PTFE) narrow-bore tube. Foret and coworkers (1986) later described a conductivity cell to which the capillary tubing was connected using a microscale molding of polyester resin to construct an on-column detector. They reported detection limits with this system in the 10^{-5} M range.

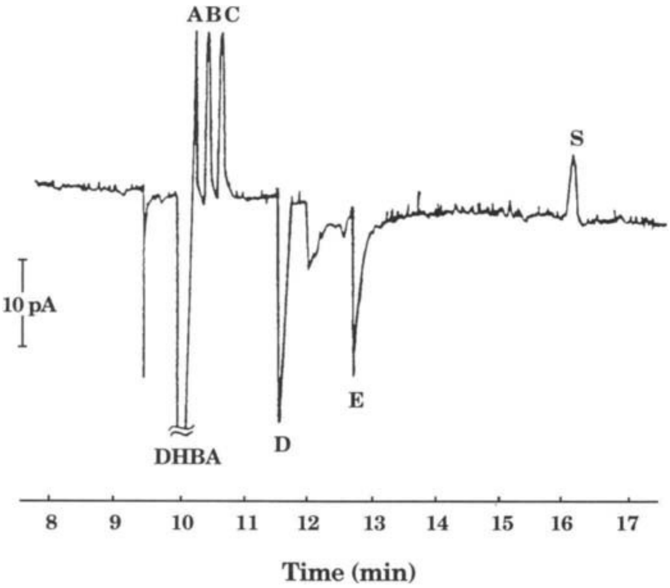

Fig. 13. Electropherogram of catecholamines and peptides with combined direct and indirect amperometric detection in a 9 μm i.d. capillary; buffer, 0.01 mM DHBA–0.025 MES (pH 5.65); 97 cm separation capillary; 1.0 cm detection capillary; injection 5 s at 25 kV; separation voltage 25 kV. Peaks: A, dopamine, (0.50 fmol injected); B, norepinephrine (0.51 fmol injected); C, epinephrine (0.50 fmol injected); D, Lys-Phe (45 fmol injected); E, His-Phe (37 fmol injected). The broad peak between D and E is an unknown impurity in the Lys-Phe. (Reproduced with permission from Olefirowicz and Ewing, 1990b.)

Huang and coworkers (1987) described a more sophisticated on-column detector design for small diameter capillaries. They used a focused CO_2 laser beam to bore 40 μm holes directly through the walls of a fused-silica capillary. Two 25 μm platinum wires were sealed into the holes on opposite sides of the capillary. An AC conductivity circuit with an oscillation frequency of 3.5 KHz was employed to measure and amplify the potential changes between the electrodes. As a result of the large voltage drop along the length of the column (of the order of 300 V/cm), the detector electrodes were required to be aligned exactly to minimize background noise from electrochemical reactions. Peak area was linear over three orders of magnitude from 2.5 μM to 2.0 mM for Li^+.

Huang *et al.* (1989) have also employed conductivity detection for the determination of low molecular weight carboxylic acids. In their study, it was found that the electrophoretic mobilities of some of the multicharged acids, under conditions in which they are largely ionized, were often greater than and opposite to the electroosmotic flow. Therefore, in order to detect these anions, it was necessary to add the surfactant tetradecyltrimethylammonium bromide to the buffer phase to control the electroosmotic flow.

In 1991, a simpler end-column conductivity detector was described by Huang *et al.* It was mounted directly at the outlet of the separation capillary of a commercial CE system. In their design, a 40 µm hole was made in a capillary near its outlet. A platinum wire was inserted along the length of the capillary close to the hole and sealed in place, as illustrated in Fig. 14. The assembly was then placed in an outlet reservoir containing the ground electrode. This ground electrode completes the electrophoretic separation circuit, and the conductivity measurement is made between the sensing and the ground electrodes. The system was applied to the analysis of several metal ions, cyclohexamine and pyridoxamine at the 10^{-4} M level.

Since the signal in conductivity detection arises from the difference in equivalent conductance between the charge carrier electrolyte ion and the analyte ion, a high background occurs which limits the sensitivity of the detector. Dasgupta and Bao (1993) addressed this limitation by utilizing a suppressed conductometric CE system. Their design incorporated a tubular cation-exchange membrane at the detection end of a capillary. A static dilute acid regenerant solution surrounded the membrane that functioned as a suppressor. With the high voltage applied and the regenerant solution grounded, effective suppression of several electrolytes including sodium borate, glycinate and cyanide was demonstrated. Electroosmotic flow carries the analyte zones past the suppressor into the conductivity detection cell, which consists of two platinum wires inserted through the wall of a PVC capillary. The system exhibited detection limits superior to those of previous conductivity approaches, in the low µg/l for a range of anions. An example of this technique is illustrated in Fig. 15. In a subsequent paper, Avdalovic and colleagues (1993) reported a similar design, employing suppressors for both anion and cation determinations, which enabled detection limits in the 10^{-8} to 10^{-7} M range for common organic and inorganic ions.

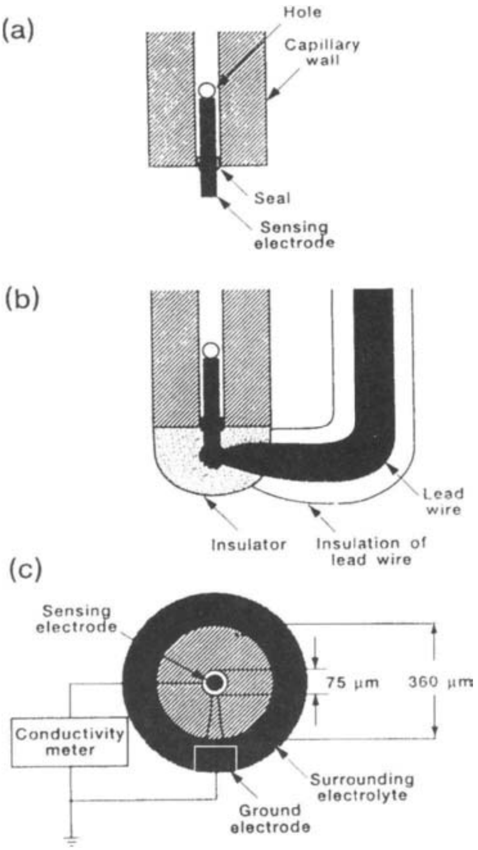

Fig. 14. End-column conductivity detector: (a) alignment of sensing electrode in capillary with eluent hole in capillary wall (not to scale); (b) same as (a) with electrical connectors; (c) horizontal cross-section. Not shown is the protective jacket, which surrounds the outlet of the capillary. Conductivity measurements are made between the sensing electrode and the ground electrode, which also acts to complete the electrophoretic circuit. (Reproduced with permission from Huang *et al.*, 1991.)

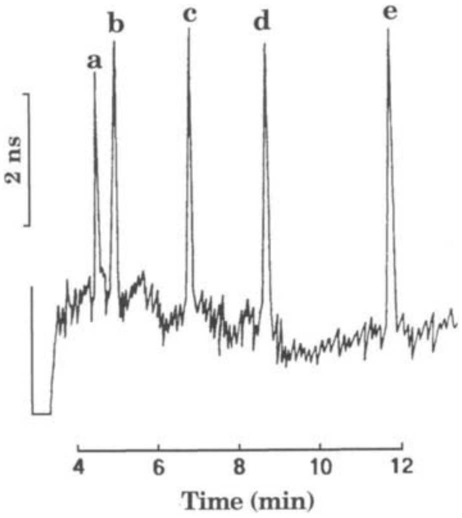

Fig. 15. Performance near detection limits: (a) unknown; (b) iodate; (c) fluoride; (d) chlorate; (e) nitrate. 50 μg/l of each ion except fluoride (10 μg/l). Injection 4 s at 5 psi. (Reproduced with permission from Dasgupta and Bao, 1993.)

2.2.5. Potentiometric detection

Potentiometric detection is based upon the measurement of the Nernst potential with respect to a reference electrode either across an ion-selective membrane or at an electrode surface. This is a type of measurement in which the controlling unit is used principally to ensure that no significant current is drawn from the cell. Therefore, detection is based on the measurement of electrode potential at zero current. The indicator electrode is chosen to respond to a particular species in solution whose activity or concentration is to be determined. The half-cell potential of the reference electrode is invariant. Historically, indicator electrodes have been made of a metal that forms a redox couple with the analyte of interest or, alternatively, an inert metal that responds to the activity ratio of a soluble redox couple. The main limitation of these electrodes is that they are prone to numerous interferences. A pivotal achievement in this area was the development of ion-selective electrodes (ISE). The response of these devices relies on the interaction of the analyte with an electrode membrane that alters the potential across the membrane. The selectivity of the response is dependent on the specificity of the interaction of the analyte with the membrane.

Potentiometric detection was first introduced for zone electrophoresis by Virtanen (1974) for 200 µm i.d. capillaries. The system consisted of a Ag/AgCl-coated platinum wire sensing electrode, which was inserted into the cathodic end of the separation capillary. Changes in potential as a result of eluting analyte zones were measured with respect to a reference electrode in a dc Wheatstone bridge. The system was used to detect inorganic ions including Li^+, K^+ and Na^+, which were reported to be linear over the range 1–6 mM.

Haber *et al.* (1991) also described a more sophisticated ion-selective microelectrode for the potentiometric detection of several alkali and alkali-earth metals. The electrode was prepared using a Ag/AgCl-coated platinum wire which was encased in a micropipet pulled to a tip diameter of ca. 1 µm. A membrane cocktail, which was drawn into the tip of the microelectrode, was specifically designed to exhibit a good response for most cations with the exception of magnesium, which was used as the electrolyte in the electrophoretic buffer. The microelectrode was placed a few microns behind the capillary outlet and, because of its high internal resistance (10^8 to 10^{11} ohms), no decoupling devices were necessary. A separation of several alkali and alkali-earth metals at the 10^{-5} M level is illustrated in Fig. 16.

3. Conclusions

CE and electrochemical detection have emerged as a powerful combination for trace measurements of electroactive compounds. Electrochemical detection has been demonstrated to be particularly well suited to CE, as it is concentration-sensitive and therefore not compromised by the small volumes associated with CE. There is little doubt that only the "tip of the iceberg" has been seen in terms of applications of CEEC. The literature now contains the basic elements for the design of such a system which should make possible a viable commercial detector in the near future. Such an instrument would certainly address the sensitivity limitations currently restricting the applications of CE.

Presently, the technology is advancing in several different areas. Detection potential is not the only means of achieving selectivity. Electrode material, pulse waveforms and electrode surface modifications have all been demonstrated to increase selectivity. However, to meet new and existing challenges posed by complex samples, it is an-

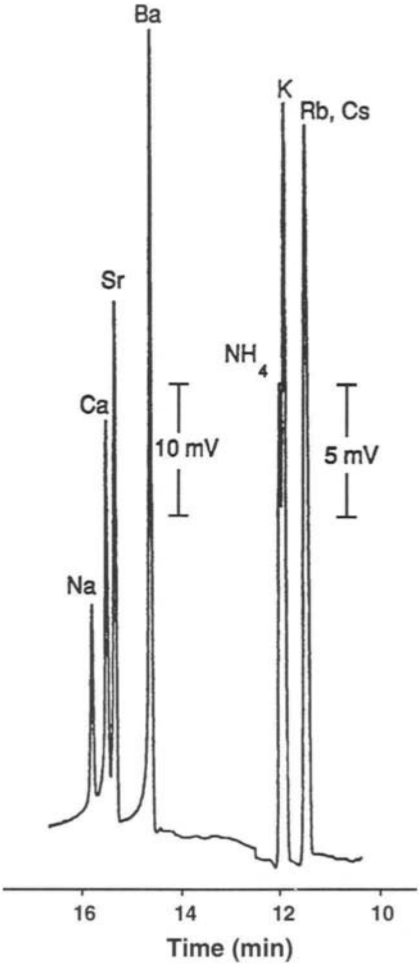

Fig. 16. Electropherogram of a solution containing 10^{-5} M alkali and alkali-earth metals. Capillary 25 μm i.d., 0.99 m long; buffer 20 mM magnesium acetate; pH 7.5; injection electrokinetically, 5000 V for 2 s; potential 15 kV; potentiometric detection postcolumn. (Reproduced with permission from Haber *et al.*, 1991.)

ticipated that electrode modification and novel derivatization schemes will play increasing roles in the enhancement of electrochemical detection for CE. A microelectrode array detector (Ewing *et al.*, 1994) has been designed for CE. Such designs will, in principle, improve the scope and selectivity of amperometric detection. Voltammetric detection is another possibility for CE. This technique, while it does not exhibit the impressive detection limits of amperometry, would offer the benefit of qualitative as well as quantitative information. For example, co-eluting peaks can be resolved if the potential at which they are electroactive differ significantly.

The excellent detection limits exhibited by suppressed conductivity should ensure its continued development and establish it as a very powerful universal detection scheme for CE.

4. References

Avdalovic. N., C. A. Pohl, R .D. Rocklin and J. R. Stillian. (1993). *Anal. Chem.*, *65*, 1470–1475.

Bard, A. J. and L. R. Faulkner (1980). *Electrochemical Methods*. Wiley, New York.

Colón, L. A., R. Dadoo and R. N. Zare (1993). *Anal. Chem.*, *65*, 476–481.

Dasgupta, P. K. and L. Bao (1993). *Anal. Chem.*, *65*, 1003–1011.

Deacon, M., T. J. O'Shea, S. M. Lunte, M. R. and Smyth (1993). *J. Chromatogr.*, 652, 377–383.

Engstrom-Silverman, C. E. and A. G. Ewing (1991). *J. Microcolumn Sep.*, *3*, 141–145.

Ewing, A. G., J. M. Mesaros and P. F. Gavin (1994). *Anal. Chem.*, *66*, 527A–537A.

Foret, F., M. Deml, V. Kahle and P. Bocek (1986). *Electrophoresis*, *7*, 430–432.

Gonon, F. G., C. M. Fombarlet, M. J., Buda and J.-F. Pujol (1981). *Anal. Chem.*, *53* 1386–1390.

Haber, C., I. Silvestri, S. Roosli and W. Simon (1991). *Chimia*, *45*, 117–121.

Huang, X and R. N. Zare (1991). *Anal. Chem.*, *63*, 2193–2196.

Huang, X., T. J. Pang, M. J. Gordon and R. N. Zare (1987). *Anal. Chem.*, *59*, 2747–2749.

Huang, X., J. A. Luckey, M. J. Gordon and R. N. Zare (1989). *Anal. Chem.*, *61*, 766–770.

Huang, X., R. N. Zare, S. Sloss and A. G. Ewing (1991). *Anal. Chem.*, 63, 189–192.

Johnson, D. C. and W. R. LaCourse (1990). *Anal. Chem.*, *62*, 589A–597A.

Kissinger, P. T. and Heineman, W. R., eds. (1984) *Laboratory Techniques in Electroanalytical Chemistry*, 1st Ed. Marcel Dekker, New York.

Kissinger, P. T. and Heineman, W. R., eds. (1996) *Laboratory Techniques in Electroanalytical Chemistry*, 2nd Ed. Marcel Dekker, New York, in press.

Kok, W. Th. (1993). *Anal. Chem.*, *65*, 1853–1860.

Kok, W. Th. and Y. Sahin (1993). *Anal. Chem.*, *65*, 2497–2501.

Lu, W and R. M. Cassidy (1993a). *Anal. Chem.*, *65* 1649–1653.

Lu, W and R. M. Cassidy (1993b). *Anal. Chem.*, *65*, 2878–2881.

Lunte, S. M. and O. S. Wong (1990). *Current Separations*, *10*, 19–26.

Lunte, S. M. and T. J. O'Shea (1994). *Electrophoresis*, *15*, 79–86.

Lunte, S. M. and C. E. Lunte (1996). In: *Laboratory Techniques in Electroanalytical Chemistry*. (P. T. Kissinger and W. R. Heineman, eds.), 2nd Ed. Marcel Dekker, New York, in press.

Mikkers, F .E. P., F. M. Everaerts and Th. P .E. M. Verheggen, (1979). *J. Chromatogr.*, *169*, 11–20.

Olefirowicz, T. M. and A. G. Ewing (1990a). *Anal. Chem.*, *62*, 1872–1876.

Olefirowicz, T. M. and A. G. Ewing (1990b). *J. Chromatogr.*, *499*, 713–719.

O'Shea, T. J. and S. M. Lunte (1993). *Anal. Chem.*, *65*, 247–250.

O'Shea, T. J. and S. M. Lunte (1994). *Anal. Chem.*, *66*, 307–311.

O'Shea, T. J., R. D. Greenhagen, S. M. Lunte, C. E. Lunte, M. R. Smyth, D. M. Radzik and N. J. Watanabe (1992a). *J. Chromatogr.*, *593*, 305–312.

O'Shea, T. J., M. W. Telting-Diaz, S. M. Lunte, C. E. Lunte and M. R. Smyth (1992b). *Electroanalysis*, *4*, 463–468.

O'Shea, T. J., P. L. Weber, B. P. Bammel, C. E. Lunte, S. M. Lunte and M. R. Smyth, (1992c). *J. Chromatogr.*, *608*,189–195.

O'Shea, T. J., S. M. Lunte and W. R. LaCourse (1993). *Anal. Chem.*, *65*, 948–951.

Ryan, M. D., E. F. Bowden and J. Q. Chambers (1994). *Anal. Chem.*, *66*, 360R–427R.

Sloss, S. and A. G. Ewing (1993). *Anal. Chem.*, *65*, 577–581.

Terabe, S., K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, (1984). *Anal. Chem.*, *56*, 111–113.

Virtanen, R. (1974). *Acta Poltech. Scand.*, *123*, 1–7.

Wallingford, R. A. and A. G. Ewing (1987). *Anal. Chem.*, *59* 1762–1766.

Wallingford, R. A. and A. G. Ewing (1988a). *J. Chromatogr.*, *441*, 299–309.

Wallingford, R. A. and A. G. Ewing (1988b). *Anal. Chem.*, *60*, 258–263.

Wallingford, R. A. and A. G. Ewing (1988c). *Anal. Chem.*, *60*, 1972–1975.

Wallingford, R. A. and A. G. Ewing (1989). *Anal. Chem.*, *61*, 98–100.

Wang, C.-W. and I.-C. Chen (1992). *Anal. Chem.*, *64*, 2461–2464.

Wang, J. (1990). *Microelectrodes*, VCH Publishers, New York.

Weber, P. L., T. Kornfelt, N. K. Klausen and S. M. Lunte (1995). *Anal. Biochem.*, *225*, 135–142.

Weber, S. G. (1986). In: *Detectors for Liquid Chromatography* (E. S. Yeung, Ed.), Wiley, New York, pp. 229–291.

Weber, S. G. and J. T. Long (1988). *Anal. Chem.*, *60*, 903A–908A.

Wightman, R. M. and D. O. Wipf (1989). *Electroanalytical Chemistry*, Vol. 15, (A. J. Bard, Ed.), Marcel Dekker, New York.

Ye, J. and Baldwin, R. P. (1994). *Anal. Chem.*, *66*, 2669–2674.

Yik, Y. F., H. K. Lee, S. F. Y. Li and S. B. Khoo (1991). *J. Chromatogr.*, *585*, 139–146.

Zhou, J. and S. M. Lunte (1995). *Anal. Chem.*, in press.

Zhou, J., T. J. O'Shea and S. M. Lunte (1994). *J. Chromatogr. A.*, *680*, 271–277.

Part Three: Applications

CHAPTER 7

Sample Preparation for Capillary Electrophoresis

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1. Introduction

In CE, as in high-performance liquid chromatography (HPLC), the matrix in which an analyte is injected onto the separation column can make a significant difference in the quality of separation achieved. The simplest case is one in which a single pure analyte is dissolved at a relatively low concentration in the same solution that fills the separation capillary. In such a case, injection of the analyte causes minimal disturbance of the equilibrium within the capillary. The analyte travels in a distinct zone along the capillary, with broadening occurring from a number of potential sources such as chromatographic interactions with the capillary surface, heating effects and diffusion. If, however, the sample zone possesses a significantly different composition than the solution filling the rest of the capillary, the equilibrium in the capillary is disturbed. This may have deleterious (or, if controlled, potentially advantageous) effects on the analysis. For example, if the sample zone has a different conductivity than the solution in the rest of the capillary, the local modification in field strength will cause either zone sharpening or broadening, and the presence of high percentages of organic solvent in the sample can cause disruption of micelles in micellar electrokinetic capillary chromatography (MECC).

Problems may also arise if the composition of the sample matrix is not consistent. For example, concentration of urine components, in particular, inorganic ions, will vary considerably from sample to sample. This may cause poor inter-sample reproducibility in resolution and in other analytical parameters. A further problem when analyzing samples in biological matrices is the possible modification of the capillary surface by proteinaceous components. The result can be loss of efficiency and resolution and poor reproducibility. Uncoated fused silica capillaries are highly susceptible to this problem, so removal of protein from the sample or inhibition of protein adhesion through the use of a suitable capillary coating or buffer system is necessary.

This chapter will be focused mainly on the sample pretreatment used with CE in the determination of endogenous compounds and xenobiotics in biological matrices. Reasons for performing a sample preparation step include:

* Sample concentration

* Removal/control of electrolyte content of the sample

* Removal of proteins

* Removal of interferents

* Removal of particulate matter

Many of these reasons are similar to those for performing sample pretreatment prior to analysis by HPLC. In comparison with HPLC, CE has two features which can be beneficial in terms of reducing the extent of sample pretreatment. Firstly, the separation generally is performed in solution in an open tube (packed-capillary electrochromatography and capillary gel electrophoresis being exceptions). Therefore, there are no problems associated with fouling of a stationary phase, only of the capillary surface. Secondly, CE offers very high efficiencies; thus, with the ability to separate a large number of components per unit time, there may be less need to use extractions to remove interferents. Under certain conditions, it is indeed possible to develop assays in which a biological sample may be injected directly onto the capillary without any pretreatment. Clearly, this situation is preferable in terms of time and effort, but direct injection assays are probably the exception rather than the rule. Without sample pretreatment, interferences often will occur when analyzing complex samples such as urine, and in many cases the sensitivity of the detector is inadequate without preconcentration. There are, therefore, many occasions where some form of sample preparation is needed. Figure 1 illustrates a possible process for deciding what route to take in developing a new assay. The potential savings of time and effort with direct injection methods are attractive, and often worth investigating.

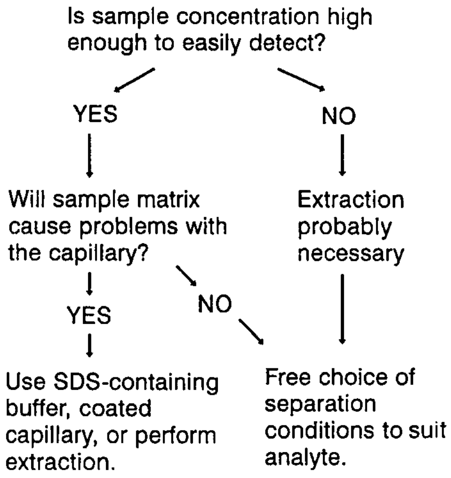

Fig. 1. Decision-making tree—can a direct injection assay be attempted?

In CE, certainly with commercial instrumentation, although the volumes injected are in the low nanoliter range, a few microliters of sample are needed for injection—a total sample amount not dissimilar to that normally injected in HPLC. Thus, in many cases, the techniques used for sample preparation for HPLC may be applied to CE with little modification. When there is abundant sample , *e.g.,* urine, and often plasma, this is a perfectly reasonable approach—after all, larger volumes are generally easier to handle than small ones. However, an area of great potential for CE is analyses in sample-limited situations. In such cases, it may be necessary to develop either microscale sample preparation techniques or assay methods where pretreatment is minimized or eliminated. Some methods of sample pretreatment which were developed for use in HPLC are now widely used for CE; these will be described only briefly here. Detailed discussions of these techniques can be found in a variety of articles such as those by McDowall (1989) and Lingemann and Tjaden (1990).

2. Removal of Particulate Matter

An almost indispensable step prior to CE analysis is removal from the sample of particulate matter which may block the capillary. This is usually the last stage of any sample preparation scheme. A microcentrifuge may adequately remove particulates in some samples. Centrifuge filters (0.2 or 0.45 μm) are useful for cleaning up microliter samples, with the added advantage over syringe filters that numerous samples can be filtered at one time.

3. Sample Cleanup and Concentration Off-Line

One of the most important reasons for performing sample preparation in CE is to concentrate the analyte(s) of interest. This is often necessary because of the limited concentration sensitivity of the most commonly used detection method for CE, UV absorbance. Typically, UV limits of detection fall in the 100 ng ml^{-1} to 10 μg ml^{-1} range. This is a limiting factor in many situations. For example, in pharmacokinetic assays and therapeutic drug monitoring, drug and metabolite concentrations are usually below this range. If the analyte is naturally fluorescent or electroactive and a suitable detector is available, achieving adequate sensitivity may not be a problem, and biological samples may be directly analyzed without preconcentration.

To achieve sample preconcentration, an extraction step is usually performed, followed by evaporation of the extraction solvent and reconstitution in a volume smaller than that of the original sample. A potential bonus may be the removal of other components in the sample. This approach has been used in a number of CE assays with either solid-phase extraction (Lloyd *et al.*, 1991; Wernly and Thormann, 1991) or liquid-liquid extraction (Meier and Thormann, 1991; Prunonosa *et al.*, 1992). These techniques were used in essentially the same way as in preparing samples for analysis by HPLC (McDowall, 1989; Lingemann and Tjaden, 1990). At least tenfold concentration of the analyte is usually possible, provided that there is adequate sample available.

In principle, there is little difference in the choice of such extraction procedures whether they are to be used for CE or HPLC. The smaller sample requirement for CE means that it may be possible to perform the whole extraction procedure with less sample, or that greater concentration can be achieved by reconstitution in smaller volumes. However, problems may occur which limit the degree to which such methods may be scaled down; for example, adsorption of the analyte onto supporting materials used in the extraction is likely to be more problematic in miniaturized system, and evaporation may also cause difficulties. Judicious choices of orthogonal extraction and separation steps can minimize problems with interferences (*e.g.*, extraction of hydrophobic compounds using a reversed-phase cartridge, followed by CE separation of ionic species).

The tendency of proteinaceous material to adsorb onto the walls of fused silica capillaries is well documented (Towns and Regnier, 1992; Wang and Hartick, 1992). Thus, proteins must either be removed from the sample prior to injection by a pretreatment step or a method to reduce adsorption and thus stabilize electroosmosis must be employed in order to achieve reproducible analyses. Reduction of adsorption is dealt with in a later section. Proteins may well be responsible for interferences in the analysis, and for this reason, too, their removal may be advantageous.

A variety of deproteinization methods are used for HPLC sample preparation and can also be employed in CE. Both solid-phase and liquid-liquid extractions can effectively remove proteinaceous components from biological fluids, and have the advantage of allowing sample concentration as well. Precipitation methods are also widely used in HPLC. Acids are quite effective precipitating agents; 0.4 ml 10% w/v trichloroacetic acid per ml of plasma precipitates >95% proteins. To achieve the same result, 0.8 ml of perchloric acid (6% w/v) is needed. Dilution of the sample is not excessive, a favorable point for these agents. Organic solvents can also be effective, but a 2:1 organic:plasma ratio is needed for acetonitrile and acetone to achieve 95% precipitation of proteins, and ethanol and methanol are even less efficient. Problems which may occur with organic solvents are excessive dilution, and alterations in resolution and peak-shape in MECC separations (Burton and Sepaniak, 1986). If the proteins themselves are the analytes of interest, it may be possible to precipitate them first using an organic solvent and then to reconstitute the precipitate in a medium suitable for injection into the electrophoresis system (Gurley *et al.*, 1991).

Ultrafiltration is another effective method for removing proteins from biological samples, and it has been applied for the preparation of plasma samples prior to CE analysis (Schoots *et al.*, 1990; Shibukawa *et al.*, 1993). This is a very simple method of sample preparation, and usually allows analytical methods developed using standard samples to be directly applied to biofluids. Only non-protein-bound small molecules pass through the filter, unless bound compounds are released (*e.g.*, using urea) before filtration. There are no beneficial concentration effects (although no unwanted dilution either), so adequate detection sensitivity must be available unless on-column concentration is possible. The other main objection to this method is that filters are rather expensive.

4. On-Capillary Chromatographic and Electrophoretic Concentration

In addition to achieving concentration in an extraction step, desalting of the sample may also be accomplished. An exception may be when a buffered wash is needed to elute the analyte from a solid phase column. The removal of electrolytes from the sample can be helpful because with a desalted sample it may be possible to use on-capillary peak-stacking to further increase sensitivity for ionic analytes (Chien and Burgi, 1992). Stacking occurs when the sample is injected in a matrix of lower conductivity than the separation buffer. Since the current is constant throughout the capillary, a local enhancement of the field strength occurs in the sample zone due to the conductivity differences. Thus, ion velocities are increased in the sample region, and the analyte ions quickly migrate from the sample zone into the separation buffer where the velocity is reduced because of the lower field strength. Ions which are initially further from the boundary migrate faster for a longer period of time and overtake the ions which were originally near the boundary; thus, a concentrating effect occurs. The degree of concentration which may be achieved is limited, since mixing effects occur because of local differences in the electroosmotic flow velocity between the sample zone and buffer.

A problem which may arise when using the stacking method is thermal degradation of sample. There is considerable excess heating in the sample zone relative to the background electrolyte because of its higher resistivity (Vinther and Soeberg, 1991). Stacking can be used quite effectively to increase sensitivity for desalted extracts of biological samples. In a validated assay for the antileukaemic agent cytosine-β-D-arabinoside, desalting was achieved (along with concentration) by solid phase extraction (Lloyd *et al.*, 1991). Analysis was performed with 7% (28 nl) of the effective separation length of the capillary initially filled with sample. The detected peak half-width volume after separation was only 3.6 nl (Fig. 2).

An enhanced field in the sample zone is necessary in order to perform sample-stacking as described above, so this method is not readily applicable to body fluids which have not been desalted. However, by judicious choice of buffer ions it may be possible to perform a transient on-capillary sample concentration step by isotachophoresis (ITP) (Foret *et al.*, 1992; Gebauer *et al.*, 1992) (coupled-column ITP-CZE systems will be considered later). Body fluids have high concentrations (hundreds of mM) of sodium and chloride ions, as well as millimolar concentrations of a number of other small, highly mobile cations and anions. This can fortuitously give rise to suitable conditions for a transient isotachophoretic concentration of less mobile organic ions in the sample during the initial part of a separation. This is illustrated in Fig. 3, which shows the migration of a minor component X^- in the presence of a large excess of a more mobile component A^-, both of which are anions of strong acids and are thus unaffected by small pH changes. If a suitable leading ion is not present, it can be added. The background electrolyte consists of anion B^- of a strong acid, and cation RH^+ of a weak base. For isotachophoretic

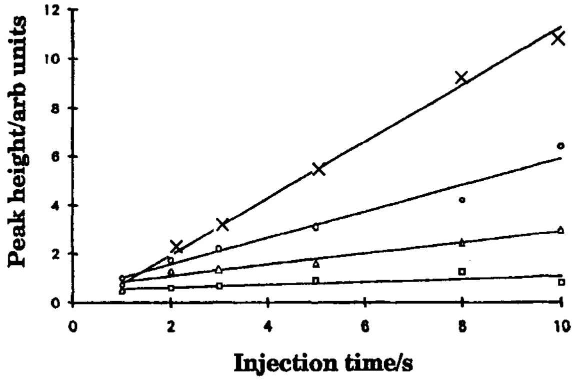

Fig. 2. Peak-stacking of ara-C analysed by CZE with a field strength of 577 V cm^{-1} using a 20 mM citrate buffer (pH 2.5) with 0 (×), 10 (○), 20 (△) and 40 mM NaCl (□) added to the sample. When the buffer conductivity is higher than the sample conductivity, peak stacking occurs; with aqueous samples the stacking effect is linear up to injection times of 10 s (injected volume 28 nl).

concentration of X^- to occur, the relative mobilities of the anions must be $\mu_A > \mu_X > \mu_B$; the buffer co-ion is the terminator, and the high-concentration sample co-ion is the leader. The starting situation is shown in Fig. 3a. After current is applied, the sample migrates out of the starting zone (past position 0, Fig. 3b). There is a region of constant concentration of A^- and X^-, and a fronting of the more highly mobile A^- as it penetrates the background electrolyte. A narrow concentrated zone of the intermediate mobility X^- begins to form behind its leader, A^-. As the zone containing A^- continues to expand, the region of constant concentration diminishes and eventually disappears, while stacking of X^- behind A^- is completed. This process is almost complete in Fig. 3c. The transition of isotachophoretic migration between B^- and A^- to zone electrophoresis in B^- occurs for X^- when A^- separates from it; the rest of the analysis then proceeds with X^- migrating as an isolated zone (Fig. 3d). With more than one minor component of interest, each component separates to form an isolated zone in order of increasing mobility. An alternative system would be one where the background electrolyte contained a co-ion with a higher mobility than the sample ions. In that instance, a

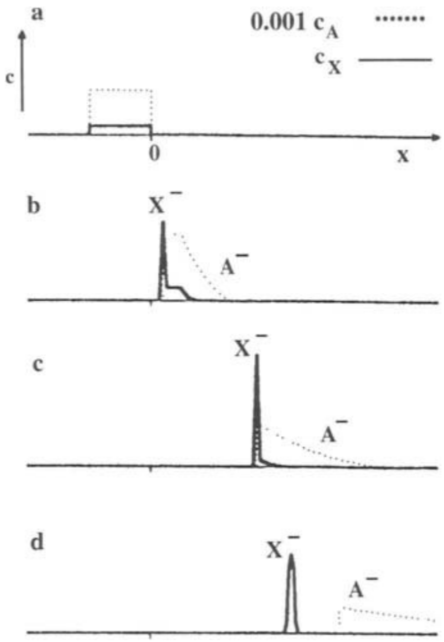

Fig. 3. Isotachophoretic concentration of a trace, low-mobility anionic component X^- in the presence of an excess of high-mobility A^- in the sample. a) Starting condition; b) concentration of X^- behind A^- and penetration of A^- into background electrolyte; c) continuation of process seen in b, separation of A^- and X^- about to occur; d) independent migration of A^- and concentrated X^-. (Reprinted with permission from Gebauer *et al.*, 1992.)

low-mobility terminator is used for a short period after sample injection, and sharpening of the sample zones occurs. This is then changed for the background electrolyte, after which the sample ions detach from the terminator zone.

Transient isotachophoretic concentration was demonstrated in the analysis of uraemic serum by Schoots *et al.* (1990), and in the analysis of urinary porphyrins by Weinberger *et al.* (1990). Figure 4 shows electropherograms of ultrafiltered serum samples diluted 40-fold with

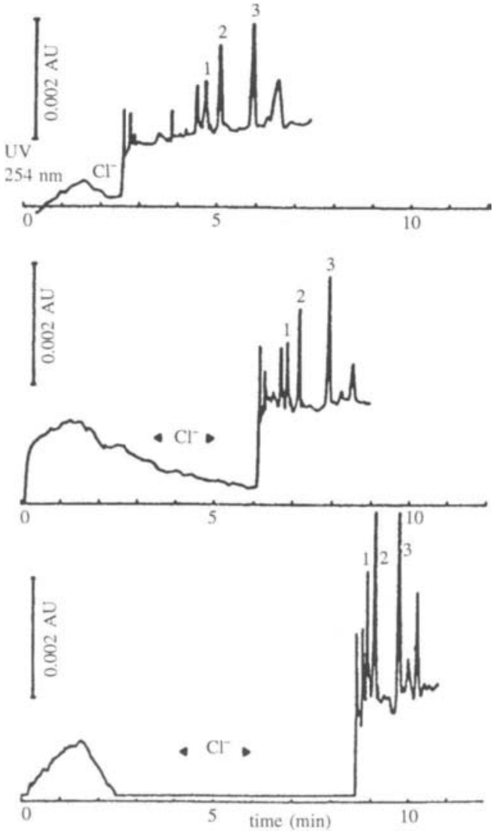

Fig. 4. Separation of 1, hippuric acid; 2, *p*-hydroxyhippuric acid; and 3, uric acid (tentative identifications) in 40-fold diluted, unfiltered uraemic serum with (top) 2.8 mM, (middle) 13.2 mM and (bottom) 54.4 mM NaCl in sample. Electrolyte, 0.01 M MES/histidine, pH 6.1 with 0.5 g l^{-1} methylhydroxycellulose. (Reprinted with permission from Schoots *et al.*, 1990.)

water (a) giving a total Cl^- concentration of 2.8 mM, and with different amounts of added sodium chloride resulting in total Cl^- concentrations of 13.2 mM (b) and 54.4 mM (c). Peak heights of the separated components increase with increasing Cl^- concentration, although migration times also increase and resolution decreases. With the higher added salt concentrations, the sample ions spend increasing amounts of time migrating with the Cl^- before separating to form individual zones. In Fig. 4 it can be seen that with higher added Cl^- concentrations the separated zones are considerably compressed. This also illustrates that variable degrees of concentration may occur if the electrolyte content of the sample matrix is not controlled. One advantage of stacking or transient ITP concentration is that it is performed using unmodified CE instrumentation. Preconcentration and selectivity can also be obtained on-capillary by chromatographic means. A small plug of chromatographic stationary phase a few millimeters long is packed into the separation capillary (usually near the column inlet) and secured by sintered frits on each side. The use of C-18 (Swartz and Merion 1993) and antibody-conjugated (Guzman et al, 1991) packings has been reported. As an alternative to using packed capillaries, small sections of the capillary wall may be coated with an adsorbent phase (Cai and El Rassi, 1992). This approach has the advantage of simplicity, and there is less chance of blockage occurring. However, capacity is limited due to the relatively small surface area coated with adsorbent, and the degree of sample concentration obtainable may not be quite as high as in packed systems.

With preconcentration capillaries, a large-volume injection is performed, using a buffer which allows the adsorption of the analytes of interest onto the stationary phase. Electromigration injection may be used; hydrodynamic injection is suitable for open-tubular systems and also packed systems if adequate flow rates can be achieved in the concentrator capillary. A wash step with run buffer usually follows, and the sample is then eluted from the chromatographic phase by injecting a short plug of solvent/buffer sufficient to cause a rapid dissociation of the analytes from the stationary phase. It is important that desorption occur as quickly as possible, since slow desorption will lead to considerable band-broadening. The plug length and type of elution solvent are experimental variables to be optimized. A drawback of this type of preconcentrator is that overloading of the stationary phase could occur if it is used with biological samples which have not already undergone extensive pretreatment. This may be a lesser problem with more analyte-specific concentrators such as antibody-conjugated phases, where direct sampling of urine has been demonstrated (Guzman *et al.*, 1991).

5. Coupled Systems

A variety of coupled electrophoretic-electrophoretic and chromatographic-electrophoretic systems have been developed. These range from sample preparation devices giving a simple sample cleanup step followed by an electrophoretic analysis, to complete two-dimensional separation systems with high resolution analyses in both dimensions (Bushey and Jorgenson, 1990). Microdialysis or capillary ultrafiltration with CE separation are other coupled systems which offer on-line analysis *in vivo.* These are described elsewhere in this volume.

Coupled ITP-CE systems use a preconcentration capillary in which the ITP step takes place, connected by some form of T-joint to a second capillary for analytical CZE (Kaniansky and Marak, 1990; Foret *et al.,* 1992; Stegehuis *et al.,* 1991, 1992). In many ways, ITP is an ideal preparation step before CZE because ions are separated into sharp zones suitable for injection into the zone electrophoresis separation system, and matrix components in high excess can be separated from the analyte zones. Using a coupled-column system it is relatively easy to optimize the electrolyte components for both the ITP and zone electrophoresis (ZE) steps, a task which is not so simple in the transient on-column approach. Greater concentration can be achieved in coupled-column than transient on-column systems; indeed, the injected sample volume on the preconcentration capillary may exceed the entire volume of the analytical capillary. The price paid for this extra flexibility and better performance is greater instrumental complexity. Figure 5 is a schematic diagram showing how a coupled ITP-CE system works. Isotachophoresis takes place in the first capillary, which is typically made of polytetrafluoroethylene (PTFE) tubing a few hundred μm in diameter. An injection valve or port is placed at the inlet end. The second capillary may be of similar material and dimensions, or it may be a smaller diameter fused silica capillary, as shown here. Two current paths are possible, via the outlet ITP system or the outlet of the CZE capillary, and two detectors are needed. The first (often a conductivity detector) is placed on the ITP capillary, just in front of the inlet to the ZE capillary, to monitor the arrival of the sample zones at the junction. This is used to determine the time at which the ITP process should be stopped and the sample introduced into the CZE system. The detector on the ZE capillary is used to quantitate the separated analytes.

Initially, the ITP capillary is filled with leading electrolyte and the ZE capillary with a buffer suitable for the ZE separation. The leading electrolyte may contain additives such as Triton X-100 or hydroxypropylmethylcellulose to minimize electroosmosis and suppress adhesion of macromolecules onto the tube wall. The sample is introduced into the ITP capillary and a current is passed through it, causing the

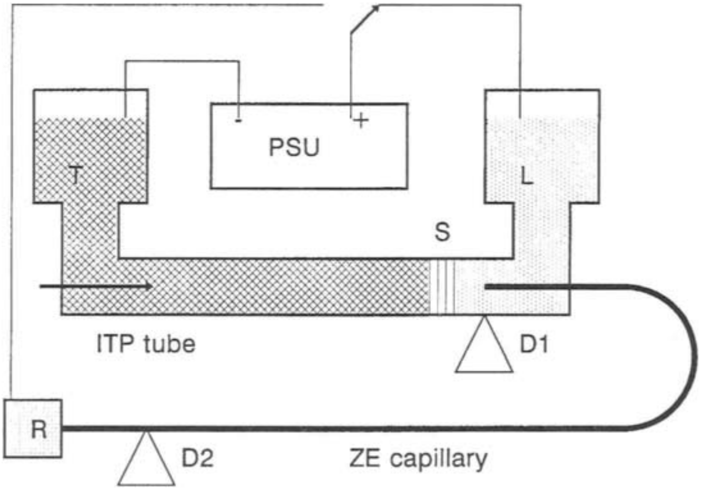

Fig. 5. Schematic diagram of a coupled ITP-CZE system. T, terminating electrolyte; L, leading electrolyte; S, sample; R, CZE electrolyte reservoir; D1, D2, detectors; PSU, power supply.

sample components to separate into discrete bands and migrate down the capillary. Unwanted components may migrate past the first detector, and the ITP process is stopped when the desired region of the analyte reaches the detector. These must then be transferred onto the ZE capillary. This can be achieved either by electrophoretic migration, transferring the electric potential to the ZE capillary, or by hydrodynamic sampling. Other unwanted matrix components are then allowed to pass the junction point before electrophoresis in the second capillary commences.

The degree of concentration possible in such systems is quite impressive. Foret *et al.* (1992) were able to preconcentrate protein samples from 1 μg ml^{-1} to 20 mg ml^{-1} in the ITP step. Stegehuis *et al.* (1992) presented quantitative data for a coupled system and reported a 1000-fold concentration. For fluorescein isothiocyanate (FITC)-derivatized angiotensin III the RSD in measured peak area was 7% when a 5 μg ml^{-1} sample was directly injected onto the separation capillary, while it was 2.9% when a 10 μl injection of the same compound at a concentration of 5 ng ml^{-1} was made onto the preconcentration system. Similar results were obtained for inter-day measurements. This group showed the application of the preconcentrator system to analyse FTIC-derivatized angiotensin III in deproteinized plasma samples.

As in electrophoresis-electrophoresis, the coupling of chromatographic and electrophoretic systems is in its infancy, and also shows considerable promise. A high degree of automation should also be possible with LC-CE systems, giving a potential significant advantage over off-line extraction methods. This type of design has advantages over on-capillary chromatographic concentrators. The chromatographic phase could be enclosed in an easily replaceable cartridge to lessen problems due to blockages and limited lifetimes, and to make quick changes between different types of chromatographic phases and capillaries easier. Debets *et al.* (1992) described an elegant device in which the chromatographic packing was contained within a small volume switching valve. This was then used in connection with either a micro-LC system or CE with a 200 μm capillary. The precolumn itself was composed of 8 μm diameter reversed-phase packing in tubing 1.5 mm × 500 μm i.d. which was inserted into the valve rotor. The valve was made of insulating materials, allowing direct connection into the CE system. This flexible arrangement allows simple washing, conditioning and sample loading onto the stationary phase. It was found that in CE the best results were obtained when the sample was switched on line with the capillary and desorbed with the electrical potential reversed from the separation condition. After partial desorption, the precolumn was switched out of line, and the electric field reversed again to the separation condition. Desorption with the potential applied in the forward direction led to excessive peak broadening, due to slow desorption of the sample (Fig. 6).

6. Direct Injection of Biological Fluids

The development of methods in which biological fluids are injected directly onto the capillary is of considerable interest, since the time taken and possible errors involved in extraction are eliminated. To achieve a successful direct injection assay, a number of criteria must be met. First, the analyte must be present at sufficient concentration for its quantitative determination with whatever detection system is available. Adequate resolution of the analyte of interest must be possible, and the sample matrix must be free of components capable of modifying the capillary surface. These last two points are interrelated, since buffer additives used to stop fouling of the capillary surface may not be compatible with successful resolution of the target compounds. If the analyte is protein-bound, the effect of the separation buffer on the binding must also be determined.

Direct analysis of urine samples can be relatively straightforward, provided the analytes of interest are present at sufficiently high concentrations. However, potential problems include interferences due

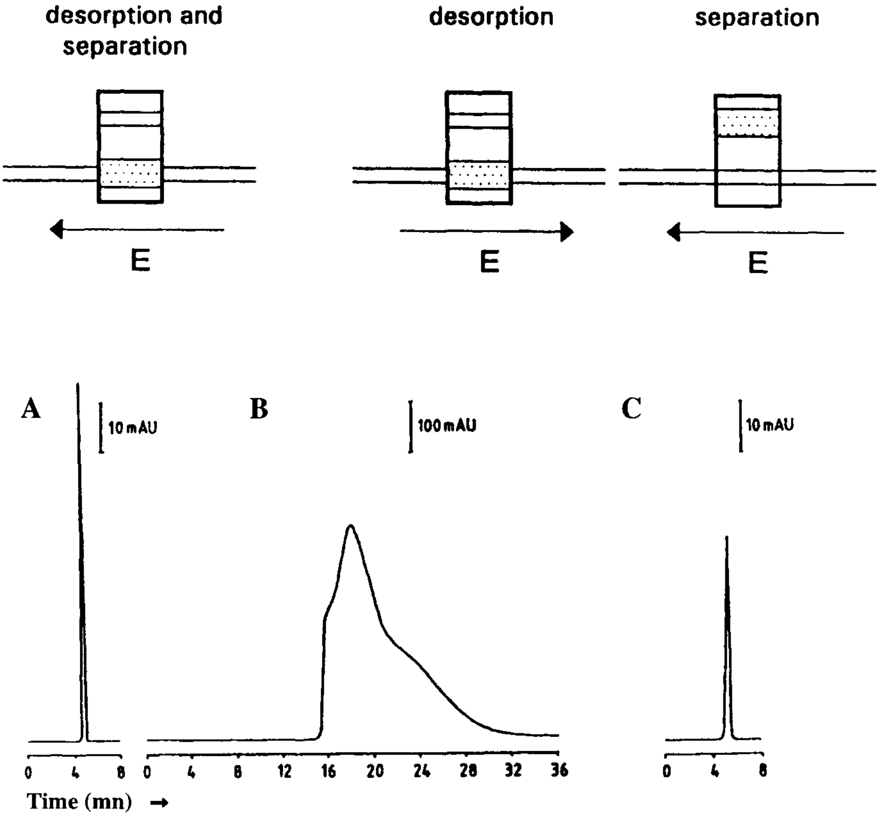

Fig. 6. Electropherograms of papaverine (0.1 mM in water-acetonitrile, 95:5, v/v) (A) after direct injection of 0.2 μl sample; (B) after 5 μl injection with valve (above left) switched to the position with the precolumn (dotted) on-line with the separation capillary during the separation, and the same field direction (E) during desorption and separation; and (C) 5 μl injection (10 μM papaverine) with field reversed for desorption (valve switched to precolumn on-line position, center), then rotation of valve straight through position, right) and normal field direction for separation. (Adapted with permission from Debets *et al.*, 1992.)

to the large number of components, demanding high resolution and good reproducibility. For example, in complex urine samples, the desire for increased sensitivity using UV detection at low-wavelengths may be incompatible with the requirements of detection selectivity using longer wavelengths. Urine also presents a particularly problematic matrix in terms of variability of its composition. One favorable characteristic of urine is a (normally) low protein content, so fouling of the capillary surface is generally negligible.

Examples of direct analysis of urine include the quantitation of dextromethorphan (DX) and its metabolite dextrorphan (DR) by CZE (Li *et al.*, 1993), and the determination of the caffeine metabolites 1-methylxanthine and 5-acetylamino-6-formylamino-3-methyluracil by MECC (Lloyd *et al.*, 1992). These methods, which involved minimal sample preparation, were developed as simplified, rapid ways to determine metabolic phenotypes. DX and DR are easily resolved from each other and from other urine components as cationic species in a pH 9.3 borate buffer. In this case, the excellent resolution obtained allowed detection at 200 nm for maximum sensitivity. A spot urine sample is collected 4 h after a single oral dose of 15 mg DX. An ideal phenotyping method would measure glucuronides as well as the parent compounds, but using CE/UV, sensitivity is too limited to allow this option. Therefore, as is common with HPLC assays, an enzymatic deconjugation was first performed. A high concentration run buffer (175 mM) was chosen to give a certain amount of on-capillary peak stacking. Separation efficiency was found to increase with increasing buffer concentration, up to a maximum around 0.15–0.175 M. By using high buffer concentrations, some stacking was achieved even for urine samples with high salt content. Detection limits of 80 ng ml^{-1} were obtained for both DX and DR. The use of high concentration buffers is most practical in situations such as this where cationic species are analyzed. This is because the magnitude of the applied field strength is limited by Joule heating, and also because both the electroosmotic flow and analyte mobilities are reduced with increasing buffer concentration. For anion separations or MECC, where analytes and electroosmotic flow are moving in opposite directions, the overall velocity could become unacceptably low.

One strategy for the direct introduction of plasma onto a HPLC column is to add a surfactant such as sodium dodecyl sulfate (SDS) to the mobile phase; this binds the plasma proteins and, in doing so, denatures and solubilizes them (Grohs *et al.* 1991). Nakagawa *et al.* (1988, 1989) showed that MECC is naturally suited to the analysis of plasma components for precisely the same reason as in HPLC. The presence of the surfactant minimizes interactions of the proteins with the capillary walls. They found that by using 10 mM SDS in a phosphate buffer as the run buffer, unextracted plasma could be directly injected onto fused-silica capillaries. The coefficients of variation for the electroosmotic flow velocity and for the migration velocities of the analyte and internal standard (cefpiramide and antipyrine, respectively) were less than 1.2% when the capillary was rinsed between separations with 1 M NaOH followed by run buffer, and 0.5% or less when the capillary was rinsed just with the run buffer between separations (7 consecutive injections). Even in the presence of SDS there may be some slight modification of the capillary surface by proteins. Lloyd (1992) found that over a sequence of 20 separations with direct plasma injection the electroosmotic flow velocity slowly decreased, before eventually stabilizing after about the 20th analysis. These separations were performed on a capillary that had previously been used for other CE separations, but never for direct injection of plasma. For these experiments, a phosphate buffer containing 170 mM SDS was used, and the capillary was rinsed only with this background electrolyte between runs.

To date, CE methods with direct plasma injection have gained only limited attention, examples being analysis of aspoxicillin (Nishi *et al.,* 1990), substituted purines (Thormann *et al.,* 1992) and the diastereoisomers of L-buthionine-(R,S) sulfoximine (Lloyd, 1992). Experience in this laboratory with direct injection methods for plasma samples indicates that validated analyses can be developed (Lloyd, 1992), but are not without difficulties. Occasional problems with capillary blockage do occur, even when plasma has been prefiltered through a 0.45 μm membrane. This difficulty seems to be sample-dependent; most plasma samples cause few blockages, but a few consistently cause blockages. Use of an internal standard is most helpful in determining when incomplete injections have occurred due to partial occlusion of the capillary.

A further point to note when using SDS is that the various commercially available grades have different UV absorbance properties, and that some may have unacceptably high absorbances at low wavelengths, leading to poorer than expected sensitivity. SDS sold as "molecular biology" grade by some manufacturers tends to have low absorbance. So far only SDS has been successfully applied for direct-injection plasma assays using fused silica capillaries. Related shorter-chain surfactants such as hexanesulfonic acid or other denaturing agents such as urea are ineffective in reducing protein adhesion to the capillary walls (Lloyd, 1994). The need to use SDS can be somewhat limiting when trying to analyse very hydrophobic compounds, or cationic solutes which are highly associated with the micelle. For neutral analytes, 25–50 mM SDS with a pH 7 phosphate buffer offers a useful starting point for direct injection separations of plasma. The same conditions are often suitable for low-mobility anions, while highly mobile anionic species generally require higher SDS concentrations (*e.g.*, >100 mM).

Instead of using bare fused silica capillaries and buffer systems capable of inhibiting protein adhesion, capillaries coated to reduce protein adhesion provide another option for direct injection of biological fluids. Since the coating rather than any additive is responsible for the elimination of solute-wall interactions, there are fewer constraints on the composition of the background electrolyte. Numerous coatings have been developed, mostly to overcome the problem of protein adhesion, and these could potentially be used advantageously for direct analysis of biological fluids with significant protein content. These capillaries are discussed in greater detail in Chapter 2. This is exemplified by the separation of serum proteins by capillary isotachophoresis with polyacrylamide-coated tubes reported by Hjertén and Kiessling-Johansson (1991). Using coated capillaries, reproducible electropherograms of serum were obtained; with bare fused-silica capillaries, resolution and reproducibility were extremely poor. Considering the rapid developments being made in coating technology, coated capillaries could well find many applications in the direct analysis of biofluids in the future.

7. References

Burton, D. E. and M. J. Sepaniak (1986). *Chromatographia, 21*, 583–586.

Bushey, M. M. and J. W. Jorgenson (1990). *Anal. Chem., 62*, 978–984.

Cai, J and Z. El Rassi (1992). *J. Liq. Chromatogr., 15,* 1179–1192.

Chien, R.-L. and D. S. Burgi (1992). *Anal. Chem., 64*, 489A–496A.

Debets, A. J. J., M. Mazereeuw, W. H.. Voogt, D. J. van Iperen, H. Lingeman, K-.P. Hupe and U. A. Th. Brinkman (1992). *J. Chromatogr., 608*, 151–158.

Foret, F., E. Szoko and B. L. Karger (1992). *J. Chromatogr., 608*, 3–12.

Gebauer, P., W. Thormann and P. Boček (1992). *J. Chromatogr., 608*, 47–57.

Grohs, R. A., F. V. Warren and B. A. Bidlingmeyer (1991). *Anal. Chem., 63*, 384–390.

Gurley, L. R., J. S. Buchanan, J. E. London, D. M. Stavert and B. E. Lehnert (1991). *J. Chromatogr., 559*, 411–429.

Guzman, N. A., M. A. Trebilcock and J. P. Advis (1991). *J. Liq. Chromatogr., 14*, 997–1015.

Hjertén, J. and M. Kiessling-Johansson (1991). *J. Chromatogr., 550*, 811–822.

Kaniansky, D. and J. Marak (1990). *J. Chromatogr., 498*, 191–204.

Li, S., K. Fried, I. W. Wainer and D. K. Lloyd (1993). *Chromatographia, 35*, 216–222.

Lingeman, H. and U. R. Tjaden (1990). In: *Detection-Oriented Derivatization Techniques in Liquid Chromatography* H. Lingeman and W. J. M. Underberg, Eds.), pp. 85–151. Marcel Dekker, New York.

Lloyd, D. K., A. M. Cypess and I. W. Wainer (1991). *J. Chromatogr., 568*, 117–124.

Lloyd, D. K. (1992). *Anal. Proc., 29*, 169–170.

Lloyd, D. K., K. Fried, K. and I. W. Wainer (1992). *J. Chromatogr., 578*, 283–291.

Lloyd, D. K. (1994). *Methodological Surveys in Biochemistry and Analysis, 23*, in press.

McDowall, R. D. (1989). *J. Chromatogr., 492*, 3–58.

Meier, P. and W. Thormann (1991). *J. Chromatogr, 559*, 505–513.

Nakagawa, T., Y. Oda, A. Shibukawa and H. Tanaka (1988). *Chem. Pharm. Bull, 36*, 1622–1625.

Nakagawa, T., Y. Oda, A. Shibukawa, H. Fukuda and H. Tanaka (1989). *Chem. Pharm. Bull, 37*, 707–711.

Nishi, H., T. Fukuyama and M. Matsuo (1990). *J. Chromatogr.,* 515, 245–255.

Prunonosa, J., R. Obach, A. Diez-Cascon and L. Gouesclou (1992). *J. Chromatogr., 574*, 127–133.

Schoots, A. C., T. P. E. M. Verheggen, P. M. J. M. De Vries and F. M. Everaerts (1990). *Clin. Chem., 36*, 435–440.

Shibukawa, A., D. K. Lloyd and I. W. Wainer (1993). *Chromatographia, 35*, 419–429.

Stegehuis, D. S., H. Irth, U. R. Tjaden and J. van der Greef (1991). *J. Chromatogr., 538*, 393–402.

Stegehuis, D. S., U. R. Tjaden and J. van der Greef (1992). *J. Chromatogr. 591*, 341–349.

Swartz, M. E. and M. Merion (1993). *J. Chromatogr., 632*, 209–213.

Thormann, W., A. Minger, S. Molteni, J. Casiavska and P. Gebauer (1992). *J. Chromatogr., 593*, 275–288.

Towns, J. K. and F. E. Regnier (1992). *Anal. Chem., 64*, 2473–2478.

Vinther, A. and H. Soeberg (1991). *J. Chromatogr., 559*, 27–42.

Wang, T. and R. A. Hartwick (1992). *J. Chromatogr., 594*, 325–334.

Weinberger, R., E. Sapp and S. Moring (1990). *J. Chromatogr., 516*, 271–286.

Wernly, P. and W. Thormann (1991). *Anal. Chem., 63*, 2878–2882.

CHAPTER 8

Capillary Electrophoresis in the Study of Amino Acids

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1. Introduction

1.1. Perspectives on Amino Acid Analysis

The large quantity of literature devoted to the development of analytical methods for the separation and quantitation of amino acids, as well as the variety of commercial analyzers and sequencers, attests to the importance of this field. Regardless of the type of instrumentation employed, these methods have been developed to analyze two basic different types of solubilized samples:

a) endogenous solutions of amino acid mixtures, often of biological origin, or
b) peptide or protein products such as hydrolysates or amino acid derivatives obtained from sequencing reactions like that used in the Edman degradation.

Peptide or protein products contain primarily only the 20 common amino acids or their derivatives and thus represent a narrower range of amino acid structures than are encountered in endogenous samples. Note that over 300 additional amino acids not found in proteins have been observed in cells, where they posses a variety of functions. For example, ornithine and citrulline are important metabolic intermediates, while γ-aminobutyric acid (GABA) is an intensely studied neurotransmitter.

Thus, methodologies developed for peptide and protein product analyses tend to be well established with respect to efficient separations and reproducible and accurate quantitation. The goal in this arena has been more sensitive methods, so that smaller amounts of peptide or protein may be analyzed with equal precision and accuracy. Indeed, it has been stated that if femtomole amounts of protein could be sequenced, rather than the picomole amounts currently required, new fields of research would be established (Smith, 1988).

On the other hand, endogenous solutions often contain amino acids mixed with a wide variety of components in the sample matrix (*e.g.*, urine, plasma or cerebrospinal fluid). Separating the non-amino acid components, such as protein, from the desired pool of amino acids can be a challenge. Also, one is often interested in the quantitation of the members of a select group of amino acids. Examples include the phosphorylated, sulfated and sulfonated amino acids, as well as amino acids that are not α-amino acids, such as GABA. These members may have properties quite similar to each other (such as size or charge) yet quite different from common amino acids. Existing methods of analysis, like those used for composition of proteins, may be altogether inappropriate. Thus, the problems faced in studying these types of samples are ones of efficient separation coupled to sensitive detection of selected amino acid analytes.

1.2. Current Methods—A Comparison of Liquid Chromatographic (LC) and Capillary Electrophoretic (CE) Techniques

Because of the success of LC in the routine analysis of amino acid products of peptide or protein origin, there is little advantage in using CE for these applications. Through years of development and refinement, LC methods have been optimized to a point that CE could achieve only through heroic efforts. However, there are a few benefits that CE operation would offer, if all other considerations were equal. Compared to CE, LC does consume large amounts of solvents, particularly organics such as methanol and acetonitrile, and concomitant waste is associated with such use. The cost and environmental benefits of such CE operation probably would not be worth the effort needed to achieve the reproducibility and accuracy attainable by LC methods. Still, CE development in this area does seem promising if the methods developed in LC analysis, including the use of existing derivatization techniques like those used in the Edman method, could be adapted to CE on a smaller scale. The low sample volume and mass requirements of CE analysis, nanoliter volumes containing low femtomole amounts of analyte, would make CE well suited to the analysis of very small amounts of protein. LC has shown little promise of further developments in this respect.

Perhaps the area in amino acid analysis where CE seems to offer the greatest potential for improvement over LC methods is that of endogenous solutions of amino acids, particularly of biological origin. Scientists, as well as medical practitioners, are often interested in particular groups of amino acids of similar size and charge, such as cysteine and homocysteine. Since the basis of LC separation rests primarily on differences in polarity rather than size and charge, resolution of such amino acid mixtures is inherently difficult. On the other hand, the simplest form of CE, capillary zone electrophoresis (CZE), separates analytes on basis of their effective size and charge and is thus ideally suited to such a task. Compared to LC, CE exhibits higher efficiencies and provides much easier means to explore different separation conditions for optimization. Also, CE runs generally take less time, especially compared to gradient LC, and may be repeated since only nanoliter volumes are used for each injection rather than the microliter volumes required in LC.

Thus, CE is emerging as a technique complementary to traditional LC methods for the analysis of amino acids. It may find its greatest practical application in certain types of analyses where select groups of amino acids need to be quantitated.

2. Methods for the Detection of Amino Acids

When considering how to employ CE for the analysis of an amino acid mixture, it is perhaps prudent to consider the method of detection that will be employed before choosing the separation mode to be used. That is, one needs to consider how the molecule will be detected before determining how to separate it from other molecules and interfering substances. This is true because often the detection method involves derivatization, and the derivatized molecule may react quite differently than the original in terms of its behavior in a CE system. Thus, a survey of the common methods employed for the derivatization of amino acids will be presented.

When determining which of the various detection methods might be most useful for a particular separation of amino acids, it helps to keep in mind the distinct advantages and disadvantages of each method. Considerations such as specificity, sensitivity, reproducibility, stability of the derivative, ease of use, and cost need to be evaluated. For example, a number of detection methods are quite sensitive, but require sophisticated and costly equipment that is difficult to use.

2.1. Direct Detection (UV and Electrochemical)

Although detection techniques involving derivatization to enhance detectability form the basis of most systems, there is interest in methods that detect amino acids without derivatization. This stems from the fact that the derivatization process is time-consuming and technically difficult to accomplish on ultramicro samples and is, thus, prone to variability. It also necessarily results in dilution of the sample and is generally unsuitable for *in vivo* analysis. To address these concerns, two methods—direct and indirect detection—have been developed.

A method that is inexpensive and simple to use is direct detection of the amino acids by monitoring the UV absorbance at a wavelength near 210 nm. Amino acids absorb light in a range near this wavelength due to the presence of the α-carboxyl group as well as groups in various side chains, particularly those side chains with aromatic residues. An example of this was reported by Bergman *et al.* (1991), in which electrophoretic direct amino acid analysis of a carboxypeptidase digest at 214 nm revealed sensitivity in the high femtomole range for strongly absorbing aromatic amino acids. However, most amino acids could be detected only in the picomole range. This typifies the major problem associated with this method, lack of sensitivity. This is not surprising in light of the widespread prevalence of substances which absorb UV light in this range and thus give high background absorbance. Even common components of the mobile phase, such as phosphate, may be expected to absorb.

The running buffers employed when using direct analysis generally have pH values significantly different from the pI of the amino acids analyzed (in the pH region between 6 and 7) in order for the amino acids to be electrophoretically mobile and thus separable. For example, a low pH (2.5) was used in the analysis by Bergman *et al.* (1991), but this resulted in poor efficiencies and separations.

Direct detection of amino acids can also be accomplished by electrochemical detection at a copper electrode. In this case, very alkaline conditions, for example, 50 mM NaOH, afforded efficient separation of 11 common amino acids (Ye and Baldwin, 1994). This is a relatively sensitive method of detection for amino acids. Detection limits varied greatly for the 20 common amino acids studied, from 0.8 fmol for tryptophan to 600 fmol for phenylalanine. In general, amino acids with hydrophobic side chains gave poor responses. Amino acids can also be detected electrochemically at a neutral pH (Engstrom-Silverman and Ewing, 1991). Through the use of zwitterionic buffers, detection limits in the low nanomolar range could be obtained for most compounds (Zhou and Lunte, 1995).

Attempts are currently underway to increase the sensitivity of direct detection methods for amino acids. Wu *et al.* (1991) used a photothermal method, laser-induced capillary vibration (CVL), to detect femtomole levels of phenylalanine and tryptophan. Other amino acids were not analyzed and would not be expected to be detectable at these levels. In general, direct detection has not been widely used for CE of amino acids, although for particular applications it may be useful.

2.2. *Indirect Detection (Fluorescence)*

Another strategy used for underivatized amino acids is indirect detection. This method is well demonstrated in a procedure by Kuhr and Yeung (1988). A fluorescent anion is utilized as the main component of the running buffer. The pH of the buffer is adjusted so that all amino acids exist as anions when present in the buffer. Thus, each amino acid displaces the anionic fluorophore and results in a negative peak when monitoring fluorescence intensity. The method is fairly sensitive (20 fmol is detectable after digital filtering) when applied to analysis of a few amino acids, although the resolution is not particularly good. The inferior separation is a result of the high concentration of buffer required to overcome noise levels inherent in the laser used in the procedure. This method, like direct detection, is not particularly selective and its usefulness seems limited.

2.3. *Derivatization Methods (Visible, Fluorescence and Electrochemical)*

It becomes apparent from the previous discussion that detection of underivatized amino acids that are adequately resolved is difficult, if not impossible, to achieve at the levels of sensitivity required by researchers in the modern laboratory. Thus, chemical derivatization of amino acids has been employed in order to generate species that are detectable at low levels, fmol and less, although derivatization necessarily causes some dilution of the sample. Since amino acids by definition contain both a carboxyl and an amino group, derivatization reagents that react with these functional groups are candidates for labeling amino acids. The specificity and reactivity of the amino group has been previously exploited by users of LC to label amino acids for sensitive detection by that method of instrumental analysis. Quite naturally, these kinds of reagents which react with amine groups have been used to derivatize amino acids for CE. Indeed, the pioneering work of Jorgenson and Lukacs (1981), in which the foundations of CE were laid, demonstrated the separation of some amino acids labeled with a fluorescent tag.

Since that time, fluorescent tags for amino acid detection in CE have become popular for a number of reasons. Fluorescent compounds can be detected with great sensitivity with relatively simple equipment. If a laser-induced fluorescence system is used, further gains in sensitivity are achieved. On the other hand, when high sensitivity is not required, and cost as well as ruggedness become important factors, fluorescent compounds can also be monitored by simple UV/visible absorption. For example, Guzman *et al.* (1992) used 214 nm detection of fluorescamine-labeled proline and hydroxyproline to improve sensitivity 800-fold. Also, since few biological compounds are naturally fluorescent, fluorescence derivatization procedures do not suffer from interference from the sample matrix, which might otherwise decrease sensitivity in real samples.

A variety of derivatizing reagents and techniques for fluorescence detection have evolved. Although derivatization increases sensitivity, it can have a negative effect on separation. Derivatization changes the size, charge and polarity of compounds, often making analytes more similar in these features and thus diminishing the differences in the compounds which facilitate efficient separation. Table 1 lists some of the more common derivatization methods along with various aspects of each that relate to the relative utility of the reagent. The following are a few observations about the information in the table.

The excitation wavelength for each is important since it should match the wavelength of the light source, which can be a particular problem when using laser light sources. Some reagents, such as 3-(4-carboxybenzoyl)-2-quinoline carboxaldehyde (CBQCA), have been designed so that the excitation wavelength of the derivative matches the output of a commercially available helium/cadmium laser. The laser systems in current use suffer from problems related to limited lifetimes and high maintenance. Higashijima *et al.* (1992) have shown that inexpensive, rugged, reliable semiconductor lasers can be employed for CE detection of amino acids labeled with 7-(diethylamino)-coumarin-3-carboxylic acid succinimidyl ester (DCCS) or Azur B.

Since the underlying reason for performing derivatization and fluorescence detection is to achieve greater sensitivity, the limit of detection (LOD) values in the table are of prime interest. However, the manner in which these values were obtained by the experimenters makes the information less comparable than it would seem. That is, many of the LOD values are obtained by derivatizing a relatively high concentration of amino acid and then using the subsequently diluted solution to determine the LOD. Obviously, this dilutes out any interfering substances that may fluoresce, such as unreacted reagent or side-products. In some literature reports, it is difficult to tell whether such a process was used or whether the amino acids were derivatized at the concentrations actually used to determine the LOD. Thus, information on the relative fluorescence of the reagent and the side-products, given in a separate column in the table, should also be considered when evaluating the sensitivity of

Table 1. Comparison of Fluorescence Derivatization Methods for Amino Acids

Reagent/ Derivative	λ_{ex}	λ_{em}	LOD mass	Fluor. Reag.	Rel. Stab.	Ease of Use	References
OPA	350	400	2.8 fmol	no	poor	good	Albin *et al.* (1991)
	365	418	300 fmol				Liu *et al.* (1988)
NDA/CN	420	490	0.8 amol	no	good	good	Ueda *et al.* (1992) Nickerson and Jorgenson (1988)
FITC[1]	490	519	2.6 fmol	yes		fair[2]	Albin *et al.* (1991)
	488		sub fmol				Waldron *et al.* (1990)
	488		10 amol				Cheng and Dovichi (1988)
Fluorescamine	390	450	15.8 fmol	no			Albin *et al.* (1991)
			2.0 amol			poor[3]	Wu and Dovichi (1989)
FMOC	260	305	0.5 fmol	yes[4]	good	fair[4]	Albin *et al.* (1991)
							Pang et al. (1990)
	248		low amol				Chan *et al.* (1993)
CBQCA	442	550	10 amol	no[5]	good	fair[6]	Liu *et al.* (1991)
TBQCA[7]	488	560	low amol	no		fair[6]	Camilleri *et al.* (1994)
Dansyl-chloride							Gozel *et al.* (1987)
DCCS	415		0.1 fmol	yes		fair[8]	Higashijima *et al.* (1992)
Azur B	670		10 pmol	yes		fair[6]	Higashijima *et al.* (1992)

[1] Fluorescein isothiocyanate

[2] Derivatization reaction is slow and amino acids degrade during derivatization

[3] Requires specially machined laser detector

[4] Fluorescent reagent extracted with pentane or reacted with 1-aminoadamantane

[5] Shows multiple reagent peaks when use at high concentrations

[6] Derivatization reagent must be synthesized by user

[7] 3-(4-Tetrazolebenzoyl)-2-quinolinecarboxaldehyde

[8] Requires a reaction time of one day

a derivative. Finally, the ease of use of the method and the relative stability of the derivatized amino acid are factors which must be considered in any real application.

There is no ideal reagent among those listed in Table 1. 9-Fluorenylmethoxycarbonyl (FMOC) has the advantage that it is one of the few reagents that reacts with secondary as well as primary amines, but, as indicated in Table 1, it is somewhat difficult to use. Naphthalene-2,3-dicarboxaldehyde (NDA) reacts only with primary amines, but exhibits several desirable characteristics (deMontigny *et al.*, 1987). Using a relatively simple method, one can make derivatives that are stable and detectable in small amounts (Lunte and Wong, 1990). Also, the reagent itself is not fluorescent, although it must be of high purity when analyzing samples containing low concentrations of amino acids. Not too surprisingly, NDA has been used in the analysis of a number of samples of biological origin (see Section 3).

It should be noted that the FITC derivatives have been developed as a means of analyzing the products of the Edman degradation scheme used commercially in sequencing proteins. Along these lines, Waldron and Dovichi (1992) have developed a thermooptical detection scheme for the determination of nonfluorescent phenylthiohydantoin (PTH) derivatives of amino acids. Although the equipment is complicated to implement, good resolution with detection limits in the 0.2–5.0 fmol range for 20 common amino acids is obtainable. Because CE has the ability to quickly separate and quantitate such products at these low levels, it presents the hope of developing a protein sequencer capable of analyzing the small amounts of protein generated in the biotechnology laboratories of today.

In all of the procedures listed in Table 1, samples are derivatized prior to injection of the sample onto the capillary. This precolumn derivatization is not subject to the technical difficulties of postcolumn derivatization, such as the demanding hardware requirements for postcolumn addition of reagent. It is quite a challenge to derivatize the small volumes (nl) emerging from the capillary without significantly increasing zone broadening and thus lowering sensitivity. However, some success in this area has been achieved by Pentoney *et al.* (1988), who derivatized amino acids on-line with OPA and achieved resolution of nine amino acids in the subfemtomole range. Also, Albin *et al.* (1991) were able to separate six amino acids by CE using postcolumn derivatization with OPA. Sensitivity in the fmol range was achieved with only 10% loss of resolution compared to separations using precolumn derivatization.

Derivatization of amino acids to permit electrochemical detection is a relatively new procedure that has awaited advances in electrochemical detectors specific for CE. While such detectors are not yet commercially available, units developed in research laboratories have resulted in detection limits in the sub-fmol range (O'Shea *et al.*, 1992).

An additional benefit of this method is the ability to identify an amino acid by voltammetric characterization. This procedure, which may be likened to obtaining an "electrochemical UV-spectrum," involves analyzing selected parts of the curve obtained from plotting the current response at different detection potentials (see Section 4.3. for an application).

3. Separation Modes for Amino Acids

As mentioned in the previous section, the suitability of a particular separation mode for amino acids depends on whether or not the amino acids have been derivatized. Since the usual protocol involves precapillary derivatization to yield a fluorescent analyte, the focus of most methods has been on the separation of these analytes.

3.1. Capillary Zone Electrophoresis (CZE)

Historically, CZE has been used as a separation mode since the groundwork for CE separation of amino acids was laid by Jorgenson's research group in 1981. Specifically, CZE permits the resolution of members of certain groups of amino acids as demonstrated in Fig. 1, in which small common amino acids as well other biologically important amino acids such as GABA are separated from other endogenous amino acids. The figure demonstrates that, in CZE, amino acid derivatives are separated based on the following parameters:

(a) The net charge of the amino acid derivative. The more negative the amino acid, the more it will oppose the endoosmotic flow, and the longer will be its migration time. Thus, amino acids with negatively charged side chains, such as Glu and Asp, have the longest migration times.

(b) The effective size and shape of the amino acid derivative. Smaller amino acids will have smaller surface areas and thus exhibit less drag as they migrate against the endoosmotic flow. As a result, they will have longer migration times. Thus, the smallest of the common amino acids, Gly, exhibits the longest migration time of any of the amino acids with uncharged side chains.

In light of these facts, it becomes apparent that amino acids of the same charge, effective size and shape (for example, the constitutional isomers leucine [Leu] and isoleucine [Ile]) cannot be resolved by CZE. In order to create slight differences in charge, one might be tempted to try separations using a buffer with a pH near 2, which is not far from the pK_a of the carboxyl groups of amino acids. However, these pK_a values for the common amino acids are often very close to each

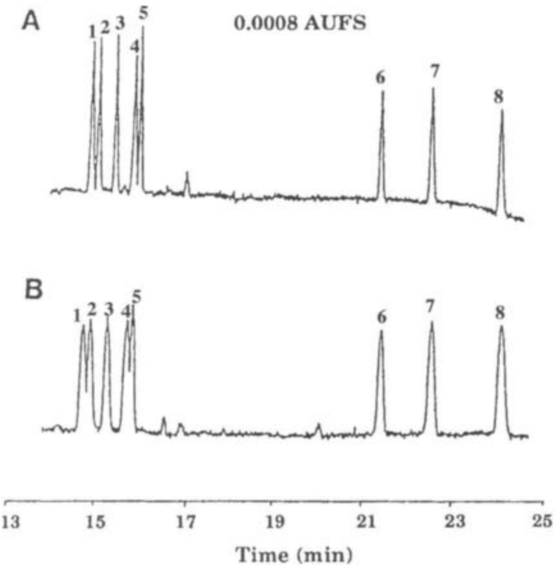

Fig. 1. Electropherogram of seven NDA-derivatized amino acids. Separation conditions: 115 cm × 50 µm i.d. column in 20 mM sodium borate (pH 9.0) operated at 30 kV; 420 nm detection. Samples derivatized in: (A) 20 mM sodium borate (pH 9.0); (B) 50 mM sodium borate (pH 9.0). Peaks: 1, GABA; 2, Ser; 3, Ala; 4, taurine; 5, Gly; 6, α-aminoadipic acid (internal standard); 7, Glu; 8, Asp. (Reproduced by permission from Weber *et al.*, 1994.)

other and will not give usable differences in charge. Also, low pH buffers do not give good endoosmotic flow and result in poor CE separations. Thus, amino acids such as Leu and Ile become inseparable by CZE. The usefulness of CZE is therefore limited to separations involving particular groups such as smaller amino acids or specific groups such as phosphoamino acids (Heber *et al.*, 1992)

3.2 Micellar Electrokinetic Capillary Chromatography

In order to overcome the inability of CZE to separate certain mixtures such as the isomers Leu and Ile, a method must be developed that can separate amino acids not just by charge, effective size and shape but by some other parameter that differs between isomers.

Since the polarity of isomers is often sufficient to allow separation, as exemplified by separations achieved by LC, a nonpolar phase is effectively introduced into the system by the addition of a detergent such as SDS to the mobile phase. This phase becomes the basis of the method known as micellar electrokinetic capillary chromatography (MEKC), which is explained in detail in Chapter 2. In recent years, MEKC has been used to separate all the common amino acids as derivatives. A good example of this type of separation can be seen in Fig. 2, in which almost all of 14 common amino acids, derivatized using NDA, are well resolved from each other. The use of other derivatizing agents such as OPA (Liu *et al.*, 1988), FMOC (Pang *et al.*, 1990) and phenylisothiocyanate (Waldron and Dovichi, 1992) has also afforded the separation of most common amino acids by MEKC.

3.3. *Additives for Chiral Separations of Amino Acids*

Another area in which CE has shown its ability to effect difficult separations is that of resolution of the D,L isomers of common amino acids. Different approaches have been used, but all share the concept of selective interaction of an isomer with a chiral moiety.

In one approach, Gozel *et al.* (1987), using dansylated derivatives, were able to resolve 14 of 18 D,L pairs attempted. This was accomplished by adding a copper(II)-aspartame support electrolyte to the mobile phase, which allows formation of diastereomers from the D,L pairs in the mobile phase and thus enables resolution.

In a slightly different variation, Ueda *et al.* (1992) added β- or γ-cyclodextrans to an SDS-containing mobile phase to cause a similar type of diastereomer formation. As can be seen in Fig. 3, resolution of six pairs of D,L isomers was obtained. Tran *et al.* (1990) used still another approach. A chiral derivatizing agent, either L- or D-Marfey's reagent, was used to produce diastereomers, which were then subsequently separated by conventional MEKC.

4. Applications

As indicated above, a variety of CE methods have been developed in the past 10 years for the high resolution and sensitive detection of mixtures of amino acids, including D,L isomers. These methods have not translated into a mature bioanalytical science in routine use in the contemporary laboratory; still, there are a number of interesting reported applications for which CE seems particularly well suited.

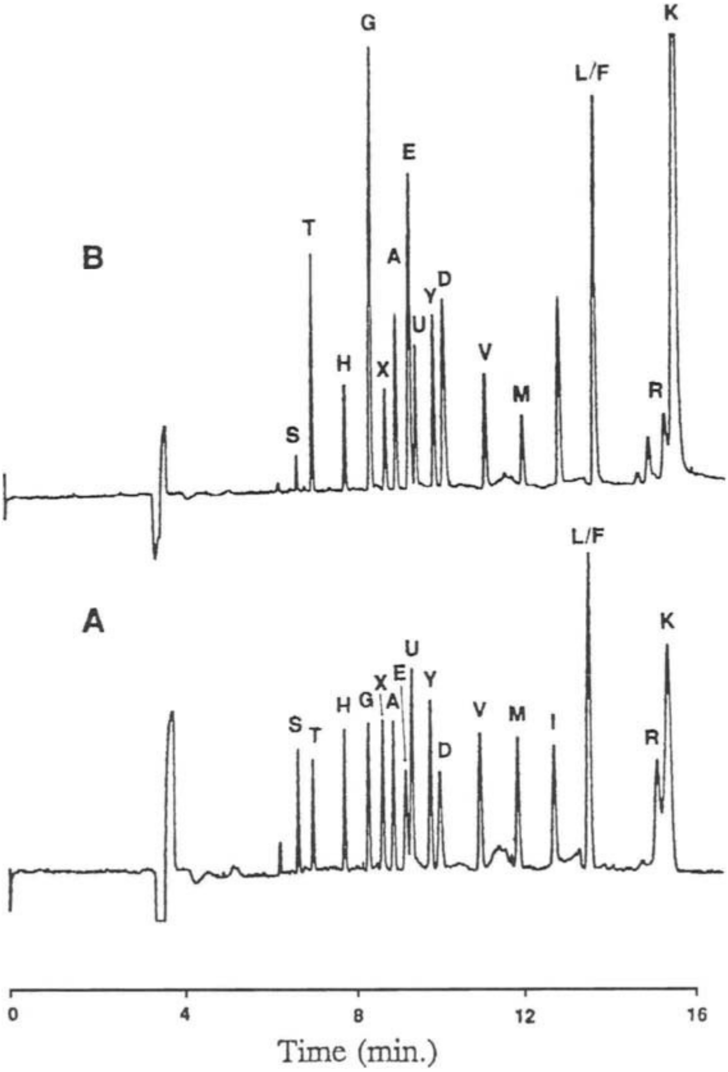

Fig. 2. Electropherogram of NDA-derivatized amino acid mixtures. Column 61 cm (36 cm to detector); applied voltage, 20.0 kV; current 33 mA; one-letter symbols used for amino acids (see Table 2); K = lysine, X = internal standard, a-aminoadipic acid, U = unidentified peak. (A) Stock solution of 15 amino acids and internal standard; (B) cytochrome *c* hydrolysate (18 h). (Reproduced with permission from Weber and Buck, 1994.)

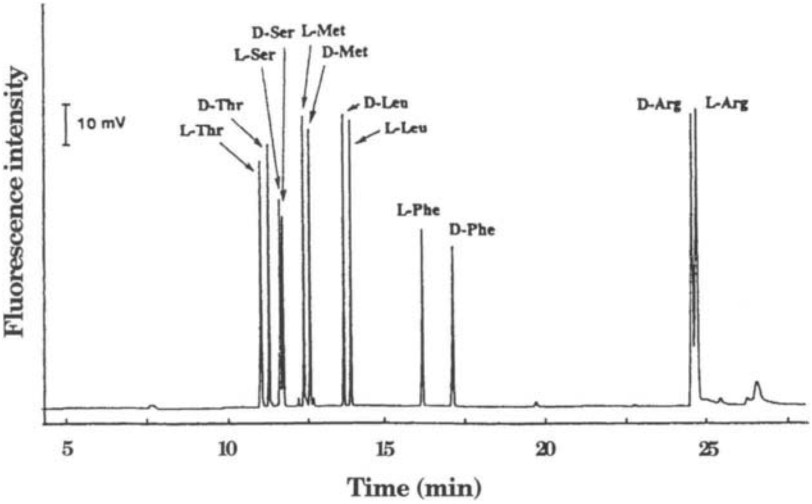

Fig. 3. Electropherogram of a mixture of six amino acids obtained by gamma-cyclodextrin-micellar electrokinetic chromatography (γ-CD-MEKC). Concentration of D,L-amino acids, 2.0 × 10^{-7} M. (Reproduced with permission from Ueda *et al.*, 1992.)

4.1. *Protein Hydrolysates*

Considering the fact that there are probably thousands of laboratories performing amino acid analysis of protein hydrolysates, it is not surprising that this is an area of major interest for researchers in CE of amino acids. Currently, amino acid analysis by other techniques is a well-established practice. Years of research have enabled LC to become the commonplace method for amino acid analysis. Yet the small volume requirements of a typical CE run, less than 20 nl, coupled with high sensitivity fluorescence or electrochemical detection, hold out the hope of using CE for the analysis of fmol or smaller quantities of protein—an accomplishment not readily demonstrable for LC! This prospect would certainly be appealing to those in the biotechnology arena who are dealing with small, precious amounts of a synthetic or recombinant protein.

Thus, for CE to become competitive with LC, it must be shown that CE can be used for routine and reliable analysis of amino acid hydrolysates at levels less than those needed for LC. To this end, Liu *et al.* (1991) have analyzed the protein lysozyme by injecting sub-fmol levels of the derivatized amino acid hydrolysate into the system. This seems impressive until one realizes that this amount is contained in nanoliter volumes and thus represents concentrations in the low nM range. Again, CE can analyze very small volumes of moderately low concentrations. The difficulty in amino acid analysis of proteins is that small volumes are difficult to manipulate quantitatively (Novotny *et al.*, 1990). Indeed, most reports of the use of CE in amino acid analysis of protein hydrolysates will present an electropherogram of the separation, but usually there is little attempt to quantitate the amino acids or determine amino acid composition from the data. If one is not particularly concerned about using small volumes, CE does provide a fast and simple method for determination of the amino acid composition of a protein as demonstrated by Weber and Buck (1994) (Table 2). The ease of use was demonstrated by the fact that the data were obtained by undergraduates with no prior experience in the use of CE.

All in all, only when the technical difficulties of handling nanoliter volumes of proteins, hydrolysates, and derivatized mixtures are overcome, can quick advances in the routine use of CE for amino acid analysis of proteins be expected.

Table 2. Amino Acid Composition of Proteins by CE[a]

	Lysozyme		Cytochrome C	
Amino Acid	Exp.	Lit.	Exp.	Lit.
Ser (S)	9.3	10	1.3	1
Thr (T)	5.1	7	8.3	8
His (H)	1.1	1	2.9	3
Gly (G)	11.5	12	13.5	14
Ala (A)	11.4	12	6.5	6
Glu (E)	5.5	5	12.0	12
Tyr (Y)	3.0	3	4.1	4
Asp (D)	15.1	21	7.4	8
Val (V)	5.3	6	3.0	3
Met (M)	1.2	2	2.1	2
Ile (I)	5.9	6	6.7	6
Leu/Phe (L/F)	11.6	11	10.6	10
Arg (R)	11.4	11	1.9	2

[a] Protein was hydrolyzed in 6 M HCl at 105°C for the time indicated. Separation conditions are given in Fig. 2.

4.2. Analysis of Compounds Containing Chiral Components

As discussed in section 3.2., MEKC has large potential for resolving a variety of mixtures in which chiral components are present, such as mixtures of D,L-amino acids. Sometimes MEKC can provide a facile means to analyze the amino acid content of pharmaceutically important peptides. An interesting example is found in the work of Okafo and Camilleri (1993), who used MEKC of both dansyl- and NDA-derivatized amino acid hydrolysates to determine the amino acid composition of D-Phe^7-bradykinin. This nonapeptide contains both D- and L-Phe, as well as other amino acids. To accomplish this analysis by LC would require multiple analyses.

4.3. Microdialysis Sampling of Biofluids

Another area of application of CE is in the analysis of samples obtained through microdialysis of living systems. The small volumes collected during a typical microdialysis procedure, often as low as 5 µl, provide limited amounts of sample for subsequent analysis. Since CE employs small sample volumes (less than 20 nl) relative to LC (generally in the microliter range), CE would seem to be an attractive method for analyzing microdialysates. O'Shea *et al.* (1992) have determined the concentrations of excitatory amino acids in samples obtained by microdialysis of the rat cortex. In this study, 5 µl samples were collected and 3 µl aliquots were derivatized by the NDA method. Figure 4 shows the release of the excitatory amino acids glutamate and aspartate upon stimulation with potassium. Electrochemical detection was used to achieve the sensitivity required for the low concentrations present in the microdialysate. Additional examples of this approach are given in Chapter 12.

4.4. Diagnosis of Human Metabolic Disorders

A number of human diseases involving errors in metabolism often result in an accumulation of some characteristic amino acid in fluids such as urine or blood. Researchers are beginning to explore the use of CE as an instrumental technique that can quickly detect abnormally high concentrations of amino acids in small samples. Jellum *et al.* (1991) have demonstrated the utility of CE for the rapid diagnosis of cystinuria and homocystinuria, diseases in which cystine and homocystine, respectively, accumulate in the urine. In this study, analyses of these two amino acids in urine samples from diseased and normal patients were performed using CE, and the results were compared to those of the current laboratory method, which employs

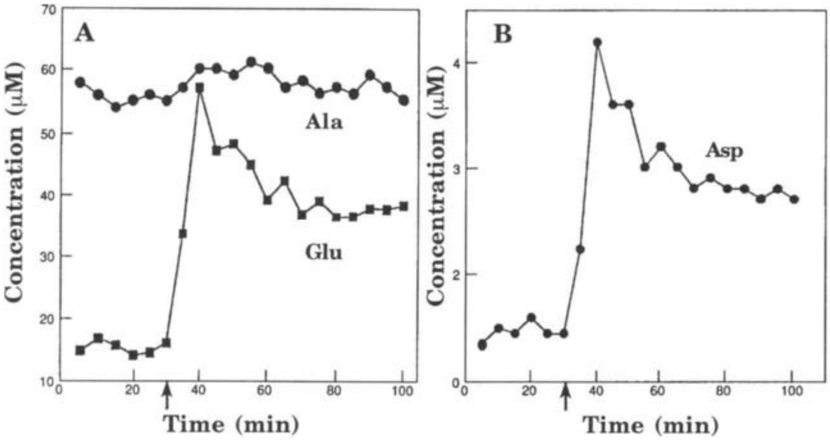

Fig. 4. Concentration-time curves of (A) alanine and glutamine and (B) aspartate in rat brain. Arrows indicated application of potassium stimulation. (Reproduced with permission from O'Shea *et al.*, 1992.

classical automated ion-exchange chromatography. Although both methods give conclusive evidence of the disease, CE analysis required less than 30 min, including less than 10 min for electrophoresis. On the other hand, the method involving traditional amino acid analysis requires 3 h, including an ion exchange separation lasting over 2 h! Thus, the rapidity of the method, combined with the relative simplicity of the instrumentation, makes CE of amino acids a promising alternative to traditional methods.

5. References

Albin, M., R. Weinberger, E. Sapp and S. Moring (1991). *Anal. Chem.*, *63*, 417–422.

Bergman, T., B. Agerberth and H. Jörnvall (1991). *FEBS Lett.*, *283*, 100–103.

Camilleri, P., D. Dhanak, M. Druges and G. N. Okafo (1994). *Anal. Proceed. Anal. Commun.*, *31*, 99–102.

Chan, K. C., G. M. Janini, G. M. Muschik and H. J. Issaq (1993). *J. Chromatogr. A*, *653*, 93–93.

Cheng, Y. and N. J. Dovichi (1988). *Science*, *242*, 562–564.

deMontigny, P., J. F. Stobaugh, R. S. Givens, R. G, Carlson, K. Srinivasachar, L. A.. Sternson and T. Higuchi (1987). *Anal. Chem.*, *59*, 1096–1101.

Engstrom-Silverman, C. E. and A. G. Ewing (1991). *J. Microcolumn Sep.*, *3*, 141–145.

Gozel, P., E. Gassmann, H. Michelsen and R. N. Zare (1987). *Anal. Chem.*, *59*, 44–49.

Guzman, N. A., J. Moschera, K. Iqbal and A. W. Malick (1992). *J. Liq. Chromatogr.*, *15*, 1163–1177.

Heber, M., C. Liedtke, H. Korte, E. Hoffmann-Posorske, A. Donella-Deana, L. A.. Pinna, J. Perich, E. Kitas, R. B. Johns and H. E. Meyers (1992). *Chromatographia*, *33*, 347–350.

Higashijima, T., T. Fuchigami, T. Imasaka and N. Ishibashi (1992). *Anal. Chem.*, *64*, 711–714.

Jellum, E., A. K. Thorsrud and E. Time (1991). *J. Chromatogr.*, *559*, 455–165.

Jorgenson, J. W. and K. D. Lukacs (1981). *Anal. Chem.*, *53*, 1298–1302.

Kuhr, W. G. and E. S. Yeung (1988). *Anal. Chem.*, *60*, 1832–1834.

Liu, J., K. A. Cobb and M. Novotny (1988). *J. Chromatogr.*, *468*, 55–65.

Liu, J., Y. Hsieh, D. Wiesler and M. Novotny (1991). *Anal. Chem.*, *63*, 408–412.

Lunte, S. M. and O. S. Wong (1990). *Current Separations*, *10*, 19–26.

Nickerson, B. and J. W. Jorgenson (1988). *J. High Resol. Chromatogr. Chromatogr. Commun.*, *11*, 533–534.

Novotny, M., K. A. Cobb and J. Liu (1990). *Electrophoresis*, *11*, 735–749.

Okafo, G. N. and P. Camilleri, (1993). *J. Microcolumn Sep.*, (5), 149–153.

O'Shea, T. J., P. L.. Weber, B. P. Bammel, C. E. Lunte, S. M. Lunte and M. R. Smyth (1992). *J. Chromatogr.*, *608*, 189–195.

Pang, J. T. K., D. J. Kramer and T. R. Tullsen (1990). In: *Current Research in Protein Chemistry: Techniques, Structures and Functions* (J. S. Villafranca, ed.), pp. 11–22. Academic Press, San Diego.

Pentoney, S. L., X. Huang D. S. Burgi and R. N. Zare (1988). *Anal. Chem.*, *60*, 2625–2629.

Smith, L. M. (1988). *Anal. Chem.* *60*, 381A–390A.

Tran, A. D., T. Blanc and E. J. Leopold (1990). *J. Chromatogr.*, *516*, 241–249.

Ueda, T., R. Mitchell, F. Kitamura, T. Metcalf, T. Kuwana and A Nakamoto (1992). *J. Chromatogr.*, *593*, 265–274.

Waldron, K. C. and N. J. Dovichi (1992). *Anal. Chem.*, *64*, 1396–1399.

Waldron, K. C., S. Wu, C. W. Earle, H.R. Harke and N. J. Dovichi (1990). *Electrophoresis*, *11*, 777–780.

Weber, P. L.. and D. R. Buck (1994). *J. Chem. Ed.*, *71*, 609–612.

Weber, P. L.., T. J. O'Shea and S. M. Lunte (1994). *J. Pharm. Biomed. Anal.*, *12*, 319–324.

Wu, J., T. Odake, T. Kitamori and T. Sawada (1991). *Anal. Chem.*, *63*, 2216–2218.

Wu, S. and N. J. Doivichi (1989). *J. Chromatogr.*, *480*, 141–155.

Ye, J. and R. P. Baldwin (1994) . *Anal. Chem.*, *66*, 2669–2674.

Zhou, J. and S. M. Lunte (1995). *Electrophoresis*, *16*, 498–503.

CHAPTER 9

Capillary Electrophoresis of Proteins and Peptides

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1. Background

The growth of the field of biotechnology has brought about a need for reliable separation methods for proteins and peptides. Because there are many instances in peptide or protein analysis in which the analyst is limited by sample size, a highly selective and sensitive analytical technique is essential. The two techniques most commonly employed for protein and peptide separations are slab gel electrophoresis and reversed-phase liquid chromatography (RP-LC). Slab gel electrophoresis is a powerful technique for the separation of complex biomolecules, but it can be manually intensive and time-consuming. In addition, the data obtained are often qualitative rather than quantitative (Wilkes, 1989), and resolution is generally poor for very small proteins and peptides. RP-LC operates by a different separation mechanism and is selective for hydrophobic and polar group differences. However, in some cases, the stationary phase can induce changes in the secondary structure of the protein or peptide, leading to adsorption (Schöneich *et al.*, 1993).

In contrast to LC, CE separates components on the basis of their charge-to-size ratio. Since separation in CE is based on the ratio of charge-to-size, it is possible to separate complex mixtures of peptides that differ only slightly in pI. This mode of separation is therefore complementary to RP-LC, where the compounds are resolved based on their hydrophobicity (Liu *et al.,* 1990, Issaq *et al.,* 1992). CE has several advantages over conventional separation methods (Table 1).

Table 1. Advantages of CE over Conventional Methods for Analysis of Proteins and Peptides

Simplicity
Small Sample Requirements
Rapid Analysis Times
High Resolution
High Separation Efficiency
Ease of Quantitation
Ease of Automation

It has minimal sample requirements, usually around 0.1–10 nl, with an actual injected volume of 100 picoliters to a few nanoliters, while LC methods generally require 1–20 μl of sample for analysis and the entire sample is used for each injection.

The use of protein and peptide separations in CE is beneficial in a large number of applications, including the analysis of pharmaceutical preparations, biological fluids, tissue samples and protein digests, and the verification of the purity of synthetic mixtures (Liu *et al.*, 1990; De Antonis *et al.*, 1994). CE has been employed for several of these applications, as well as for peptide mapping of recombinant proteins (Bongers *et al.*, 1992; Cobb and Novotny, 1992b; Amankwa *et al.*, 1993; Chang and Yeung, 1993; Rudnick *et al*, 1994; Wahl *et al.*, 1994); analysis of insulin (Klyushnichenko *et al.*, 1994; Nashabeh *et al.*, 1994) and other recombinant proteins such as human growth factor (Shimura and Karger, 1994); glycopeptide separations (Landers *et al.*, 1992; Rush *et al.*, 1993; Weber *et al.*, 1995); and determination of proteins and peptides in single cells (Hogan and Yeung, 1992; Kristensen *et al.*, 1994; Xue and Yeung, 1994).

Totally automated systems are commercially available, and detectors designed for LC often can easily be modified for CE. The use of small diameter capillaries permits efficient heat dissipation and, thus, many biomolecules can be separated without the use of a gel. An aqueous medium is conducive to peptide and protein separations because it minimizes the possibility of denaturation of the analyte.

2. Strategies for Basic Protein and Peptide Separations

Most peptides contain an amine terminus and many also contain other basic functional groups such as lysine and arginine. Peptides with a high pI are the most difficult to analyze by CE due to the electrostatic attraction of these cationic residues to ionized silanols on the capillary surface. Several techniques can be used to prevent interaction between the capillary wall and protein surface. These can be classified into two categories: (1) the use of additives such as carrier electrolytes or pseudophase additives (Liu *et al.*, 1990; Cole *et al.*, 1991; Corradini *et al.*, 1994), and (2) permanent modification of the capillary wall (Song *et al.*, 1993). Both of these minimize surface-analyte interactions.

Most macromolecules, including large peptides and proteins, have very low diffusion coefficients and, therefore, could ideally have theoretical plate numbers in the millions. However, experimentally, the efficiencies are much lower (Song *et al.*, 1993). This is due primarily to hydrophobic and coulombic interactions between the analyte and the surface of the capillary wall. Some molecules have greater affinity for the surface, depending on their natural, physicochemical properties and structural conformation (Guzman *et al.*, 1992). The adsorption of positively charged compounds through electrostatic interactions has been shown to lead to decreased separation efficiencies or loss of analyte (Emmer *et al.*, 1991). Adsorption of analytes to the wall also changes the zeta potential, which can lead to peak tailing, changes in the electroosmotic flow (EOF), and irreproducible migration times. The build-up of positively charged analytes after repeated injections along the surface of the capillary can eventually result in reversal of the EOF (Song *et al.*, 1993).

Simple adjustments of the ionic strength of the background electrolyte (BGE) are sometimes sufficient to prevent peptide and protein adsorption to the capillary wall. For example, Chen *et al.* (1992) were able to significantly reduce peptide and protein absorption using run buffers of high ionic strength. As a general rule, the ionic radius of the analyte is smaller in high ionic strength run buffers because the molecule is more constricted due to interactions with ions in the BGE, making possible a more efficient separation. With low ionic strength buffers there is a diffuse atmosphere surrounding the analyte, and the efficiency is slightly lower. Also, increased buffer concentration and ionic strength often make it possible to obtain higher resolution due to suppression of electrophoretic dispersion.

It is important to monitor the effects of Joule heating that sometimes result when the ionic strength of the BGE is in excess (Kuhn and Hoffstetter-Kuhn, 1993). The major complication that arises from Joule heating when dealing with high ionic strength buffers is the subsequent high currents. Therefore, short capillaries of small i.d. (25–50 μm) are often used to minimize Joule heating. However, the disadvantage of this approach is that the small pathlength characteristic of these capillaries leads to a large decrease in sensitivity when using UV detection (Swedberg, 1990). Also, an efficient capillary cooling system is often necessary when working with higher currents (Chen *et al.,* 1992). Lower applied voltages are generally employed when using highly conductive buffers because higher field strengths can lead to arcing (Cifuentes *et al.,* 1993).

Very high salt concentrations also alter the double layer on the surface of the capillary and reduce the overall EOF (Nielsen and Rickard, 1990). Swedberg (1990) analyzed various proteins such as lysozyme, ribonuclease, trypsinogen, myoglobin and carbonic anhydrase B and observed that the use of high ionic strength and/or increased buffer concentrations led to increased migration times due to decreased EOF. One should also be aware that under high ionic strength conditions, migration order is no longer a predictable function of pI for peptides, and the interpretation of the data may become more complex. Also, the composition of the sample is important in that too large a difference in conductivity between the sample and BGE could lead to the formation of air bubbles in the run buffer and subsequent loss of current flow. On the other hand, low ionic strength sample matrices are useful for focusing or sample stacking (Swedberg, 1990). As a general rule, the ionic strength of the analyte should be less than 1% of the ionic strength of the BGE to minimize peak distortion (Lee and Desiderio, 1994).

2.1. Basic pH

When developing any CE separation, it is desirable to keep the method as simple as possible. Unnecessary additives or features that complicate the method should be avoided whenever possible. Another way of reducing protein and peptide adsorption and improving separation efficiency is through manipulation of the pH of the background electrolyte.

At pH values above 2.0, the silanol groups on the fused silica capillary wall are negatively charged (Lauer and McManigill, 1986). Therefore, if the pH of the run buffer is below the pI of the peptide or protein, adsorption of the peptide or protein to the capillary surface will probably occur. Since most peptides and proteins are amphoteric, it is possible to work in a range where both the capillary surface and the protein bear a negative charge (Deyl *et al.,* 1989). This results in maximum coulombic repulsion and improved separation effciencies (Lauer and McManigill, 1986). Adsorption of many basic peptides and proteins can be overcome by operating in the pH range of 8–11 because they are negatively charged at this pH (Lauer and McManigill, 1986).

Caution is required, however, in cases where the pH of the background electrolyte and the pI of the protein or peptide are considerably different. In extreme cases, this situation can result in structural damage or hydrolysis of the analyte (Lauer and McManigill, 1986). Conversely, protein precipitation can result in cases where the pH of the buffer is too close to the pI (Emmer *et al.,* 1994). However, the occurrence of these phenomena depends solely on the nature of the particular analyte.

At pH values above 11, additional problems with Joule heating and arcing can occur and are the result of the high currents typically observed at this pH (Okafo *et al.,* 1994a). Therefore, it is usually necessary to work at reduced voltages at this pH range. In addition, the high EOF at this pH can lead to a loss in resolution. Another problem resulting from the use of run buffers with pH above 11 is that the fused silica is slowly dissolved by the buffer (Issaq *et al.,* 1992). In general, background electrolytes of pH less than 10 are preferred for protein and peptide separations.

2.2. *Acidic pH*

Many peptide and protein separations can be carried out successfully in bare, fused silica capillaries with run buffers in an acidic pH range (2.5–5.0). This is the most flexible range in which to manipulate charge differences between the protein and the capillary surface (Issaq *et al.,* 1992). In this range, although most proteins and peptides are positively charged, the net wall charge is approaching zero and, ideally, minimal adsorption occurs. One disadvantage of operating in the acidic pH range is that due to protonation of the carboxy terminus there is a lower mass-to-charge ratio for peptides and, therefore, a decreased chance of achieving resolution of compounds of similar structure (Song *et al.,* 1993). The pH range 2–2.5 would appear to be best since below 2.0 the capillary walls should be essentially neutral in charge and, therefore, there is little or no electrostatic interaction with the wall. However, at pH values below 2, the capillary wall may not be stable (Cifuentes *et al.,* 1993).

Although it is often satisfactory simply to optimize the pH of the run buffer to obtain the desired separation, this does not always work. It may be necessary to use an additive to the background electrolyte to improve the separation. Alternatively, permanent modification of the capillary surface may be employed to reduce surface-analyte interactions, as described in Chapter 2 of this book.

2.3. *Buffer additives*

Metal ions and zwitterionic salts: Metal ions and zwitterionic salts are commonly used in the separation of basic proteins and peptides.

These substances are intended to reduce or suppress the interactions of the protein or peptide with the capillary wall by competing for the negatively charged sites on the fused silica capillary (Muijselaar *et al.*, 1992). Bullock and Yuan (1991) used various metal salts such as Na_2SO_4, K_2SO_4, NaCl, KCl and K_2HPO_4 as additives for the separation of various proteins. Issaq *et al.* (1992) used zinc salts to resolve two peptides that had a similar peptide sequence, and were able to obtain a successful separation. Lithium chloride and tetramethylammonium chloride have also been used as buffer additives for the determination of a model therapeutic protein, humanized anti-TAC monoclonal antibody (Guzman *et al.*, 1992).

Zwitterionic salts exhibit a lower conductivity and are, therefore, a good alternative to conventional buffer systems for CE. However, some zwitterions have low water solubility, which causes difficulties when dealing with aqueous run buffers and samples. This could also ultimately lead to the denaturation of proteins or peptides (Cifuentes *et al.*, 1993). However, zwitterionic salts permit the use of high electric fields and maintain good selectivity for peptide and protein separations (Nashabeh *et al.*, 1994). Nashabeh *et al.* (1994) used a zwitterionic detergent, N-dodecyl-N,N-dimethyl-3-amino-1-propanesulfonate (DAPS), as a hydrophobic selector to separate variants of insulin growth factor I. Utilization of this detergent permitted the use of a wide range of buffer pH without alteration of the zwitterionic character of the detergent.

Dynamic coatings: A dynamic coating is one that is contained in the run buffer and consists of an ionic, zwitterionic, or non-ionic molecule that is strongly absorbed onto the walls of the capillary, forming either a monolayer or a bilayer on the capillary surface (see Fig. 1 for a schematic of the bilayer formation). This coating also causes reversal of the EOF, resulting in a larger separation window for cationic analytes. Assuming that the electrophoretic conditions remain constant throughout the run, the system should remain in equilibrium (Gilges *et al.*, 1994). Fluorad, or FC-135, is a commercially available fluorinated, cationic surfactant that forms a positively charged dynamic coating along the capillary surface. It is a useful additive for repelling basic proteins (Emmer *et al.*, 1991). This coating has been shown to significantly reduce the adsorption of basic peptides and proteins to the capillary wall (Emmer *et al.*, 1994). Muijselaar *et al.* were also able to separate four highly basic proteins, α-chymotrypsinogen, ribonuclease A, cytochrome *c* and lysozyme, as shown in Fig. 2. The "separation" obtained without a dynamic coating is shown in Fig. 3.

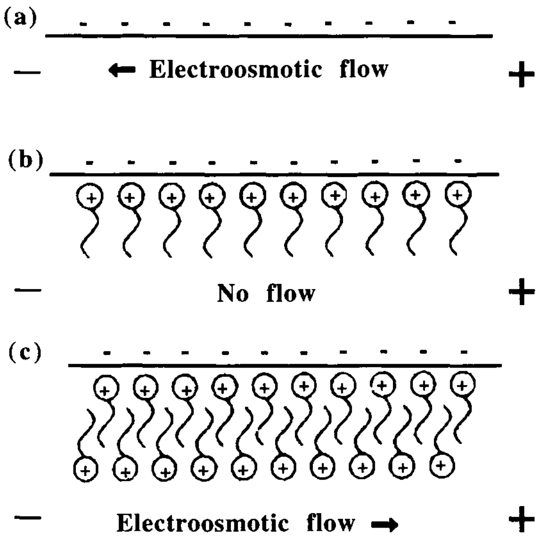


Fig. 1. (a) No surfactant added, electroosmotic flow in normal direction. (b) Electrostatic absorption of positively charged surfactant headgroups to the negative silanol groups on the silica surface of the capillary inner wall. (c) Admicellar bilayer formation by hydrophobic interaction between the apolar chains, resulting in a reversal of the electroosmotic flow. (Reproduced with permission from Emmer *et al.*, 1991.)

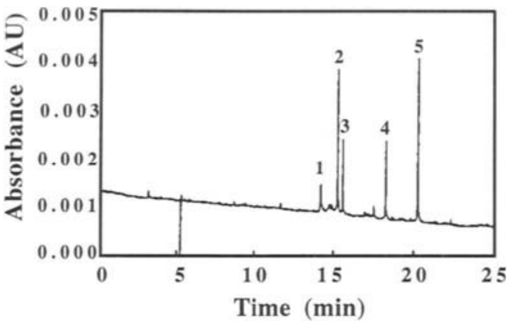


Fig. 2. Electropherogram for the separation of (1) mesityl oxide, (2) α-chymotrypsin, (3) ribonuclease, (4) cytochrome *c* and (5) lysozyme. Capillary l_1 (length to detector) = 99.65 cm and l_2 (total length) = 87.28 cm; injection 5 s at 5 kV; separation voltage, 25 kV; buffer, 10 mM phosphate (pH 7.0) with 50 μg/ml of FC135 added. (Reproduced with permission from Muijselaar *et al.*, 1992.)

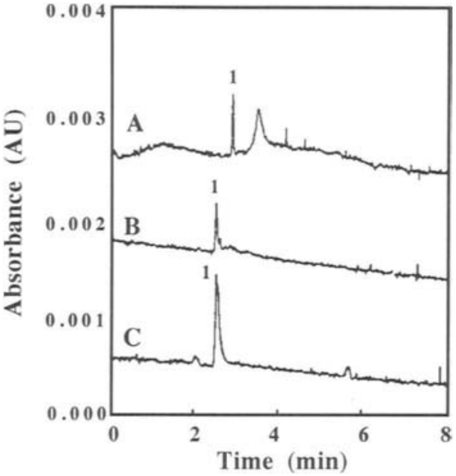


Fig. 3. Electropherograms obtained under conditions without a dynamic coating. Capillary, l_1 (length to detector) = 42.15 cm and l_2 (total length) = 34.60 cm. Anode at the inlet side and cathode at the outlet side, respectively. Injection 5 s at 5 kV; separation voltage 15 kV; buffer, 10 mM borate at (A) pH 11.0, (B) pH 9.0 and (C) pH 7.0. Sample same as in Fig. 2. (Reproduced with permission from Muijselaar *et al.*, 1992.)

In contrast to the use of salt additives, which leads to Joule heating due to the high conductivity of the buffer, the fluorad system yields good separation efficiency under conditions of low ionic strength. The short- and long-term reproducibilities are excellent as long as the dynamic equilibrium at the surface of the wall is maintained throughout the run (Emmer *et al.,* 1991).

Amine additives: Several amine additives have been shown to improve the separation of basic proteins and peptides by masking the exposed silanol groups on the fused silica as shown in Table 2. This results in the formation of a cationic surface that repels positively charged molecules. Several amine additives have been evaluated, including 1,3-diaminopropane (DAP), ethylenediamine (ED), 1,4-diaminobutane phosphate (DAB), 1,5-diaminopentane triethylamine and triethanolamine (Song *et al.,* 1994). The amines absorb as an immobilized electrical double layer of the capillary, and the remainder of the ions will be distributed throughout the free solution. When the specific adsorption of the additives is sufficiently large, the direction of the EOF is often reversed (Corradini *et al.,* 1994; Song *et al.,* 1994).

Table 2. Common Amine Additives for Basic Protein and Peptide Separations

Type of amine additive	Abbreviation	Application	Ref.
ethylenediamine	ED	Separation of basic proteins such as lysozyme, cytochrome *c*, ribonuclease, trypsinogen, and α-chymotrypsin using 60-80 mM ED	Song *et al.,* 1993
ethylenediamine, 1,3-diaminopropane, 1,4-diaminobutane	ED, DAP, DAB	Comparison of these amine additives were evaluated for the separation of basic proteins: lysozyme, cytochrome *c*, ribonuclease, and α-chymotrypsin	Song *et al.,* 1994
triethylamine, triethanolamine, galactosamine, glucosamine		Excellent separation was obtained for these amine additives for the separation of lysozyme, ribonuclease A, cytochrome *c*, and α-chymotrypsin	Corradini *et al.,* 1994
tetradecyltrimethylammonium bromide	TTAB	Analysis of a single erythrocyte and their components	Xue and Yeung, 1990
hexadecyltrimethylammonium bromide, dodecyltrimethylammonium bromide	DTAB, HTAB	These two additives were used in the separation of angiotensin peptide variants	Liu *et al.,* 1990
tetramethylammonium chloride, trimethylammonium propylsulfonate	TMAC, TMAPS	Used for the separation of a humanized monoclonal antibody in bulk and therapeutic formulation	Guzman *et al.,* 1992

ED was shown to produce a higher EOF than most amines; this was probably due to weak adsorption or interaction with the silanol groups.

Corradini *et al.* (1994) looked at various cationic amine additives, including triethylamine, triethanolamine, glucosamine and galactosamine, for protein separation by free zone CE. A separation of four basic proteins using glucosamine as an additive is shown in Figure 4.

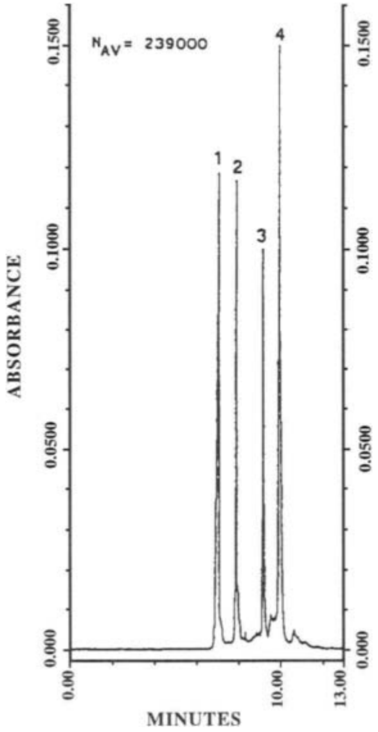


Fig. 4. Electropherogram of standard basic proteins obtained with addition of 40 mM glucosamine, pH 2.5. Proteins: 1, cytochrome *c*; 2, lysozyme; 3, ribonuclease A; 4, α-chymotrypsinogen A. (Reproduced with permission from Corradini *et al.*, 1994.)

These workers were able to obtain excellent resolution and good selectivity of these model basic proteins using these cationic additives to reduce the adsorption of the proteins to the capillary wall.

Tetraalkyl amines have been shown to have an additional "blocking power," which is due to the presence of both hydrophobic and polar regions of the molecule (Guzman *et al.*, 1992). Tetradecyltrimethylammonium bromide (TTAB), dodecyltrimethylammonium bromide (DTAB), hexadecyltrimethylammonium bromide (HTAB) and cetyltrimethylammonium bromide (CTAB) are all surfactants that have very low critical micellar concentrations (CMC). The CMC is defined as the concentration at which the surfactant molecules spontaneously aggregate to produce spherical molecules due to the increased hydrophobic interactions at the higher concentrations (Kuhn and Hoffstetter-Kuhn, 1993). When a surfactant is above the CMC, smaller molecular weight compounds can partition between the pseudostationary phase, which is the micelle, and the aqueous buffer phase to give added selectivity. In the case of ionic micelles, it is possible to have ion pairing with the protein or peptide. In addition, the charged micelle can aid in the separation mechanism by introducing charge to an otherwise neutral analyte (Nashabeh *et al.*, 1994). Liu *et al.* (1990) investigated both HTAB and DTAB for the separation optimization of eight underivatized angiotensin analogs. Huang *et al.* (1989) found that, although the CMC for TTAB was determined to be 3.5 mM, it is possible to have a reversed EOF at concentrations below the CMC. This is believed to be due to the complexation of the TTAB molecules with the capillary wall.

Neutral additives/organic solvents: Most background electrolytes in CE utilize only aqueous-soluble salts. Although organic solvents may be used, they are normally employed only as additives to optimize a specific separation. Organic solvents affect the polarity, conductivity and the viscosity of the BGE. Several groups have investigated the use of organic solvents in CE separations (Cole and Sepaniak, 1991; Idei *et al.*, 1992; Langenhizen *et al.*, 1993). Organic solvents can influence peptide separations by direct interaction with the capillary wall or through alteration of the electrophoretic mobility of the analyte and subsequent changing of the EOF of the separation (Idei *et al.*, 1992). The addition of organic solvent to the BGE also leads to reduced currents and, consequently, decreased Joule heating.

Although the exact mechanism by which uncharged organic modifiers participate in CE separations is not well understood, it is thought that they produce a decrease in the zeta potential that results in a change in the dielectric properties of the Stern layer (Stefansson and Westerlund, 1993). This leads to a reduction in the EOF. EOF, the zeta potential and the viscosity of the solution are related as shown below:

$$\mu_{eo} = \frac{\zeta\varepsilon}{4\pi\eta} \tag{1}$$

where: μ_{eo} = effective electrophoretic mobility
ζ = zeta potential
ε = permittivity of the medium
η = viscosity of the solution

The addition of organic solvents also affects the viscosity and ionic strength of the BGE (Kuhn and Hofstetter-Kuhn, 1993). As can be seen in Eq. (1), this will also affect the EOF. The combination of the decreased ionic strength of the run buffer, upon addition of the organic modifier, and the lower viscosity of the solvent results in increased mobility of the analyte and, therefore, decreased migration times when compared to aqueous buffers (Idei *et al.,* 1992). In one study, it was found that the effect of alcohols on migration time was opposite to that of acetonitrile (Idei *et al.*, 1992). There was an inverse relationship between the strength of the organic modifier, measured with respective dipole moments, and the migration times of the analytes. As the strength of the organic solvent is increased, the viscosity is decreased and, thus, the overall mobility is also decreased, as shown in Eq. (1). The order of organic modifiers with respect to decreasing dipole moments is:

$$\text{acetonitrile} > H_2O > \text{MeOH} > \text{EtOH} > \text{isopropyl alcohol}$$

where acetonitrile has the lowest viscosity in the run buffer when compared to other organic solvents such as MeOH, EtOH and isopropyl alcohol.

Within the alcohol category, the longer the carbon chain, the greater the migration time of the analyte with respect to aqueous run buffers (Idei *et al.,* 1992). This migration trend among organic modifiers also holds true when implemented in micellar CE. Cole and Sepaniak (1991) found that MeOH and 2-propanol were useful in extending migration times in micellar electrokinetic chromatography (MEKC) because these organic solvents interact with the capillary wall, thus decreasing the wall charge and increasing the amount of time that the micelle spends in the capillary. Acetonitrile reduces the EOF to a lesser extent, due to decreased interaction with the capillary wall. Also, since the elution window is sandwiched between the column void and the micelle migration time, a "stacking" effect of hydrophobic compounds sometimes results. This is due to the increased association time of the hydrophobic species with the micelle itself. As the overall polarity of BGE is decreased, solutes that are insoluble in water will spend less time in the micelle and more time in the "free BGE." On the other hand, the organic modifier also interacts with the capillary wall, decreasing the EOF but increasing the migration time of the micelle (Cole and Sepaniak, 1991). The combination of these two effects yields the overall net migration times of the analytes.

In some instances, however, organic modifiers have been shown to be ineffective for the separation of proteins and peptides. Langenhizen *et al.* (1993) saw no improvement or change in the separation of adrenocorticotropic hormone (ACTH), endorphins or cholecystokinins or fragments of these pharmaceutical peptides with 10% MeOH or acetonitrile in various BGEs, including phosphate buffer, formic acid and Tris buffer. It was found that if the organic concentration was too high (<15%), efficiency was reduced and the analysis time increased, which led to distorted peaks.

Cyclodextrins: Cyclodextrins (CDs) are cyclic oligosaccharides that consist of six, seven or eight glucose units, corresponding to the α, β, and γ-CD, respectively. CDs are basket-shaped molecules with a hydrophobic interior cavity and hydrophilic external cavity (Kuhn and Hoffstetter-Kuhn, 1993). The entrance to the cavity is also hydrophilic since it is lined with primary and secondary hydroxyl groups.

CDs enhance both resolution and selectivity through judicious partitioning of guest molecules or ions into the host hydrophobic cavity (Snopek *et al.*, 1994). The hydrophobic cavity can form inclusion complexes with aromatic or alkyl moieties of proteins and peptides, as long as a favorable match is made between the size and shape of the entering solute and the cavity size of the CD. Although unmodified CDs are neutral in charge, they can still significantly improve resolution. In this case, the separation is due to an alteration in electroosmotic mobility of the peptide or protein due to differences in MW between the complexed and free molecules. Liu *et al.* (1990) investigated the use of α-CD and β-CD as buffer additives for the separation of 3-(4-carboxybenzoyl)-2-quinoline carboxaldehyde (CBQCA)-derivatized angiotensin variants and fragment peptides; they observed increased resolution due to the presence of the CDs. Figure 5 shows a model mixture of nine peptides with the addition of β-CD to the run buffer.

The use of CDs can also result in increased fluorescence for derivatized peptides, since molecules of this type sometimes exhibit a quenched fluorescence in the absence of a complexing agent (Liu *et al.*, 1990). In particular, CDs have been shown to enhance the fluorescence intensity of peptides and amino acids derivatized with naphthalene-2,3-dicarboxaldehyde/cyanide (NDA/CN) and CBQCA (Liu *et al.*, 1990; Lunte and Wong, 1990; Schwartz *et al.*, 1991).

Anionic additives: Phytic acid is an anionic ion pairing reagent that has been proven to be effective in separating very basic proteins or peptides. Phytic acid is highly charged and water-soluble; it contains six phosphate groups with pK_a values that range from 1.9–9.5. Okafo *et al.* (1994a) found that the sodium salt form of phytic acid gave a better resolution for basic peptide separations than did the free acid. Positively charged proteins or peptides ion pair with the polyanionic phytic acid to prevent interactions of the analyte with the capillary wall. Overall, a lower but constant EOF is observed with this additive. It would be expected that this polyanionic species would

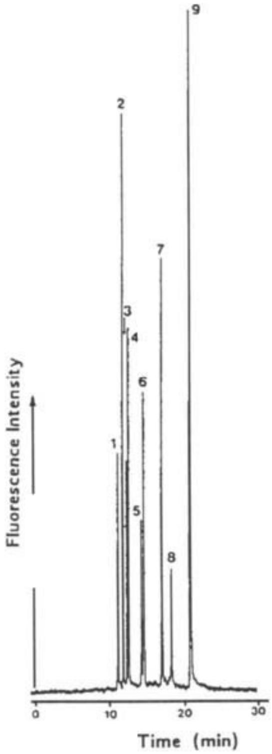


Fig. 5. Electropherogram of fluorescamine-derivatized peptides, using β-cyclodextrin as a buffer additive. Model mixture of 9 peptides. Peaks: 1 = angiotensin III; 2 - Gly-Gly-Tyr-Arg; 3 = angiotensin I; 4 = [Val^5]-angiotensin II; 5 = Met-Leu-Phe; 6 = Gly-Leu-Tyr; 7 = Val-Gly-Ser-Glu; 8 = Ala-Gly-Ser-Glu; 9 = Val-Gly-Asp-Glu. Capillary: 80 cm in length (50 cm to detector), 50 µm i.d. Separation buffer: 0.05 M borate buffer (pH 9.50)–20 mM β-cyclodextrin–15% methanol–1% THF. Operating voltage: 20 kV. (Reproduced with permission from Liu *et al.*, 1990.)

produce much higher currents than monoionic electrolytes. However, experimentally, this is not the case. The reason for this is most likely the increased ionic radius of the hydrated form compared to that of the monoionic salts (Okafo *et al.,* 1994a). Figures 6a and 6b show the separations of two amino acids and six dipeptides with and without the addition of phytic acid at pH 7.5. Phytic acid has also been used as a buffer additive for peptide mapping of hemoglobin by CE (Okafo *et al.* 1994b).

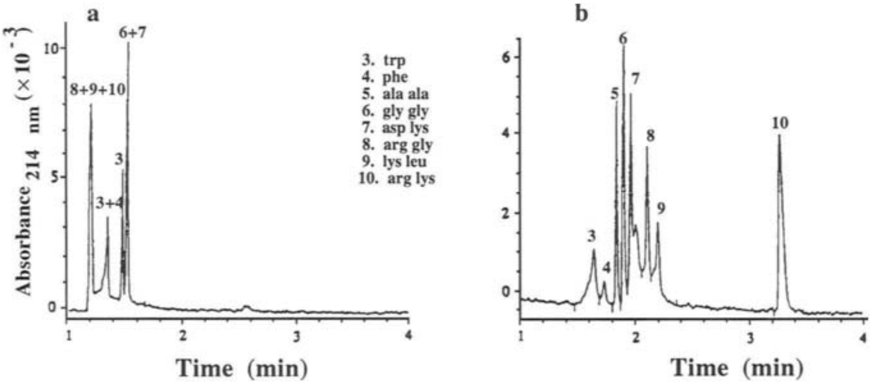


Fig. 6. Electropherograms of six dipeptides and two amino acids in the (a) absence and (b) presence of the sodium salt of phytic acid (10 mM). Electrophoresis conditions: capillary, fused silica; L = 27 cm, l = 20 cm, i.d., 50 µm; electrolyte, 300 mM sodium borate adjusted to pH 7.5, 8.2 and 9.2; voltage 10 kV; temperature, 25°C. (Adapted from Okafo *et al.*, 1994b.)

Bile salts: Bile salts are biological surfactants with a substituted steroidal structure. These salts are postulated to form small, primary micelle aggregates, composed of up to 10 monomers, that are both rigid and helical. In these micelles, the hydrophobic portion of the monomer that is in contact with the aqueous solution and the hydrophilic portion forms the internal section of the micelle aggregate (Terabe *et al.,* 1989; Cole and Sepaniak, 1991; Wu *et al.*, 1994). In some respects it can be thought of as an "inverted micelle." Bile salts are most successful for the separation of hydrophobic compounds (Wu and Sweedler, 1994). Polar bile salts differ significantly from long alkyl chain micelles such as sodium dodecyl sulfate (SDS) in structure and in the nature of the separation. They are far more useful for hydrophobic separations than are anionic surfactants such as SDS because it is possible to utilize both the positive and negative charges on the bile salt molecule (Cole and Sepaniak, 1991). In one example, Beijersten and Westerlund *et al.* (1993) used 4.0 mM taurodeoxycholic acid (TDC) in an acidic phosphate buffer in combination with coated polyacrylamide capillaries for the separation of enkephalin-related peptides. In aqueous solutions, TDC forms helical "reversed micelles," which leads to increased selectivity. Increased electroosmosis was observed for the met-enkephalins in the presence of TDC, which may be the result of ion pairing or an electrostatic interaction of TDC with the basic arginine group.

3. Separations of Acidic and Hydrophobic Proteins and Peptides

In the majority of cases, the approach to the separation of acidic and hydrophobic proteins and peptides in CE is quite straightforward. Separations can be optimized by simple manipulations of buffer type and adjustment of the ionic strength of the background electrolyte. However, in those instances where the peptides are highly anionic in character, *i.e.*, acidic analytes, the separation can sometimes be improved by addition of bile salts and adjustment of run buffer pH (see the section on buffer additives in this chapter). For example, Langenhizen *et al.* (1993) found the separation of the acidic peptide cholecystokinin (CCK) to be inefficient at a low pH, but much better at neutral or basic pH, where there was a repulsion with ionized silanol groups on the capillary wall. Electrostatic interactions were eliminated at low pH due to protonation of the free silanols. Therefore, the low efficiency exhibited at low pH can be attributed primarily to hydrophobic interactions of the peptides with the silanol groups.

The main difficulty encountered with hydrophobic proteins and peptides is that many nonpolar species suffer from low water solubility. Therefore, it may be necessary to dissolve the sample in an organic solvent prior to analysis (Sweeney *et al.,* 1993). Under these conditions, a large conductivity difference between the sample matrix and the BGE may result, which can lead to boiling of the run buffer and subsequent formation of air bubbles in the capillary. This is usually accompanied by a rapid, spontaneous drop in current. In addition, the use of buffers containing large amounts of organic solvent in a high electric field can lead to a temperature gradient due to the change in the dielectric constant that again can lead to boiling of the solvent in the capillary during the separation (Sweeney *et al.,* 1993). Using high performance CZE and high performance capillary isotachophoresis (CITP), Josic′ *et al.* (1990) investigated the separation of hydrophobic membrane proteins, both extrinsic and intrinsic, including calcium binding proteins from liver plasma membranes and Morris hepatoma 7777 plasma membranes, and a mixture of three different intrinsic protein glycoproteins. They were able to prevent the precipitation of these proteins, which often occurs in aqueous media, through the use of detergents such as Triton X-100 and denaturing reagents such as urea.

In those cases where there are only subtle differences in the hydrophobicity and ionic character of the analytes being separated, the resolution of these analytes can be particularly challenging. One example of a difficult separation is that of insulin-like growth factor variants (Nashabeh *et al.* 1994). In this case, the use of a capillary coated with a neutral hydrophilic surface was necessary. The use of the modified capillary led to an overall reduction in EOF and prevented unwanted adsorption of the protein or peptide to the capillary surface. Weinmann *et al.* (1994a) developed a pressure-assisted CE method to elute a highly hydrophobic protein, lung surfactant protein SP-C, from the capillary and detect it by mass spectrometry. However, without this pressure-assisted step, the mass flow would not be large enough for subsequent MS analysis. Other approaches that had been successful for hydrophobic proteins and peptides include the use of MEKC (Wu *et al.,* 1994) (see section on MEKC in this chapter).

4. Glycoproteins

Glycoproteins are those proteins that possess covalently attached carbohydrates with varying degrees of heterogeneity. Most glycoproteins contain oligosaccharides composed of four sugars—two neutral sugars, mannose and galactose, and two amino derivatives, N-acetylglucosamine and N-acetylgalactosamine—and a peptide chain (Liu *et al.,* 1991b).

Carbohydrate analysis of glycopeptides usually involves the release of the neutral sugar residues via hydrolysis with 2.0 M formic acid and the release of the hexosamines through exposure to more strongly acidic conditions, such as 4.0 M HCl (Liu *et al.,* 1991b). However, the carbohydrate composition of glycoproteins tends to be very heterogeneous. Hexosamines can be derivatized by reagents that react with primary amines and analyzed using fluorescence detection. Neutral sugars lack both the amine moiety and a distinct chromophore and display no native fluorescence (Stefansson and Westerlund, 1993). Alternate modes, for example, indirect detection (Liu *et al.,* 1991b), pulsed electrochemical detection (PED) (Weber *et al.,* 1995) or derivatization of an alternative moiety such as the carboxyl group followed by UV or fluorescence detection (Liu *et al.,* 1991a,b), are thus necessary for the determination of these sugars.

Thin layer chromatography (TLC), gas chromatography (GC), liquid chromatography (LC), gel permeation (GP), supercritical fluid chromatography (SFC), ion exchange (IE) and slab electrophoresis have all been used for the separation of glycopeptides. However, the use of LC or CE is preferred for glycoproteins and glycopeptides due to the high separation efficiencies and tremendous resolution capabilities of these systems (Stefansson and Westerlund, 1993). Rush *et al.* (1993) used affinity high performance CE, which uses a molecule with affinity for the analyte in order to capture the desired species onto the capillary and increase selectivity, for the analysis of recombinant human erythropoietin (rHuEPO); they were able to separate both glycosylated and non-glycosylated peptide fragments (Fig. 7).

An alternative method for the separation of glycoproteins and glycopeptides involves the use of boric acid. Boric acid, in its open-chain form, is known to preferentially complex with diols, polyols and carbohydrates and transforms the sugar/alcohol from the neutral form into a anionic species that can be electrophoretically separated by CE (Stefansson and Westerlund, 1993, Hoffstetter-Kuhn *et al.,* 1991). The resulting sugar borate complex is then detectable in the low UV range (Hoffstetter-Kuhn *et al.,* 1991).

Landers *et al.* (1992) resolved five major protein peaks of ovalbumin, a 43,000 MW glycoprotein of avian egg white, using borate buffer as a complexing agent. In an attempt to increase resolution, phosphate and borate buffers were evaluated as complexing agents for carbohydrates and glycoproteins. Ovalbumin was also used as a model in these studies to compare the separation obtained with borate to that with other buffers, such as phosphate. Inferior results were obtained with phosphate, and this was postulated to be due to steric differences between the two molecules. For example, better resolution of fluorescently labeled glycosamine and galactosamine was obtained using borate rather than phosphate (Landers *et al.*, 1992).

Another important factor in the use of borate buffers is the effect of pH. In cases where the analyte concentration was held constant, increasing the pH of the run buffer increased the number of ionized acidic groups on the boric acid molecule available for complexation. High pH run buffers also had more effect on migration time because of an overall increase in EOF (Hoffstetter-Kuhn *et al.,* 1991).

Stefansson and Westerlund (1993) investigated alternatives to the use of boric acid complexation as a means of separating glycopeptides and glycoproteins. These included the separation of glycoconjugates at alkaline pH using MEKC. The addition of 40 mM CTAB reversed the migration order of the analytes by reversing the EOF. They also evaluated the effect of organic modifiers, which decreased the overall EOF and ultimately resulted in a decrease in the overall efficiency.

Weber *et al.* (1995) used CE with a BGE containing 42 mM sodium tetraborate (pH 9.3) to separate several glycopeptides isolated from recombinant coagulation factor VII (rcf VII). They used both UV and

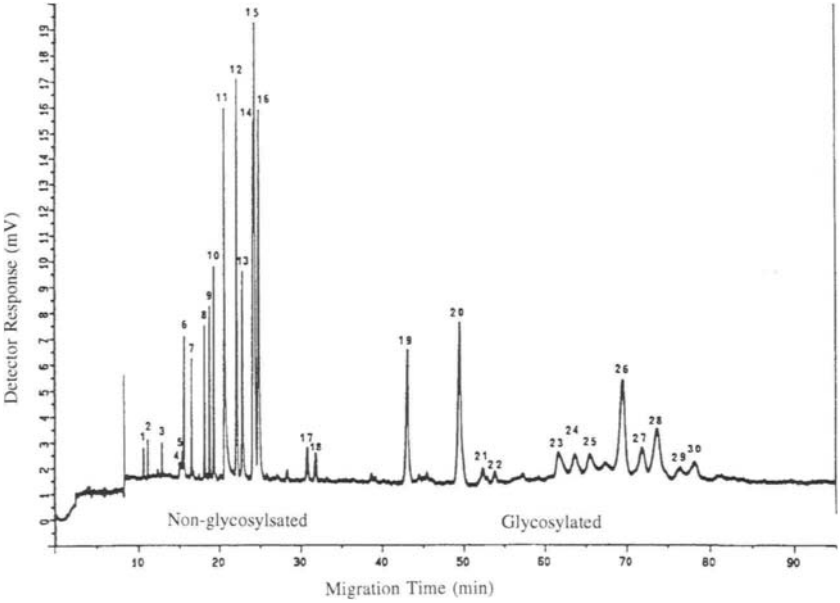


Fig. 7. HPCE profile of trypsin-digested rHuEPO (3.75 mg/ml), vacuum injected for 2 s into a 50-μm i.d., 360 μm o.d. capillary of total length (L) to effective length (l) of L/l75/50 cm, respectively. The sample was dissolved in 10% glacial acetic acid for electrophoresis. Electrophoresis was conducted at 16,000 V at 30°C (electric field 213 V/cm) in 40 mM sodium phosphate buffer, pH 2.5, containing 100 mM heptanesulfonic acid ion pairing agent; the profile was monitored on line at 200 nm, 0.025 AUFS at a data collection rate of 15 Hz for 95 min. The current level was about 110 μA for a power load of about 2.35 W/m. The nonglycosylated peptide and the glycopeptide sections of the map are designated and the corresponding HPCE peaks are numbered according to migration time. (Reproduced with permission from Rush *et al.*, 1993.)

PED detection for this analysis. With PED, simultaneous detection of the oligosaccharides and the peptide fragments of the molecule was possible.

5. Additional Strategies

5.1. Micellar Electrokinetic Chromatography (MEKC)

Micelles are aggregates of amphiphilic compounds with a hydrophobic interior and a hydrophilic exterior. Depending on the exposed functional group of the hydrophilic moiety on the exterior, micelles may be one of the four types: anionic, such as SDS, cationic, such as CTAB, zwitterionic, such as sulfobetaine, and nonionic, such as Brij-35 (Kuhn and Hoffstetter-Kuhn, 1993).

The addition of micelle-forming surfactants above the CMC has been reported to increase selectivity and resolution by introducing a pseudophase into which the analytes can partition (Schwartz *et al.*, 1991). This partitioning between the BGE and the micelle can aid in the separation of neutral compounds by CE under these conditions.

The separation is based on the relative distribution of analytes between the buffer phase and the micelle (Liu *et al.*, 1990). Micelles migrate at a different velocity than the BGE; thus, analytes included in the micelle will have migration times closer to that of the micelle, while analytes not associated with the micelle will have migration times based on their electrophoretic mobility. Most analytes will fall between these extremes. MEKC can be used to exploit subtle changes in hydrophobicity, size, charge, and/or shape to differentiate charged species that are normally difficult to separate (Liu *et al.*, 1990).

MEKC is most useful in cases where the charge-to-size differences of the analytes are similar (Issaq *et al.*, 1992), for example, for separations of peptides with similar net charge. MEKC can also be used to improve resolution of ionic peptides (Liu *et al.*, 1990; Kuhn and Hoffstetter-Kuhn, 1993). However, when attempting to separate larger polypeptides and proteins by MEKC, poor selectivity sometimes results from the inability of the solute to partition into the micelle (Nashabeh *et al.*, 1994). Some additives are not compatible with MEKC; for example, chaotropic ions are not useful in MEKC because they destabilize the micelle systems and decrease the overall polarity of the system (Kuhn and Hoffstetter-Kuhn, 1993).

SDS is the surfactant most commonly used in this technique. SDS micelles possess a negative charge and, therefore, a negative electrophoretic mobility results. SDS is frequently used as a buffer additive for the separation of basic proteins and peptides. It competes with the negatively charged capillary wall for the positively charged sites on the protein (Liu *et al.*, 1990).

5.2. Capillary Isoelectric Focusing (CIEF)

Isoelectric focusing (IEF) separates molecules on the basis of their neutral isoelectric points or pIs. The premise by which IEF works is that a protein or peptide will migrate under the influence of an electric field as long as it maintains a charge at a given pH. As the analyte begins to lose charge with changing pH, and it becomes more neutral, the velocity of the analytes begins to decrease and eventually migration ceases when pH is equal to pI (Hjertén and Zhu, 1985; Weinberger, 1993).

The main difference between conventional IEF and capillary isoelectric focusing (CIEF) is that conventional IEF uses an anticonvective medium such as a slab-gel as a separation medium, while CIEF uses an open tubular capillary. Also in IEF, a mobilization or elution step is not necessary as it is in CIEF, since detection is performed online through a staining procedure.

In CIEF, various zwitterion reagents known as carrier ampholytes are used to establish a pH gradient across the capillary (Hjertén and Zhu, 1985; Weinberger, 1993). Under the influence of an electric field the positive ampholytes migrate to the cathode and the negative ampholytes migrate to the anode until they reach their neutral pI. The pH gradient range can be large or small depending on the nature of the ampholyte blend. It is possible to create a "smoother" gradient by using a larger number of ampholytes (Xu, 1993). CIEF can be performed with or without the influence of EOF; however, most separations are satisfactory without the presence of EOF, which can be eliminated through the use of coated capillaries.

Sample introduction in CIEF is unique in that the sample and ampholyte solutions are normally mixed into a blend prior to introduction to the capillary. Upon application of the field, the gradient is formed and the analytes migrate to their neutral pIs.

Both Mazzeo *et al.* (1993) and Vonguyen *et al.* (1994) examined a protein digest of cytochrome *c* using CIEF to determine the isoelectric points for the various tryptic digest fragments. Mazzeo and his colleagues were able to use uncoated capillaries to identify the pIs of various cytochrome *c* tryptic digest fragments.

5.3. Capillary Gel Electrophoresis

Although capillary gel electrophoresis (CGE) is frequently used for protein separations, this chapter is concerned primarily with capillary zone electrophoresis (CZE)-based methods. See Chapters 2 and 10 for more detailed discussions of techniques based on CGE.

5.4. Two-Dimensional (2-D) Techniques Combined with CE

When dealing with extremely complex samples such as biological fluids, LC or CE alone are frequently not sufficient to achieve successful separations of all peptide and protein components. Giddings (1984, 1987) reviewed the application of multidimensional separation systems. In 1990, Bushey and Jorgenson elaborated on this type of 2-D separation system, which incorporated LC and CE. Larmann *et al.* (1993) later developed automated size exclusion chromatography (SEC)-CZE and reversed-phase LC-CZE coupled systems for peptide mapping of horse cytochrome *c* following precolumn derivatization with tetramethylrhodamine isothiocyanate isomer-5 (TRITC-label). In another study, shown in Fig. 8, a mixture of proteins including thyroglobin (THYRO), chicken egg albumin (OVA), bovine serum albumin (BSA), and horse heart myoglobin (MYO) in a reconstituted lyophilized human serum sample was analyzed by SEC-CZE (Lemmo and Jorgenson, 1993). Two- dimensional CE techniques have also been reported. A two-dimensional CE-CE map of glycoprotein digests using two unique CE separations that incorporate two different carrier systems was reported by Suzuki *et al.* (1992).

Although two-dimensional systems are capable of providing tremendous resolving power for proteins and peptides, there are some drawbacks. Run times are generally long and can last 2–12 hours. This is due, in part, to the need to sample several points in each peak migrating from the first dimension. Another frequently encountered difficulty concerns compatibility of the mobile phase or run buffer used in the two separation systems. Solutions of high salt concentration are generally used to eliminate undesirable interactions of positively charged analytes with the silica-based packing material used in SEC (Lemmo and Jorgenson, 1993). However, these high ionic strength solutions are usually not compatible with CE and can lead to very high currents and Joule heating. In the case of RP-LC, a large quantity of organic modifier in the LC mobile phase can also interfere with subsequent CE separations. This could result in a large conductivity difference between the low ionic strength sample being introduced and the normally high ionic strength BGE. Therefore, with 2-D systems, compromises often must be made with respect to the type of LC packing material and the ionic composition of the mobile phase or run buffer. The LC-CZE interface must also be designed so that both flow rates and the diameters of the LC column and the CE separation capillary are compatible, in order to avoid producing a large back pressure in the system (Lemmo and Jorgenson, 1993). Another problem frequently encountered with 2-D systems is caused by the difference in pathlength for UV detection in LC and CE. In order to obtain adequate sensitivity for trace analysis,

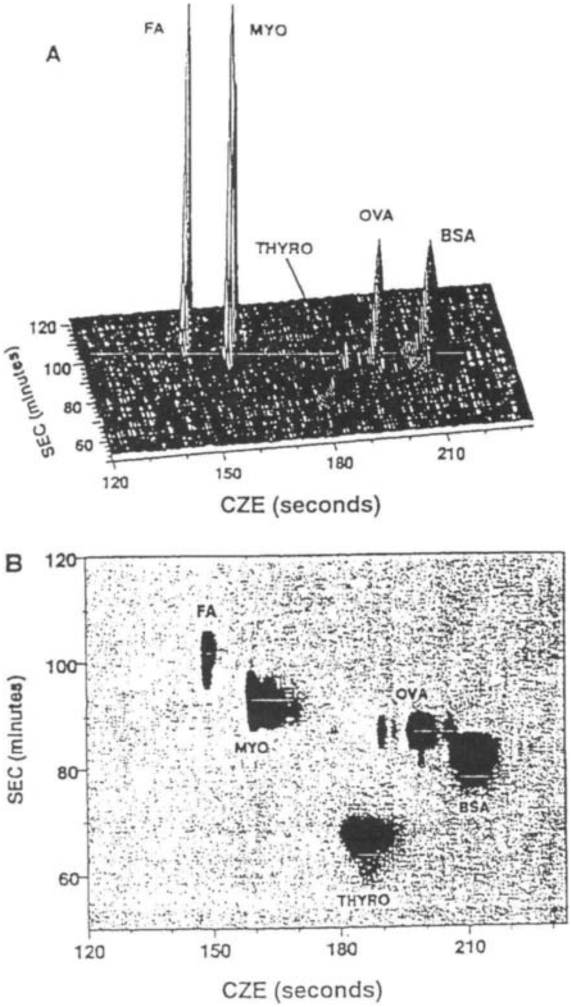


Fig. 8. Separation of protein standards by 2D SEC-CZE. SEC injection was 10 min at 7 bar (100 p.s.i.). Head pressure of 55 bar (800 p.s.i.) was applied to generate a flow-rate of 360 nl/min for the chromatographic separation. The electrophoresis capillary was 38 cm long, 20 cm to the detection window. CZE conditions are 5-s electromigration injection at –3 kV and 4-min runs at –8 kV. Data collection was 2 points/s. Shown here: (A) Surfer-generated 3D chromatoelectropherogram and (B) Spyglass-generated grayscale image. (Reproduced with permission from Lemmo and Jorgenson (1993).

samples must almost always be derivatized for fluorescence detection. The derivatization process makes the compounds look "more alike" and, therefore, some of their intrinsic differences that would enhance the separation are lost. Future work on on-line or postcolumn derivatization systems should circumvent this problem.

5.5. Detection Techniques

5.5.1. UV Detection

UV detectors are robust, inexpensive and versatile, and almost all biological and chemical compounds absorb to some extent in the UV range (Grossman and Colburn, 1992). Proteins and peptides containing aromatic amino acids such as phenylalanine, tyrosine and tryptophan possess absorption maxima at 280 nm and the amide bonds absorb around 200–210 nm, whereas those containing aliphatic amino acids usually also absorb more specifically around 215 nm. However, the LOD for UV is not quite as sensitive as for other methods such as electrochemical and fluorescence detection.

It is also possible to determine proteins and peptides that lack a suitable chromophore through the use of indirect UV detection. However, the LOD for indirect UV detection is not as great and is directly proportional to the amount of background chromophore in the run buffer (Xue and Yeung, 1994). In addition, this method is not very selective for many biological samples.

5.5.2. Electrochemical Detection

Capillary electrophoresis with electrochemical detection (CEEC) is a sensitive technique that is gaining popularity (see Chapter 6). Although CEEC has not been employed for the determination of proteins, several strategies have been developed for the detection of peptides. Most of these are based on the complexation of peptides with copper to yield an electroactive complex.

Baldwin's group has used CEEC with a copper electrode for the detection of N-blocked peptides (Ye and Baldwin, 1994). These compounds are not easily derivatized and are therefore difficult to determine by other means. In one example, the cyclic peptides Leu-Trp-Asp-Pro-Val and Leu-Tyr-Asp-Pro-Val were detected in solid phase synthesis products.

An alternative method, also based on reaction with copper, is the use of the Biuret reaction (Deacon *et al.*, 1993). In this case, the copper is first placed in the capillary via a rinsing procedure. The peptide sample is introduced into the capillary at a high pH; there it reacts with the Cu^{2+} on the capillary wall to form a Cu^{2+}-peptide complex. This complex can then be oxidized at a moderate potential at a carbon fiber microelectrode.

For peptides containing specific amino acids such as cysteine, other detection approaches can be employed. Zhou *et al.* (1994) used a mixed valent RuCN-modified electrode for the detection of glutathione and glutathione disulfide. Other approaches that have been used for the detection of cysteine-containing peptides include the use of a gold/mercury electrode (amalgamated gold fiber) (O'Shea and Lunte, 1993) and cobalt phthalocyanine-modified electrodes (O'Shea and Lunte, 1994).

Pulsed amperometric (PAD) can be used for the direct detection of peptides. Weber *et al.* (1995) were able to detect both the peptides and carbohydrates released from recombinant coagulation factor VIIa following treatment with glycopeptidase F (PNGase F).

It should also be possible to detect tyrosine- and tryptophan-containing peptides directly by CEEC, although there have been few reports of this. A more comprehensive review of electrochemical detection methods for CE is given in Chapter 6.

5.5.3. Mass Spectrometric Detection

Mass spectrometry (MS) has not always been considered sensitive enough for protein and peptide detection following CE separation due to the small amount of mass introduced from the CE column. However, in cases where there are limitations with detection techniques such as UV and fluorescence due to the restricted pathlength, MS can be extremely useful (Wahl *et al.*, 1993). Identification of compounds by both UV and fluorescence is limited to measurements of electrophoretic mobility and wavelength λ-ratioing. CE-MS, however, provides an accurate determination of the molecular weight, which can be used in conjunction with the electrophoretic mobility of the analyte for positive identification (Wahl *et al.*, 1993; 1994). MS is ideal in terms of selectivity since very exact measurements of MW can be determined. Only enantiomers cannot be distinguished by MS. Another advantage of using MS is in the determination of the nature and extent of protein denaturation that has occurred, including covalent modifications (Burlingame *et al.*, 1994; Wahl *et al.*, 1994).

There are practical considerations concerning the compatibility of CE with MS detection that must be addressed when dealing with protein and peptide separations. These include system suitability, high salt concentration in the sample matrices and flow rate differences at the interface (Burlingame *et al.*, 1994) (see Chapter 5).

Continuous flow fast atom bombardment mass spectrometry (cfFAB-MS), in conjunction with an aminopropyl-modified capillary, has been used for the identification of basic peptides. Efficiencies of hundreds of thousands of theoretical plates were obtained with detection limits around 10 μM (Mosley *et al.*, 1991). Wahl *et al.* (1993) found that smaller diameter capillaries increased sensitivity for CE-MS peptide analysis 25–50-fold as a result of controlling the number of ions that entered the system.

Rosnack *et al.* (1994) utilized CE-UV-electrospray ionization (ESI)-MS to determine the presence of a dehydration product of the synthetic peptide heregulin-β (Fig. 9a,b,c). A dehydration product (M-18) was exhibited in the ESI-MS spectrum of the heregulin-β sample. To determine if this product was an impurity in the sample or if it was due to the electrospray process itself, the sample was analyzed by both LC-UV and CE-UV. LC-UV was incapable of discriminating between the two products since only one slightly broad peak was present. However, two peaks were seen by CE-UV, indicating that there was indeed a dehydration impurity in the heregulin-β sample. Peak identity was verified using CE-UV-ESI-MS.

Peptide mixtures generated by tryptic digests of cytochrome *c* were analyzed by CE-ES-MS (Wahl *et al.* (1994). This method was found to be suitable despite the wide range of masses and hydrophobicities of the peptides. Loo *et al.* (1989) used ESI-MS to detect positively charged peptides and proteins in buffers at high pH; however, they experienced the loss of over an order of magnitude in their LOD when working above the pI of the fragment since at this stage there is a loss of protonation. Higher molecular weight peptides or proteins can be analyzed by electrospray MS or matrix-assisted laser desorption (MALDI), but oxidation of methionine can take place during ESI (Bornsen *et al.*, 1990; Schnolzer *et al.*, 1992; Burlingame *et al.*, 1994).

Weinmann *et al.* (1994b) coupled CE to MALDI off-line with a coaxial sheath flow isolation for structural determinations of proteins and peptides. Figure 10 shows a schematic of the CE-MALDI set-up with which Weinman was able to successfully separate six peptides, as shown in Fig. 11. Due to their similar inherent electrophoretic mobilities, the peptides accumulated into three groups as they comigrated in the CE. However, using MALDI, these peptides were all easily identified.

5.5.4. Fluorescence Detection

Problems can arise with UV for CE if extremely sensitive analyses are required. Because of the small volumes injected (from picoliters

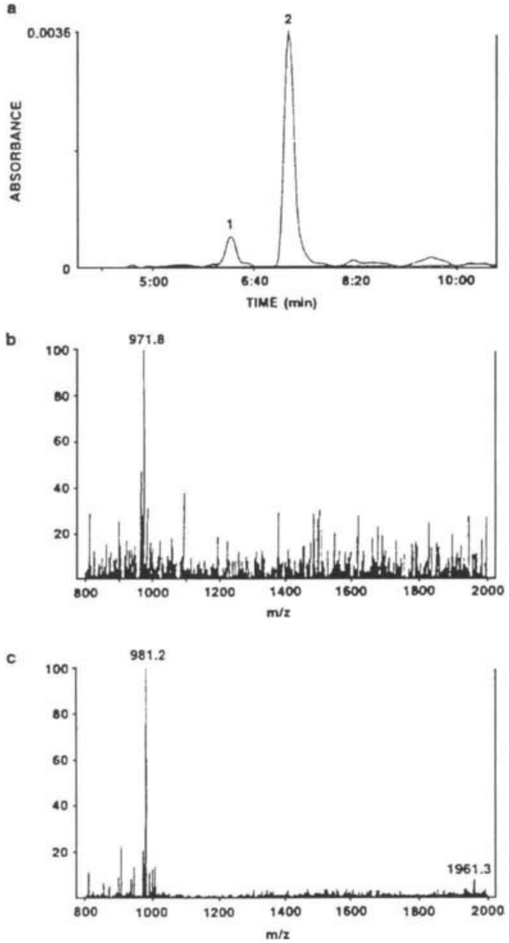


Fig. 9. (a) CE-UV electropherogram of the heregulin-β fragment (ca. 5 pmol). CE analysis was performed using a 110-cm untreated fused-silica column with 10 mM ammonium acetate (pH 5.5) as the background electrolyte. (b) Averaged mass spectrum of peak 1 from the CE-UV-ES-MS analysis of the heregulin-β sample. The observed M_r based on the double charged ion is 1941.6 and corresponds to a dehydration product. (c) Averaged mass spectrum of peak 2 from the CE-UV-ES-MS analysis of the heregulin-β fragment sample. The observed M_r was 1960.4 and corresponds to the intact peptide. (Reproduced with permission from Rosnack *et al.*, 1994.)

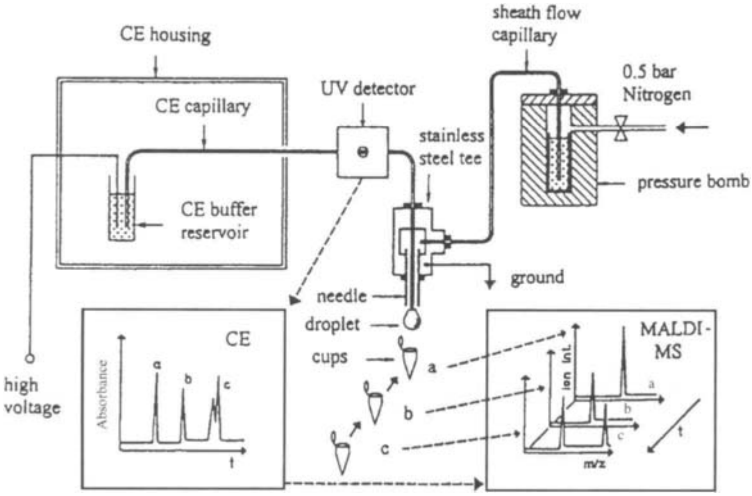


Fig. 10. Scheme of the CE-MALDI-MS interface using a sheath flow. (Reproduced with permission from Weinmann *et al.*, 1994b.)

to nanoliters) and the short optical pathlengths characteristic of CE, it may not be possible to reach the limits of detection necessary for the determination of proteins and peptides in biological samples. The most convenient and reliable method for using fluorescence detection takes advantage of the native fluorescence properties of the analytes (Gilman and Ewing, 1995). Peptides and proteins containing tryptophan, tyrosine and phenylalanine exhibit native fluores-cence when excited at 275 nm and thus can be detected using laser-induced native fluorescence (LINF). However, considerations such as acid and base quenching of fluorescence must be monitored since quenching of these residues is a well-know phenomenon (White, 1959). Most peptides are not naturally fluorescent. Therefore, in many cases, it is necessary to derivatize the protein or peptide with a fluorophore in order to improve sensitivity through the use of fluorescence or laser-induced fluorescence (LIF) detection (Grossman and Colburn, 1992; Guzman, 1993; De Antonis *et al.*, 1994; Kuhn and Hoffstetter-Kuhn, 1993). An outline of commonly used derivatization reagents is given in Table 3.

One difficulty experienced with derivatization is that most readily available reagents are relatively non-selective when it comes to protein and peptide analyses. The most common approach for these analyses is derivatization of the primary amino group, since this functionality is present on almost all proteins and peptides. OPA, NDA

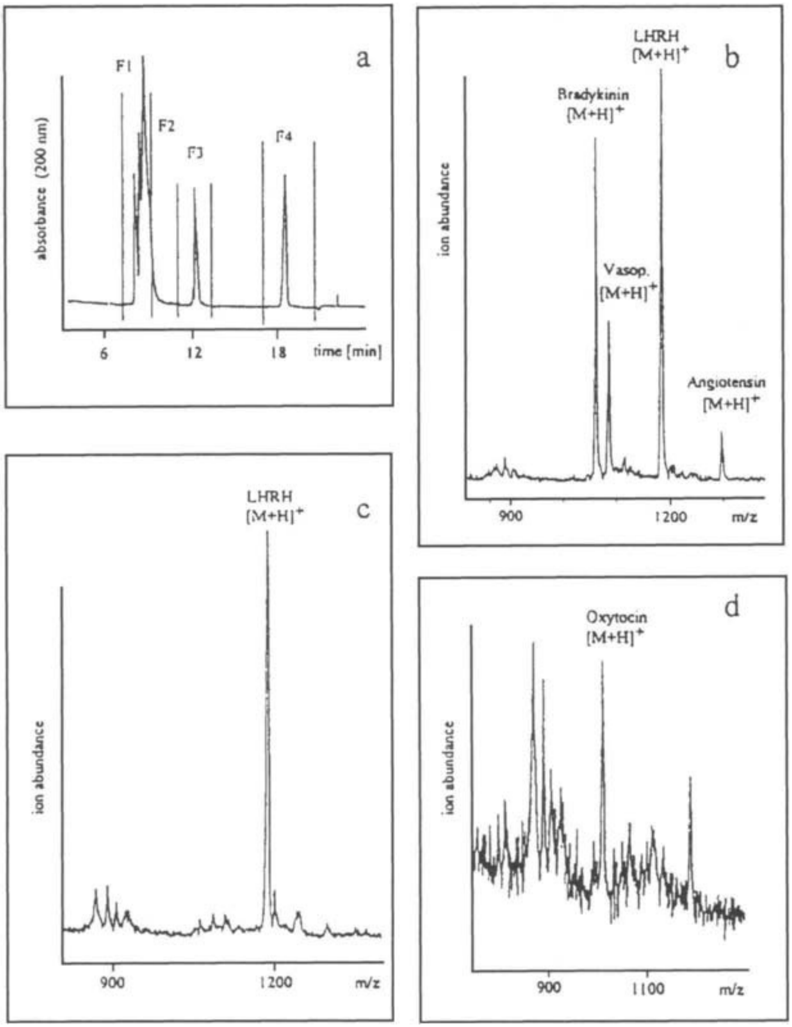


Fig. 11. CE-MALDI-MS of a mixture of six peptides. (a) CE separation of six peptides (angiotensin, bradykinin, Arg^8-vasopressin, luteinizing hormone-releasing hormone (LHRH), oxytocin and sleep-inducing peptide) on an underivatized fused-silica capillary; (b–d) MALDI spectra of (b) unresolved fraction F1 containing the most basic peptides, bradykinin, angiotensin, Arg^8-vasopressin and LHRH; (c) fraction F2 containing LHRH; (d) fraction F3, oxytocin. (Reproduced with permission from Weinmann *et al.*, 1994b.)

Table 3. Properties of UV/fluorescent Derivatization Reagents for Peptide Analysis by CE

Derivatization Reagent	Abbreviation	UV- λ_{max} FL-$\lambda_{ex}/\lambda_{em}$ (nm)	Reacts with:	References
3-(4-carboxy-benzoyl)-2-quinoline carboxaldehyde	CBQCA	FL-442/550	primary amines	Kuhn and Hofstetter-Kuhn, 1993; Toulas and Hernandez, 1992
ortho-phthalaldehyde	OPA	FL-340/450	primary amines	De Antonis *et al.*, 1994; Kuhn and Hofstetter-Kuhn, 1993; Toulas and Hernandez, 1992
naphthalene-2,3-dicarboxaldehyde	NDA	FL-440/490	primary amines	De Antonis *et al.*, 1994; Kuhn and Hofstetter-Kuhn, 1993; Toulas and Hernandez, 1992
4-phenylspiro[furan-2(3H), 1'-phthalan]-3,3'-dione	fluorescamine	FL-390/450	primary amines	De Antonis *et al.*, 1994; Kuhn and Hofstetter-Kuhn, 1993; Xue and Yeung, 1994
fluorescein isothiocyanate	FITC	FL-494/525	primary and secondary amines	Kuhn and Hofstetter-Kuhn, 1993; Toulas and Hernandez, 1992
9-fluorenylmethyl-chloroformate	FMOC	FL-260/305	primary and secondary amines	De Antonis *et al.*, 1994
ninhydrin	ninhydrin	FL-390/475	primary and secondary amines	De Antonis *et al.*, 1994
dansyl chloride	Dns-Cl	FL-325/600	primary and secondary amines	De Antonis *et al.*, 1994; Kuhn and Hoffstetter-Kuhn, 1993
6-aminoquinolyl-N-hydroxysuccin-imidyl carbamate	AQC	FL-250/395	primary and secondary amines	De Antonis *et al.*, 1994
benzoin	benzoin	FL-325-440	arginine-containing peptide	Cobb and Novotny, 1992a
4-methoxy-1,2-phenylenediamine	N/A	FL-330-438	tyrosine-containing peptide	Cobb and Novotny, 1992a

and CBQCA react selectively with primary amines to produce fluorescent derivatives, whereas 9-fluorenylmethoxycarbonyl (FMOC), ninhydrin, phenyl isothiocyanate (PITC), fluorescein isothiocyanate (FITC), dansyl and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) react with both primary and secondary amines (Toulas and Hernandez, 1992).

If the protein or peptide being analyzed contains a tyrosine or arginine group, other reagents can be employed. Benzoin reacts selectively with arginine, and the derivative can be detected using laser-induced fluorescence (LIF) with an excitation wavelength of 325 nm and an emission wavelength at 440 nm. This makes it compatible with the He-Cd laser. Table 4 shows lasers commonly used in fluorescence detection and their available excitation wavelengths. Proteins and peptides containing tyrosine can be selectively detected using 4-methoxy-1,2-phenylenediamine, which reacts in $CHCl_3$ with tyrosine to give a stable derivative with an excitation wavelength at 330 nm and an emission wavelength at 438 nm. It can also be readily detected by CE-LIF with the He-Cd laser (Cobb and Novotny, 1992a). Another fluorescent derivatization reagent, tetramethyl rhodamine isothiocyanate isomer-5 (TRITC), is useful since it reacts primarily with primary amines found on peptides and proteins. However, care must be taken to avoid reaction with amine contaminants. Larmann *et al.* (1993) derivatized tryptic peptides of horse heart cytochrome *c* with a TRITC label. This label gives an excellent match for the 543.5 nm line of the green He-Ne laser.

Table 4. Common Lasers Used for LIF Detection of Derivatized Peptides (Guzman, 1993)

Laser Type	Abbreviation	λ_{ex} available (nm)
Helium-Neon	He-Ne	543, 633
Argon ion	Ar	257, 275, 305, 334, 350, 364, 459, 488, 514
Helium-Cadmium	He-Cd	325, 442

The most commonly employed reagents for CE-LIF are NDA, CBQCA, OPA and FITC, since they are compatible with the laser wavelengths available. However, OPA derivatives are not particularly stable and, therefore, the reactions must be carefully timed to obtain accurate results (De Antonis *et al.*, 1994). OPA is most useful for analytes containing lysine because N-terminus-labeled derivatives of peptides are not fluorescent and yield very low quantum efficiencies (deMontigny *et al.*, 1987; Lunte and Wong, 1990; Soper *et al.*, 1990). Although the excitation maximum for OPA is close to the 325 line of the He-Cd laser, it is not exact and, therefore, not extremely sensitive for LIF detection (Toulas and Hernandez, 1992). This reaction is also pH dependent and conditions tend to vary from analyte to analyte (De Antonis *et al.*, 1994). FITC is known for its high quantum yield; however, it can sometimes be unstable in the derivative form (De Antonis *et al.*, 1994; Xue and Yeung, 1994). It is compatible with the 488 nm argon ion laser; however the large reagent peak can sometimes interfere with the separation.

Many of the reagents form reaction side products that can interfere in CE separations. NDA, in the presence of cyanide, reacts with peptides to form stable fluorescent derivatives; however, when excess NDA is present in the reaction, complicated byproducts sometimes result (Schöneich *et al.*, 1993; Toulas and Hernandez, 1992; Xue and Yeung, 1994). Fortunately, most of these byproducts are neutral. One additional problem with the use of OPA and NDA with lysine-containing peptides is that derivatization often results in a "double tag." With NDA, this results in a decreased quantum yield value of $\phi_f = 0.02$, whereas when OPA has reacted with lysine, it has a quantum efficiency of one-tenth that of the reaction with other amino acids (deMontigny *et al.*, 1987; Oates and Jorgenson, 1990). However, this "quenching" of fluorescence can be controlled by optimization of reaction kinetics. FMOC, FITC and dansyl react with water to give a large, sometimes interfering hydrolysis peak (De Antonis *et al.*, 1994).

Depending on the kinetics of the reaction, it is possible to tag an analyte with a fluorogenic agent either pre- or postcolumn. However, postcolumn CE detection is not readily available, although some systems have previously been described (Pentoney *et al.*, 1988; Rose and Jorgenson, 1988; Albin *et al.*, 1991; Rose, 1991). Since ninhydrin is unstable and can only be derivatized postcolumn, it is not useful for CE separations (De Antonis *et al.*, 1994, Xue and Yeung, 1994).

5.5.5. Chemometric predictions of migration times

Deyl *et al.* (1989) established an empirical relationship relating relative migration times (t_m) with the pI and molecular mass of a compound. This relationship, $t_m = M^{2/3}/z$, was found to be almost linear with a relatively wide range of proteins (100,000–600,000 daltons. However, it was found that the model was less useful for larger, more complex peptides and proteins. Chen *et al.* (1993a) described a relationship, shown in Eq. (2), that could be applied to peptides of up to four residues.

$$t_m = 6\pi L\eta b \frac{M^{1/2}}{Eq} \tag{2}$$

where:
L = capillary length
η = buffer viscosity
b = a constant
M = molar mass of the solute
E = electric field strength
q = net charge of the ionic cloud

Chen further refined this relationship using 37 model peptides varying in length from two to four amino acids. This relationship correlates migration time with the physiochemical properties of peptides:

$$t_m = m_0 + m_1 \frac{(M^{1/2})}{q} \tag{3}$$

where:
m_1 = $6\pi\eta bL/E$
m_0 = migration time for $M = 0$ species

The above relationship appears to work well for small peptide chains of only a few amino acid residues. However, in the case of more complex proteins and polypeptides, there is often increased interaction between the analyte and the electrical double layer along the capillary surface, and the extent of hydration and conformation of the peptide also begin to play a role (Chen *et al.,* 1993). Altogether, these features make the prediction of migration times more challenging. For precise predictions, the net charge of the peptide at a given pH must be known exactly. The Henderson-Hasselbach equation can be used to predict this if the exact pH of the run buffer and accurate pK_a values for the ionizable groups are known; however, the pK_a information is not always readily available.

Finally, Chen developed a relationship for the prediction of migration times of slightly more complex peptides. This equation relates the migration time to the number of positively charged ionizable groups on the molecule and takes into account electrostatic interactions with the fused silica (Chen *et al.,* 1993)

$$t_m = m_0 + m_1 \frac{M^{1/2}}{Q} \tag{4}$$

where Q = number of positively ionizable groups.

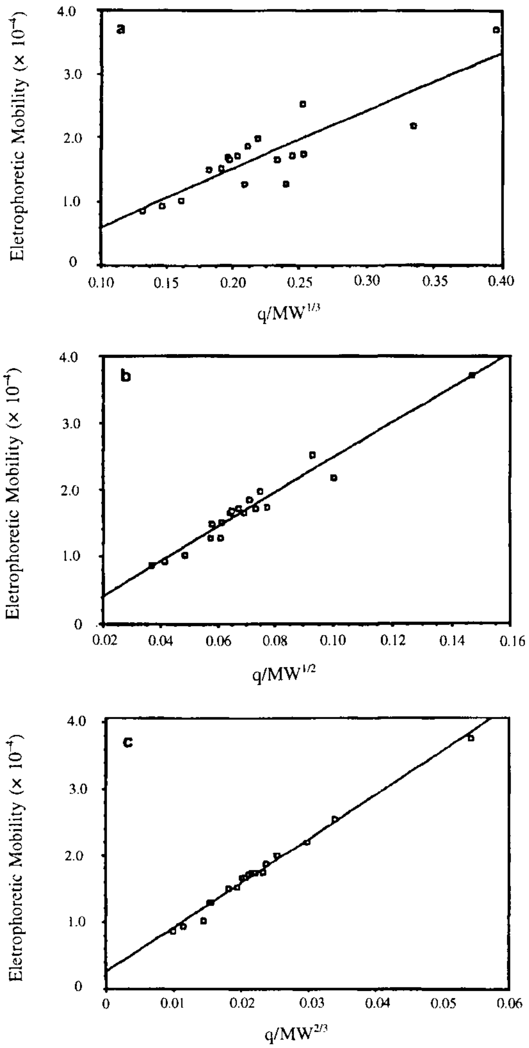
Rickard *et al.* (1991) also investigated the relationship of electrophoretic mobility with the physiochemical properties of peptides with the goal of predicting the migration times of peptides in protein digests. Three possible relationships were evaluated with respect to electrophoretic mobility. These were based on the radius of gyration, which relates chain length to MW, Stokes' law, which relates the radius of the sphere to MW, and Offord's relationship. As shown in Fig. 12, the best fit for peptide separations was found to be that based on Offord's relationship, where $q/MW^{2/3}$ is proportional to electrophoretic mobility. In this case, the assumption is that the peptide is spherical in shape. Tryptic digests of human growth hormone (hGH) were used to verify Offord's relationship for prediction of migration times (Rickard *et al.,* 1991). Offord's relationship was found to be the most accurate in predicting migration times when compared to the other methods tested.

5.6. *Recombinant Protein and Peptide Analysis*

Recombinant proteins: Biotechnology provides a powerful means by which to generate larger quantities of therapeutic proteins. However, problems with incorrect folding, improper formation of the native disulfide bonds or the formation of degradation products (such as oxidized methionine residues) may occur. In the latter case, the impurities formed can be extremely difficult to detect (Nashabeh *et al.*, 1994).

Nashabeh *et al.* (1994) analyzed IGF-I, a basic polypeptide with 70 aa and 3 disulfide bonds. An aqueous/organic run buffer and both zwitterionic additives and hydrophobic selectors were added; a neutral hydrophilic capillary coating was employed in order to improve the separation quality. Shimura and Karger (1994) used an "affinity probe" to separate the singly and doubly deamidated species from the native met-rhGH. In this case, a kinetically stable fluorescent complex is formed between the hinge thiol of the Fab fragment of a mouse monoclonal antibody and the fluorescent label tetramethylrhodamine-iodoacetamide. The sample is then capable of reacting with the analyte and can be separated by CE and detected by LIF. Nielsen *et al.* (1989) separated 19 peptide fragments after protein digestion of human growth hormone (hGH). In Fig. 13, tryptic maps of hGH by LC and CZE are compared.

Peptide mapping: Peptide mapping is a very useful method for structural determination of proteins. It involves the use of enzymes or chemicals to cleave a protein at a specific site, breaking it up into smaller segments for determination of the integrity of the peptide fragments. The smaller the peptide fragment, the more sensitive the



(a) mobility vs. $q/MW^{1/3}$ $y = 9.09e - 4x - 3.17e - 5$ $r = 0.768$
(b) mobility vs. $q/MW^{1/2}$ $y = 2.58e - 3x - 1.11\,e - 5$ $r = 0.955$
(c) mobility vs. $q/MW^{2/3}$ $y = 6.50e - 3x + 2.45e - 5$ $r = 0.989$

Fig. 12. Fit of electrophoretic mobility (cm^2/Vs) vs charge to size parameter for hGH digest separated in pH 2.35, 0.1 M glycine buffer. Equations and correlation coefficients for the linear least squares lines are given. Data are plotted for various mobility models using Shields' values. (Reproduced with permission from Rickard *et al.*, 1991.)

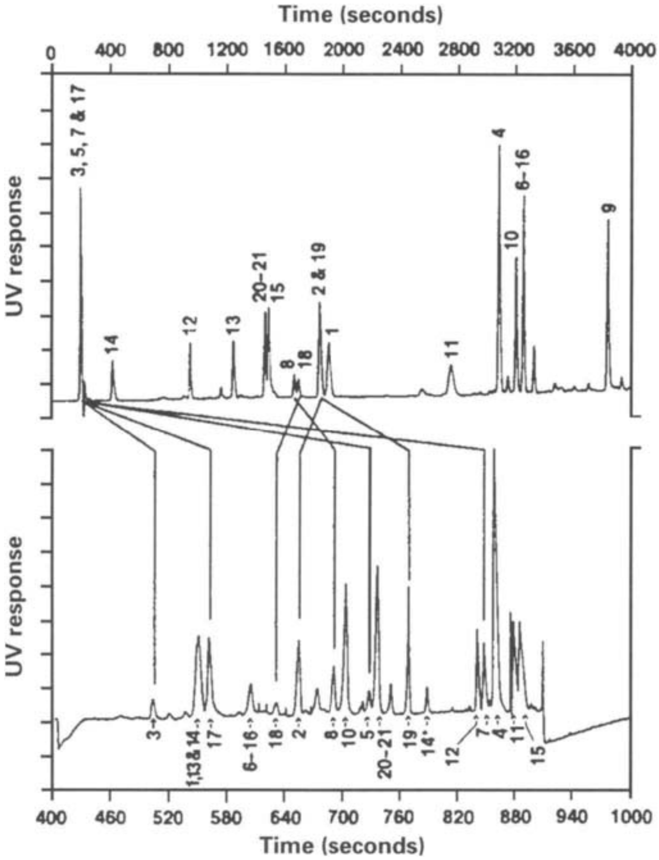


Fig. 13. Comparison of RP-HPLC and CZE separation. Differences in selectivity for these two techniques are illustrated. (Top) Tryptic map by RP-HPLC; (bottom) tryptic map by CZE. (Reproduced with permission from Nielsen *et al.*, 1989.)

the technique will be to subtle differences in structure (Nielsen *et al.*, 1989). Since it is well established that large proteins absorb to the walls of capillaries and cause band broadening, tailing, and variations in migration times, protein digestion simplifies the analysis of proteins. The peptide map is characteristic of the particular protein and can be used for a qualitative determination of the primary structure of the protein as well as its purity (Cifuentes and Poppe, 1994).

Peptide mapping has been successfully performed by both RPLC and CE. However, since RPLC is dependent on the hydrophobic character of the analyte and proteins and peptides are generally intrin-

intrinsically amphipathic (Rudnick *et al.*, 1994), the separation is often dependent on a small region of the molecule. This means that it may be impossible to separate similar proteins and peptides by means of RP-LC. CE, with its minimal sample requirement and high efficiency, is well suited for proteolytic mapping of proteins. In CE, the migration order is dependent on the charge/mass ratio, making it complementary to RP-LC (Bongers *et al.*, 1992).

The main disadvantages of protein digestion for protein structure determination is poor reproducibility. Therefore, it is critical that the conditions be well defined in order to avoid incomplete and/or overdigestion of peptides where there are too many fragments. Other crucial parameters that must be carefully controlled to obtain reproducible results include pH, temperature, protein:enzyme ratio, and reaction time (Chang and Yeung, 1994). An on-line approach to peptide digestion has been shown to maintain higher reproducibility in the system (Amankwa *et al.*, 1993).

In peptide mapping, the protein is denatured in order to expose reaction or cleavage sites. Then the disulfide bonds are eliminated via reduction, alkylation, or oxidation. It should be noted that if this step is incomplete, the third step of the fragmentation will probably also remain incomplete. The next step is the fragmentation of the protein by an enzyme or a chemical agent. Reagents used for this purpose include trypsin, chymotrypsin, cyanogen bromide and pepsin (Cobb and Novotny, 1992a).

Amankwa *et al.* (1993) immobilized trypsin on the interior of a fused silica capillary and monitored on-line protein digestion using CE. This approach has several advantages: the microreactor can be utilized for multiple injections, the sample is free from contamination because of the enclosed system, and there is minimal sample handling. Also, unlike previous systems, such as that developed by Guzman (1992), this technique utilizes different buffer components and pH values during the enzymatic reaction and the separation phase. This is done by coupling the two capillaries via a fluid joint, allowing a second buffer system to be used. Chang *et al.* (1994) looked at the separation of nine fragments obtained from a tryptic digest of recombinant porcine pro-growth hormone-releasing hormone (rp-GHRH) and obtained excellent resolution.

UV is the most common mode of detection for peptide mapping; however, it can result in very complex maps. Because many species absorb at 210–220 nm, it is not very selective and the results can be difficult to interpret. Therefore, derivatization of selected fragments gives a much cleaner electropherogram from which it is easier to elucidate protein structure (Cobb and Novotny, 1992a). For example, a selective derivatization reagent with benzoin can be used to identify arginine-containing peptides with fluorescence detection. Native fluorescence can also be used for Trp-, Tyr- and Phe-containing peptide fragments. More recently, Chang and Yeung (1993) used pepsin for the on-line digestion of β-lactoglobulin and CZE-LINF to obtain the high sensitivity and selectivity needed for the analysis.

5.7. Analysis of Single Cell Constituents

The capability of CE to combine small sample injections with high efficiency and low detection limits makes it attractive for the analysis of single cell (Kristensen *et al.*, 1994). The possibility of determining the chemical and biochemical composition of single cell could aid in the discovery of the functions of organisms, the morphology and physiology of identical cells, the diagnosis of early stages of diseases, specific cellular traits such as disease resistance, cell-to-cell variation, and the presence of alternative cellular subclasses (Hogan and Yeung, 1992; Xue and Yeung, 1994).

However, one of the main challenges in cellular analysis is that the majority of cellular components are not naturally fluorescent. The low degree of selectivity of indirect detection thus leads one to investigate more sensitive and selective detection methods. A few alternatives to indirect detection include electrochemical detection of electroactive cell components or microderivatization of components combined with UV or fluorescence detection (Hogan and Yeung, 1992; Gilman and Ewing, 1995).

Hogan and Yeung (1992) were able to detect both fluorescent and nonfluorescent components within a single cell utilizing both electrochemical detection and microderivatization of intracellular components coupled to LIF. Using fluorescence detection, they were able to detect levels of a physiological peptide, glutathione. They noted that when the cell came into contact with the low ionic strength run buffer, the cells would automatically lyse and release their intracellular fluid, which then permitted separation of the distinct the ionic species within the cell. More information on the analysis of single cell is found in Chapter 12.

6. Conclusion

Capillary electrophoresis is a useful technique for peptide separations, which is complementary to current LC methodologies such as RP-LC. The applications of CE to protein and peptide analysis include separations of glycopeptides, pharmaceutical peptides and recombinant proteins for peptide mapping.

Although analysis of basic peptides can sometimes be complex and cumbersome compared to that of typical hydrophobic or acidic peptides, the use of run buffer additives such as dynamic coatings or micellar pseudophases can be used to reduce adsorption.

UV detection of peptides following CE separation is known to have fairly low sensitivity. However, other detection techniques such as electrochemistry, mass spectrometry and fluorescence have been used with and without precolumn derivatization for the analysis of proteins and peptides.

7. References

Amankwa, L. N. and W. G. Kuhr (1993). *Anal. Chem., 65,* 2693–2697.

Beijersten, I. and D. Westerlund (1993). *Anal. Chem., 65,* 3484–3488.

Bongers, J., T. Lambros, A.M. Felix and E. P. Heimer (1992). *J. Liq. Chromatogr., 15,* 1115–1128.

Bornsen, K. O., M. Schar and H. M. Widmer (1990). *Chimia, 44,* 412–416.

Bullock, J. A. and L.C. Yuan (1991). *J. Microcol. Sep., 3,* 241–248.

Burlingame, A. L., R. K. Boyd and S. J. Gaskell (1994). *Anal. Chem., 66,* 634R–683R.

Bushey, M. M. and J. W. Jorgenson (1990). *Anal. Chem., 62,* 978–983.

Chang, H. and E. S. Yeung (1993). *Anal. Chem., 65,* 2947–2951.

Chang, J. P., D. L. Smiley and M. R. Coleman (1994). *J. Liq. Chromatogr., 17,* 1899–1916.

Chen F. A., L. Kelly, R. Palmieri, R. Biehler and H. Schwartz (1992). *J. Liq. Chromatogr., 15,* 1143–1161

Chen, N., L. Wang and Y. K. Zhang (1993). *Chromatographia, 37,* 429–432.

Cifuentes, A. and H. Poppe (1994). *J. Chromatogr. A, 680,* 321–340.

Cifuentes, A., M. de Frutos, J. M. Santos and J. C. Diez-Masa (1993). *J. Chromatogr. A, 655*, 63–72.

Cobb, K. A. and M. V. Novotny (1992a). *Anal. Biochem.,, 200,* 149–155.

Cobb, K. A. and M. V. Novotny (1992b). *Anal. Chem., 64,* 879–886.

Cole, R. O. and M. J. Sepaniak (1991). *J. Chromatogr., 557,* 113–123.

Corradini, D., A. Rhomberg and C. Corradini (1994). *J. Chromatogr. A, 661,* 305–313.

Deacon, M., T. J. O'Shea, S. M. Lunte and M. R. Smyth, *J. Chromatogr., 652*, 377–383.

deMontigny, P., J. F. Stobaugh, R. S. Givens, R. G. Carlson, K. Srinivasachar, L. A. Sternson and T. Higuchi (1987) *Anal. Chem., 59*, 1096–1101.

De Antonis, K. M., P. R. Brown Y. Cheng and S. A. Cohen (1994). *J. Chromatogr. A, 661,* 279–285.

Deyl, Z., V. Rohlicek and R. Stuzinsky (1989). *J. Liq. Chromatogr., 12,* 2515–2526.

Emmer, A., M. Jansson and J. Roeraade (1991). *J. Chromatogr., 547,* 544–550.

Emmer, A., M. Jansson and J. Roeraade (1994). *J. Chromatogr. A, 672,* 231–236.

Giddings, J. C. (1984) *Anal. Chem., 56,* 1258A–1264A.

Giddings, J. C. (1987). *High Res. Chromatogr. Chromatogr. Commun., 10,* 319–323.

Gilges, M., M. Kleemiss and G. Schomburg (1994). *Anal. Chem., 66,* 2038–2041.

Gilman, S. D. and A. G. Ewing (1995).. *Anal. Chem., 67,* 58–64.

Grossman, P. D. and J. C. Colburn (1992). *Capillary Electrophoresis, Theory and Practice,* Academic Press, New York.

Guzman, N. A. (1992). *Sixth Annual Symposium of the Protein Society,* T-92.

Guzman, N. A. (1993). *Capillary Electrophoresis Technology, Chromatographic Series Technology, Volume 64,* Marcel Dekker, New York.

Guzman, N. A., J. Moschera, K. Iqbal and A. W. Malick (1992). *J. Chromatogr., 608,* 197–204.

Harris, D. C. (1991). *Quantitative Chemical Analysis,* pp. 195–196. W. H. Freeman, San Francisco.

Hjertén, S. and M. Zhu (1985) *J. Chromatogr., 346,* 265–270.

Hoffstetter-Kuhn, S. H., A. Paulus, E. Gassmann and H. M. Widmer (1991). *Anal. Chem., 63,* 1541–1547.

Hogan, B. L. and E. S. Yeung (1992). *Anal. Chem., 64,* 2841–2845.

Huang, X., J. A. Luckey, M. J. Gordon and R. N. Zare (1989). *Anal. Chem.,* 61, 766–770.

Idei, M., I. Mezo, Z. S. Vadasz, A. Horvath, I. Teplan and G. Y. Keri (1992). *J. Liq. Chromatogr., 15,* 3181–3192.

Issaq, H. J., G. M. Janini, I. Z. Atamna, G. M. Muschik and J. Lukszo (1992). *J. Liq. Chromatogr., 15,* 1129–1142.

Josic′, D., K. Zeilinger, W. Reutter, A. Böttcher and G. Schmitz (1990) *J. Chromatogr., 516,* 89–98.

Klyushnichenko, V. E., D. M. Koulich, S. A. Yakimov, K. V. Maltsev, G. A. Grishina, I. V. Nazimov and A. N. Wulfson (1994). *J. Chromatogr. A, 661,* 83–92.

Kristensen, H. K., Y. Y. Lau and A. G. Ewing (1994). *J. Neurosci. Meth., 51,* 183–188.

Kuhn, R. and S. Hoffstetter-Kuhn (1993). *Capillary Electrophoresis, Principles and Practice,* pp. 191–205. Springer-Verlag, Berlin, New York,.

Landers, J. P., R. P. Oda, B. J. Madden and T. C. Spelsberg (1992). *Anal. Biochem., 205,* 115–124.

Langenhizen, M. H. J. M. and P. S. L. Janssen (1993). *J. Chromatogr. A, 638,* 311–318.

Larmann Jr., J. P., A. V. Lemmo, A. W. Moore Jr. and J. W. Jorgenson (1993). *Electrophoresis, 14,* 439–447.

Lauer, H. H. and D. McManigill (1986). *Anal. Chem., 58,* 166–170.

Lee, H. G. and D. M. Desiderio (1994). *J. Chromatogr. B, 655,* 9–19.

Lemmo, A. V. and J. W. Jorgenson (1993). *J. Chromatogr., 633,* 213–220.

Liu, J., K. A. Cobb and M. Novotny (1990). *J. Chromatogr., 519,* 189–197.

Liu, J., O. Shirota and M. Novotny (1991a). *Anal. Chem., 63,* 413–417.

Liu, J., O. Shirota, D. Wiesler and M. Novotny (1991b). *Proc. Natl. Acad. Sci. USA, 88,* 2302–2306.

Loo, J. A., H. R. Udseth and R. D. Smith (1989). *Anal. Biochem., 179,* 404–412.

Mazzeo, J. R., J. A. Martineau and I. S. Krull (1993). *Anal. Biochem., 208,* 323–329.

Mosley, M. A., L. J. Derterding, K. B. Tomer and J. W. Jorgenson (1991). *Anal. Chem., 63,* 109–114.

Muijselaar, W. G. H. M., C. H. M. M. de Bruijn and F. M. Everaerts (1992). *J. Chromatogr. A, 605,* 115–123.

Nashabeh, W., K. F. Greve, D. Kirby, F. Foret, B. L. Karger, D. H. Reifsnyder and S. E. Builder (1994). *Anal. Chem., 66,* 2148–2154.

Nielsen, R. G. and E. C. Rickard (1990). *J. Chromatogr., 516,* 99–114.

Nielsen, R. G., R. M. Riggin and E. C. Rickard (1989). *J. Chromatogr., 480,* 393–401.

Okafo G. N., H. C. Birrell, M. Greenaway, M. Haran and P. Camilleri (1994a). *Anal. Biochem., 219,* 201–206.

Okafo, G. N., D. Perrett and P. Camilleri (1994b). *Biomed. Chromatogr., 8,* 202–204.

O'Shea, T. J. and S. M. Lunte (1993). *Anal. Chem., 65*, 247–250.

O'Shea, T. J. and S. M. Lunte (1994). *Anal. Chem., 66*, 307–311.

Rickard, E. C., M. M. Strohl and R. G. Nielsen (1991). *Anal. Biochem., 197,* 197–207.

Rosnack, K. J., J. G. Stroh, D. H. Singleton, B. C. Guarino and G. C. Andrews (1994). *J. Chromatogr. A, 675,* 219–225.

Rudnick, S. E., V. J. Hilser and G. D. Worosila (1994). *J. Chromatogr. A, 672,* 219–229.

Rush, R. S., P. L. Derby, T. W. Strickland and M. F. Rohde (1993). *Anal Chem., 65,* 1834–1842.

Schöneich, C., S. K. Kwok, G. S. Wilson, S. R. Rabel, J. F. Stobaugh, T. D. Williams and D. G. Vander Velde (1993). *Anal. Chem., 65,* 67R–84R.

Schnolzer, M., A. Jones, P. F. Alewood and S. B. H. Wood (1992). *Anal. Biochem., 204,* 335–343.

Schwartz, H. E., R. H. Palmieri, J. A. Nolan and R. Brown, (1991). *Introduction to Capillary Electrophoresis: Proteins and Peptides* Beckman Instruments, Vol. 2.

Shimura, K. and B. L. Karger (1994). *Anal. Chem., 66,* 9–15.

Snopek J., H. Soini, M. Novotny, E. Smolkova-Keulemansova and I. Jelinek (1994). *J. Chromatogr., 559,* 215–222.

Song, A. L., Q. Ou and W. Yu (1993). *J. Chromatogr. A, 657,* 75–183

Song, L., Q. Ou and W. Yu (1994). *J. Liq. Chromatogr., 17,* 1953–1969.

Soper, S. A., S. Chamberlin, C. K. Johnson and T. Kuwana (1990) *Appl. Spectrosc., 44,* 858–863.

Stefansson, M. and D. Westerlund (1993). *J. Chromatogr., 632,* 195–200.

Suzuki, S., K. Kakehi and S. Honda (1992). *Anal. Biochem., 205,* 227–236.

Swedberg, S. A., (1990). *Anal. Biochem., 185,* 51–56.

Sweeney, P. J., J. G. Darker, W. A. Neville, J. Humphries and P. Camilleri (1993). *Anal. Biochem., 212,* 179–184.

Terabe, S., M. Shibata and Y. Miyashita (1989). *J. Chromatogr., 480,* 403–411.

Toulas, C. and L. Hernandez (1992). *Analusis, 20,* 583–585.

Vonguyen, L., J. Wu and J. Pawliszyn (1994). *J. Chromatogr. B, 657,* 333–338.

Wahl, J. H., D. R. Goodlett, H.R. Udseth and R. D. Smith (1993). *Electrophoresis, 14,* 448–457.

Wahl, J. H., D. C. Gale and R. D. Smith (1994). *J. Chromatogr. A, 659,* 217–222.

Weber, P. L., T. Kornfelt, N. K. Klausen and S. M. Lunte (1995). *Anal. Biochem., 225,* 1–7.

Weinberger, R. (1993). *Practical Capillary Electrophoresis,* Academic Press, Boston.

Weinmann, W., C. Maier, K. Baumeister, M. Przybylski, C. E. Parker and K.B. Tomer (1994a) *J. Chromatogr. A, 664,* 271–275.

Weinmann, W., C. E. Parker, L. J. Deterding, D. I. Papac, J. Hoyes, M. Przybylski and K. B. Tomer (1994b). *J. Chromatogr. A, 680,* 353–361.

White, A. (1959) *Biochem. J., 71,* 217–220.

Wilkes, D. (1989). *Laboratory Equipment Digest,* April, 17–18.

Wu, N., B. Li and J. V. Sweedler (1994). *J. Liq. Chromatogr., 17,* 1917–1927.

Xu, Y. (1995). *Anal. Chem., 67,* 463R–473R.

Xue, Q. and E. S. Yeung (1994). *J. Chromatogr. A, 661,* 287–295.

Ye, J. and R. P. Baldwin (1994) *Anal. Chem., 66,* 2669–2673.

Zhou, J., T. J. O'Shea and S. M. Lunte (1994). *J. Chromatogr., 680,* 271–277.

CHAPTER 10

Separation of Nucleic Acids and Oligonucleotides and Sequencing of DNA Fragments by Capillary Electrophoresis

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1. Introduction

The separation of molecules in capillary electrophoresis (CE) is based on the electrically driven flow of ions in solution. In most cases, CE exhibits greater speed and better efficiency and is easier to automate than are conventional slab-gel electrophoresis or liquid chromatography (LC).

Two competing forces, electroosmotic flow (EOF) and electrophoretic mobility (μ_{ep}), are involved in conventional capillary zone electrophoresis (CZE). The EOF is generated by the ionized silanol groups on the fused silica capillary and moves in the direction of the cathode. The electrophoretic mobility is dependent on both the size and the charge of the analyte. The migration time is a result of the sum of these two forces. The effective electrophoretic mobility (μ_{eff}) is the sum of the EOF and the μ_{ep}. For cations, the μ_{ep} is positive; therefore, these molecules migrate faster. In the case of anions, the μ_{ep} is negative, causing these molecules to migrate slower than neutral compounds. Therefore, one finds a reduction in μ_{eff} in the case of nucleotides separated by CZE due to the presence of the negatively charged phosphate groups.

Micellar electrokinetic capillary chromatography (MEKC) involves the addition of a surfactant such as sodium dodecyl sulfate (SDS) to the CE run buffer. This produces micelles with hydrophobic interiors and charged exteriors. Neutral and some charged molecules, such as bases and nucleotides, can be resolved by MEKC due to differences in the partition coefficients between the bulk solution and the micelles. This technique is discussed in greater detail in Chapter 3.

Capillary gel electrophoresis (CGE) can be considered a hybrid of slab-gel electrophoresis and CE since it consists of a solid gel network covalently anchored to the capillary surface. The separation of polynucleotides in CGE is based on a combination of molecular sieving and electrophoretic movement toward the anode. However, in CGE, the electrophoretic mobility contributes very little to the separation of oligonucleotides because, as the molecular weights of DNA fragments increase, the charge on the molecule also increases proportionately. As a result, the overall *charge*/mass ratio remains nearly constant. Therefore, the primary mechanism of separation in CGE of oligonucleotides is molecular sieving, and smaller oligomers emerge prior to the larger ones.

There have been many advances in the area of nucleic acid research. The ability to sequence DNA segments of several thousand base pairs (bp) was made possible by the development of a chemical-cleavage sequencing method (Maxam and Gilbert, 1977). The range was further extended to several hundred thousand bp by the chain-termination procedure employed by Sanger *et al.* (1977). The next challenge was to overcome the difficulties encountered in the separation of the large DNA fragments produced by these methods. This was accomplished by the development of pulsed-field gel electrophoresis (Schwartz and Cantor, 1984; Carle, 1992), which permitted the separation of DNA fragments of up to 10 million bp. Other important advances include the discovery of a heat-stable DNA polymerase as well as a useful cloning vector (M13) for DNA sequencing. Finally, the invention of the polymerase chain reaction for amplification of DNA has made possible the generation of large quantities of DNA from a single strand (Mullis and Falcona, 1987).

Current research is focused on methods for rapid separation of the large DNA fragments found in higher organisms and the analysis of minute amounts of DNA. Some degree of success has been achieved using agarose gel electrophoresis; however, this technique has many limitations. CGE appears ideally suited to this type of analysis because of its low sample volume requirements and ability to separate molecules based on their size. The efficiency of heat dissipation in capillaries is much higher than in slab gels; therefore, the ability to use higher electric fields in CGE speeds up the separation process. However, this is accompanied by a loss of resolution of large DNA fragments. Linear polyacrylamide in CE has been successfully applied to separate DNA fragments (Pentoney *et al.,* 1992; Ruiz-Martinez *et al.,* 1993). The ultimate solution will depend on the integration of separation methods developed for chain-terminated fragments with the procedures that provide simultaneous on-line identification of the terminal residues. Several research groups are currently working in this area (see Cohen, 1993; Luckey *et al.*, 1993, Manabe *et al.*, 1994; Bruin and Paulus, 1995 for recent review articles on this subject).

Purine and pyrimidine bases cannot be separated by CZE because these molecules bear no charge. However, nucleotides can be successfully resolved by MEKC because they exhibit varying degrees of hydrophobicity. Nucleotides have also been separated using CZE through the addition of polymers to the separation medium. Nucleoside phosphates carrying one or more negative charge have been resolved by both CZE and MEKC, as well as by addition of polymers in the separation medium. Smaller oligonucleotides can be resolved by MEKC; however, larger oligonucleotides, polynucleotides, and DNA fragments can be separated only by CGE or polymer solution capillary electrophoresis (PS-CZE).

In this chapter, applications of CGE and PS-CZE are described in sections 2 and 3, respectively, and the effect of electrical potential and detector flow cells on resolution in sections 4 and 5, respectively. The separation of bases, nucleosides, including anti-viral drugs, and nucleotides is summarized in section 6 while examples of oligonucleotide separations are given in section 7. The separation of DNA fragments derived from restriction endonucleases by different modes of CE is described in section 8, and contemporary approaches to DNA sequence analysis by CE are described in section 9.

2. Capillary Gel Electrophoresis

The separation of many important biomolecules including large DNA fragments and SDS-proteins is not possible by free solution CE (Cohen *et al.*, 1987b, 1988a). However, CGE using cross-linked rigid gel matrices has been successfully employed for many of these separations. CGE has several advantages over conventional slab gel electrophoresis for biopolymers. These include automated injection, high separation efficiencies and ease of quantitation. It also has advantages over conventional CZE for the separation of biopolymers, including the absence of EOF, reduced adsorption of solutes to the capillary surface, and size selectivity. Pioneering research in this area was performed by Karger *et al.* (1989), Guttman *et al.* (1990), Drossman *et al.* (1990) and others (Swerdlow and Gesteland, 1990; Zagursky and McCormick, 1990; Smith, 1991). It is now possible to sequence DNA fragments by CGE. However, there are also some drawbacks to the use of CGE, including restrictions on sample size, variable day-to-day performance and limited life span of the gels, and increased cost of operation compared to slab gel electrophoresis. (For review articles on this subject refer to Li, 1992; Guttman, 1992; Cohen *et al.*, 1993; Schomburg, 1993.)

2.1. Polyacrylamide Gel Electrophoresis

Most CGE separations use capillaries filled with cross-linked polyacrylamide gels. This material is most popular because of its electrical neutrality. Generally, the composition of the gel is described by %T, the total amount of acrylamide (on a weight basis) and %C, the amount (on a weight basis) of the cross-linking agent. These values are calculated as follows:

$$\%\mathrm{T} = [\text{acrylamide (g)} + \text{cross-linking agent (g)}]/100$$

$$\%\mathrm{C} = \{[\text{cross-linking agent (g)}]/\text{acrylamide (g)} + [\text{cross-linking agent (g)}]\} \times 100$$

Bisacrylamide is the most commonly used cross-linking agent, although many other materials are available. By varying the %T and %C, significantly different gel properties may be generated. Gels with lower %T (and or %C) will have larger pore size and provide greater speed of migration; on the other hand, as %T (or %C) decreases, so also does the resolution. Even polyacrylamide gels with relatively low monomer content (%T) provide separations of very high efficiency. For example, 30×10^6 plates per meter were reported for a 160-mer oligonucleotide (Guttman *et al.*, 1990).

In CGE, the gel matrix effectively abolishes electroosmotic flow; therefore, separations are based primarily on charge and mass differences. Due to the high separation efficiencies obtainable with this technique, it is often possible to separate oligonucleotides that differ by only a single base-pair (Drossman, 1990; Guttman, 1992). Therefore, CGE provides the opportunity for direct sequence analysis of oligonucleotides. This is also useful for the analysis of antisense oligonucleotide drugs. Phosphorothioate oligonucleotides have been analyzed in biological fluids (Bourque and Cohen, 1994) and in pharmaceutical formulations by CGE (Srivatsa *et al.*, 1994; Vilenchik *et al.*, 1994). Detection of low-level impurities in polynucleotide samples is becoming routine (Cohen *et al.*, 1988a). It is also possible to determine molecular size by CGE. Linear plots of migration time *vs.* molecular size have been obtained over the range of 30–160 bases (Paulus *et al.*, 1990).

2.2. *Comparison of Capillary Gel Electrophoresis with LC and Slab Gel Electrophoresis*

The separation of oligoadenylates by reversed-phase chromatography (Singhal *et al.*, 1976) and CGE (Baba *et al.*, 1991a,b, 1992) has been reported. However, the resolution achieved by CGE was found to be far better than that which could be obtained by LC. Figure 1 shows a separation of a mixture of polyadenylates (up to 60-mer) differing by only one residue using CGE. Baseline resolution of all the oligonucleotides was achieved in approximately 35 min (Baba *et al.*, 1991a).

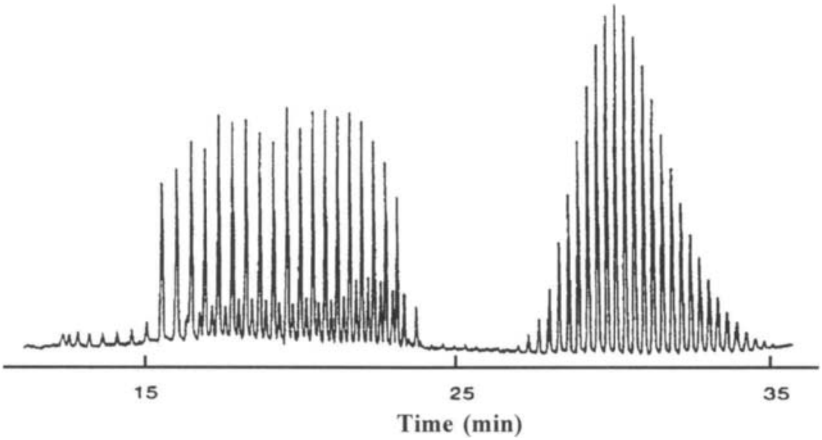

Fig. 1. Single base resolution of oligoadenylates containing from 12 to 60 nucleotides by capillary-gel electrophoresis (capillary, 72 cm × 100 mm i.d.; effective length, 42 cm; field 225 V/cm; detection at 260 nm). (Adapted with permission from Baba *et al.*, 1991a).

The size limitations for baseline separation of oligonucleotides differing by a single base-pair are 250 (CGE), 30 (reversed-phase), and 40 (ion-exchange or mixed-mode chromatography) (Baba *et al.*, 1991a). Plate numbers obtained using CGE (3×10^6 to 7×10^6 plates/m) were an order of magnitude higher than those obtained by LC (4×10^4 to 8×10^5 plates/m) (Baba *et al.*, 1991a).

The mechanism of separation using gel-filled capillaries is similar to that of slab gel electrophoresis (Paulus and Ohms, 1990). However, in contrast to slab-gel electrophoresis, CGE offers on-line sample detection, quantitation, and automation. Commercial PAGE-CE capillaries have been used for 50 consecutive injections (for more than 20 h of run time) by these workers. In general, these capillaries remain viable for approximately 40 1–h runs and are available from several vendors (ABI, Beckman, BioRad, J&W Scientific). However, it should be noted that while only one sample can be analyzed at a time using CGE, several samples can be evaluated simultaneously using slab-gel electrophoresis. Both slab-gel and capillary electrophoresis provide higher resolution for oligonucleotides than does LC.

2.3. Effect of pH on the Separation of Oligonucleotides by Capillary Gel Electrophoresis

Guttman *et al.* (1992) studied the influence of pH on the migration of single-stranded oligonucleotides in CGE. Homooligomers of equal chain length but different base composition exhibited significant differences in relative migration times when the pH of the gel and run buffer were varied from pH 6 to 8. Similar variations in migration times were observed when a gel-filled capillary column was electrophoretically equilibrated with a pH 8 buffer and then run using a buffer at pH 6. In the latter case, the current reached its maximum value after 20 min due to the long equilibration time of the gel, and a migration order identical to that obtained at pH 6.0 was obtained only after 4 h of electrophoresis. This demonstrates the importance of preparing gel-filled capillary columns in the same buffer (composition and pH) that will be used for the separation to avoid extensive time delays due to column equilibration.

2.4. The Effect of Operating Temperature

If there is insufficient heat dissipation within the capillary, the gel matrix can be destroyed. However, temperatures slightly above room temperature can be used safely for dehybridization of double-stranded DNA. Inadequate control of the temperature of the CGE system can lead to several detrimental processes. These include a non-linear dependence of the separation current on the applied voltage, variations in separation current, and a decrease in plate numbers. At high currents the electrophoretic process becomes unstable (Nelson *et al.*, 1989).

Several practical guidelines have been proposed to minimize temperature effects (Bello and Righetti, 1992). The use of a constant current for the separation has been suggested for systems lacking forced cooling because this approach minimizes Joule heating. Narrow bore capillaries are also recommended to reduce radial temperature fluctuations. A large temperature difference between the axis and the periphery is usually found in capillaries of greater than

500 µm i.d., which results in zone broadening (loss in plate numbers) because of radial viscosity and pH gradients.

2.5. The Effect of Capillary Wall Modifications

At high field strengths, the electroosmotic flow produced by the ionized silanol groups present on the inner surface of capillary can cause gels to migrate out of the tubes. However, the use of low field strengths substantially increases the analysis time. Efforts have been made to eliminate the electroosmosis by chemically bonding polyacrylamide monomer to the fused silica surface via Si-C bond (Cobb *et al.*, 1990) and via Si-O-C bond (Hjertén, 1985).

About 20% of the time, air bubbles form in the capillary during polymerization. These bubbles originate near the injection end of the capillary and can seriously disrupt the separation. In order to avoid bubble formation during gel preparation, polymerization *in situ* using a radical surface treatment has been proposed (Baba *et al.*, 1992). Another method for the preparation of bubble-free polyacrylamide gels consists of photopolymerization at a low temperature with riboflavin as the photo-initiator (Wang *et al.*, 1991). Alternatively, non-cross-linked gels can be employed (Paulus and Ohms, 1990). This includes the use of low-viscosity entangled-polymer solutions such as hydroxyethylcellulose (Grossman and Soane, 1991; Poli and Schure, 1992).

2.6. Sample Application

Electrokinetic injection is the only method that can be used with CGE. Vacuum or pressure-based injectors will force the gel out of the capillary. In CGE, samples are generally prepared in water or in solutions of low ionic strength in order to enhance their detection by peak stacking (Nathakarnkitkool *et al.*, 1992). Addition of 7 M urea in both the gel and the sample buffer inhibits hybridization of complementary single-stranded oligonucleotides. Large complementary fragments may hybridize if mixed in water at ambient temperature (Paulus and Ohms, 1990). However, if double-stranded DNA is being analyzed, urea must be eliminated from the sample, the run buffer and the gel. The size of the sample injected onto the gel directly influences the efficiency of the separation (Huang *et al.*, 1989).

There are simple procedures for concentrating dilute samples before an electrophoretic separation. For example, field enhancement can be used to stack ions in a large plug of low-conductivity buffer which precedes the high-conductivity buffer (Chien and Burgi, 1992). It has been suggested that the chaotropic effect of urea in the electrolyte is inversely related to the size of the aqueous sample slug (Paulus and Ohms, 1990).

3. Polymer Solution Capillary Zone Electrophoresis (PS-CZE)

Although non-cross-linked polymers have been used as a sieving media in centrifugation (Langevin and Rondelez, 1978) and in electrophoresis (Tietz *et al.*, 1986), PS-CZE has not been fully exploited for the separation of proteins and nucleic acids. In this case, the gel is a physical gel. Separations of DNA fragments have been achieved using both dilute solutions of linear chain polymers (Zhu *et al.*, 1989; Heiger *et al.*, 1990; Rodriguez *et al.*, 1992) and entangled polymer solutions (Barron *et al.*, 1994; Bruin and Paulus, 1995). Polyacrylamide (Heiger *et al.*, 1990), methylcellulose, and hydroxypropylmethylcellulose (Zhu *et al.*, 1989) have all been employed as sieving agents in PS-CZE for the separation of oligonucleotides and DNA fragments. These polymers produce highly viscous solutions that are not easily introduced into small diameter capillaries (50–75 μm); this is a serious drawback of PS-CZE. Polymer molecules with spherical shapes would be expected to have lower intrinsic viscosity, which would allow higher concentrations of the polymer to be introduced into the capillary.

3.1. Separation of Homooligomers vs. Heterooligomers

It is much easier to separate homopolymers than heteropolymers by CGE. For homopolymers, migration times have been found to increase linearly with the nucleotide number between 15 and 50 basepairs. However, the separation of heterooligomers is dependent on their specific base composition and, in particular, on their purine content (Baba *et al.*, 1991a).

3.2. The Influence of Linear Polymer Solution

Separations of DNA restriction fragments have been accomplished using capillaries filled with polyacrylamide with little or no cross-linking (Heiger *et al.*, 1990; Pariat *et al.*, 1993; Paulus and Husken, 1993). The separation mechanism for macromolecules using linear polyacrylamide is similar to that observed when using cross-linked polymer gels. The process could be viewed as molecules migrating through *dynamic pores* produced by the fluctuating polymer chain network (Bode, 1977a,b; Manabe *et al.*, 1994; Cheng and Mitchelson, 1994). The extent of sieving increases with increasing amount of linear polymer. However, with large amounts of polymer, increased viscosity can adversely affect the separation and lead to difficulties in handling the gel. A polyacrylamide gel consisting of 9%T was used for separation of DNA restriction fragments up to 12,000 bp (Heiger *et al.*, 1990). Capillaries with higher %T polymers have been found to be useful for the separation of single-stranded oligonucleotides (Heiger *et al.*, 1990). Pariat *et al.* (1993) reported the use of 1.5–6% linear polyacrylamide for the rapid separation of double-stranded DNA fragments ranging from 20–23,000 bp. Linear polyacrylamide gel-filled capillaries have been found to be stable for several weeks without degradation of performance. The mobility behavior of DNA fragments in capillaries filled with linear polyacrylamide has been studied and CE conditions for DNA sequencing optimized (Manabe *et al.*, 1994). Additives such as (hydroxyalkyl)methylcellulose to borate buffers have been used to create physically entangled solutions for separation of the DNA fragments (Cheng and Mitchelson, 1994).

3.3. Non-Gel-Sieving CE

Non-gel-sieving CE offers a convenient and economical alternative to CGE for many separations. The technique is simple, and the capillaries are easily filled and long-lasting. Samples can be analyzed using different injection methods with a variety of sieving systems without requiring a change of the capillary. These include agarose and cellulose. These polymers absorb very little in the short UV range and, therefore, the background is low if one is using spectroscopic detection. Separations can be carried out using either coated or uncoated capillaries. Rodriguez *et al.* (1992) explored the use of non-gel sieving CE for a variety of applications, including determination of the purity of synthetic oligonucleotides and characterization of DNA restriction fragments.

The separation of DNA fragments (up to 1353 bp) by CE using agarose solutions was reported by Bocek and Chrambach (1991). These workers could resolve even larger DNA fragments (1200 bp) by increasing the agarose concentration (Bocek and Chrambach, 1992). It was found that the agarose solutions produced fewer air bubbles than polyacrylamide gels and exhibited greater solubility and stability than cellulose-based gels.

Schwartz *et al.* (1991) investigated the use of polysiloxane-coated capillaries and polymeric buffer additives for the separation of DNA restriction fragments and polymerase chain reaction products. They proposed that the polymer produces a dynamic coating on the capillary surface, which suppresses the electroosmotic flow in addition to producing the sieving effect. Addition of ethidium bromide to the entangled polymer solution led to longer migration times and better resolution. Intercalation of GC base pairs by ethidium bromide was believed to alter the DNA conformation by opening up the helical structure.

3.4. Capillary Zone Electrophoresis—Counter-migration Hypothesis

A separation mechanism for polymer-solution CE has been proposed by Chin (1990). In highly viscous polymer solutions, small particles move faster through the polymeric mesh than do larger ones. Migration order is determined by the presence or absence of electroosmotic flow. According to the "counter-migration" hypothesis, DNA fragments migrate against the electroosmotic flow due to the large anionic character of these molecules. The two forces (electromigration and electroosmotic flow) are of unequal strength. In uncoated capillaries, the electroosmotic flow exerts a greater velocity and tends to *sweep* the molecules toward the detector (cathode). Consequently, larger fragments exit prior to the smaller ones, even though the larger fragments exhibit a greater degree of negative (phosphate) charge (Singhal and Xian, 1992; Bruin and Paulus, 1995). In the case of coated capillaries, where there is no electroosmotic flow, fragments move according to their molecular size, and small DNA fragments exit first (detection is at the anode in this case) Bruin and Paulus, 1995).

4. Control of Electrical Potential in Capillary Gel Electrophoresis

4.1. Application of Pulsed-Field Voltage

It has been shown that separations of oligonucleotide mixtures and DNA fragments can be improved by employing pulsed-fields. Pulsed field gel electrophoresis (PFGE) was first described by Cantor's group (Schwartz and Cantor, 1984) and extends the fractionation of DNA fragments to 10 million base pairs. In PFGE, different electrode (array) pairs are sequentially pulsed. Large elongated DNA molecules wriggle through the porous network of the gel toward the anode. When the direction of the electric field is changed abruptly, the DNA molecules must reorient their long axes along the new direction of the field before they can continue passing through the gel.

The time required for this reorientation depends upon the size of the DNA fragments. Large fragments require more time to reorient because they are more restricted in the gel due to their larger surface area. Consequently, small molecules migrate faster than larger molecules.

4.2. *Effect of Applied Electric Field on Mobility in CGE*

The separation of DNA by CGE is influenced by both the applied electric field and the size of the molecules being separated. For DNA fragments larger than 1,000 bp, lower fields give better resolution than higher fields, but lead to longer analysis times. To reduce the time required for analysis of DNA, pulsed-field electrophoresis force has been explored (Heiger *et al.*, 1990). In slab gel electrophoresis, it was found that DNA can align parallel to the applied electric field, and migration of aligned species results in a serpentine, *reptation* movement (Slater and Noolandi, 1989). In CGE, DNA fragments larger than those normally isolated by gel sieving can be separated due to their ability to wriggle through the gel matrix. This means that there is a size-independent migration in the gel matrix.

A combination of high and low fields can be employed to achieve separations of mixtures of small and large molecules with high efficiency. First, small fragments are separated by application of a high field, after which the larger fragments are separated by a gradual decrease of the applied field. A 20% increase in resolution was observed using pulsed-field (100-Hz) operation compared to that found for continuous field operation (Heiger *et al.*, 1990).).

An increase in resolution and a decrease in analysis times for both large and small DNA fragments have also been achieved by a sinusoidal variation of the electric field (Demana *et al.*, 1991). This appears to cause reorientation of fragments in a manner similar to PFGE.

4.3. *The Effects of Other High Field Parameters*

Separation efficiencies may be increased by reducing the amount of Joule heating caused by high currents (Chu *et al.*, 1986; Lai *et al.*, 1989). Computer-controlled high voltage equipment has been developed for both capillary electrophoresis and electrochromatography (Kansal *et al.*, 1991). This instrumentation permits the generation of various voltage waveforms with a wide range of *frequencies*, *amplitudes* and *duty cycles* (the ratio of time for high *vs.* low voltage).

The migration of charged species in electrophoresis is governed by the applied potential difference between two electrodes. In the isoelectrostatic (constant applied electric field) and isorheic (constant current) modes, the current or voltage, as required, are determined by the resistance of the capillary gel column at a given temperature. The effect of temperatures between 20°C and 50°C on the separation of restriction fragments using both modes was examined by Guttman and Cooke (1991a). They found that in the isoelectrostatic mode the plate height decreased as the temperature increased due to increased Joule heating. At low temperatures (~20°C), the gel viscosity was increased, resulting in increased resistance. According to Ohm's law, when the potential is held constant, an increased resistance is offset by a decrease in current. The lower current results in the slow migration of the analyte. At higher operating temperatures (30–40°C), the viscosity is lower, resulting in faster migration times for all analytes. In the isorheic separation mode, increasing the column temperature resulted in the fastest separations and the best resolution for the lower molecular weight fragments (< 200 kD).

5. Detection of Oligonucleotides in CGE

Detection of nucleic acids is most frequently accomplished using UV detection at 260 nm due to the high molar absorptivity of the nucleobases (pyrimidines, 10,000; purines, 14,000). Typical spectrophotometric detectors used for CE are described in detail in Chapter 4 of this book. In order to increase the effective pathlength and, thus, the sensitivity for detection of oligonucleotides, a z cell can be employed (Singhal *et al.*, 1992; Xian, 1994). This design increases the sensitivity, but also results in a large loss in efficiency and resolution for the CE separation.

A laser-induced fluorescence detector employing a sheath flow cuvette has been used by Swerdlow *et al.* (1990) and others (Manabe *et al.*, 1994) for DNA sequencing. This configuration minimizes the background noise generated by scattered light from both the gel and the capillary. A serious problem of this method is band-broadening, which was found to produce a significant loss of resolution for DNA fragments above 140 bp. However, the sensitivity is quite good, and a mass detection limit of 10^{-20} mole for fluorescein-labeled DNA fragments has been reported (Swerdlow *et al.*, 1990; for a recent review of laser-induced fluorescence detection, see van de Nessa *et al.*, 1995).

Fluorescent phosphorothioate oligonucleotides, ranging from 10 to 25 bases in length, have been separated in less than 45 s with up to 200,000 theoretical plates using a planar system (Effenhauser *et al.*, 1994). In this example, a highly viscous entangled polymer solution is polymerized into channels etched in a glass plate (Fig. 2). Advances such as this will play an important role in DNA sequencing.

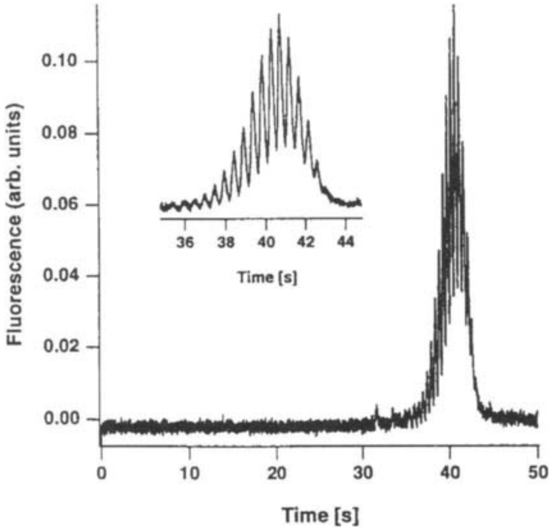

Fig. 2. Electropherogram of a fluorescein-labeled phosphorothioate oligonucleotide mixture (PS pd(T)$_{10-25}$ recorded at 2000 V/cm in a 10% T non-cross-linked polyacrylamide matrix, buffer: 100 mM Tris, 100 mM boric acid, 2 mM EDTA, 7 M urea, pH 8.5. Separation length, 3.8 cm. (Reproduced with permission from Effenhauser *et al.*, 1994.)

6. Separation of Bases, Nucleosides and Nucleotides

6.1. Capillary Zone Electrophoresis

Tsuda *et al.* (1983) were the first to report the separation of nucleotides by CZE. A complete separation of nucleoside mono-, di-, and triphosphates and cyclic nucleotides was achieved by adding 0.5% ethylene glycol to the run buffers (Tsuda *et al.*, 1988). The use of D_2O rather than H_2O in the run buffer further improved the resolution of these compounds (Camilleri and Okafo, 1991). However, because of the higher viscosity of D_2O, the analysis time is increased.

The use of CZE for the analysis of nucleotides in biological samples has been reported (Grune *et al.*, 1993), including the detection of degradation products of ATP in fish tissues (Nguyen *et* *al.*, 1990). Analysis of free intracellular ribonucleotides by CE has been attempted by Ng *et al.* (1992). Differences in the nucleotide profile were observed between cells derived from peripheral blood lymphocytes and those obtained from human leukemic cells. CZE has also been applied to the separation of cyclic nucleotides (cAMP, cGMP, and cIMP) (Hernandez *et al.*, 1990). Two simple, on-line radioactivity detectors have been described for CZE work, and separation of trinucleotides with a detection limit of low nCI range has been demonstrated (Pentoney *et al.*, 1989).

Li *et al.* (1992) studied the separation of fluorescein-ethylenediamine conjugates of the four common deoxynucleotides using CZE. They found that buffer pH was an important parameter in controlling resolution of these nucleotide derivatives. The method shows promise for the detection of DNA adducts. A more comprehensive review of the use of CE for the separation of nucleosides and nucleotides is given in Table 1.

Table 1. Separation of Nucleosides and Nucleotides

Sample	Buffer composition	Capillary length (cm)	V/cm	Reference
Mixture of nucleosides and nucleotides[a]	pH 8.5 phosphate-borate buffer + 75 mM SDS	not given	146	Row *et al.* (1987)
Mixture of nucleosides and nucleotides[a]	pH 7 phosphate-borate buffer + 0.1 mM SDS	80	175	Cohen *et al.* (1987a)
Mixture of nucleosides and nucleotides[a]	pH 7 phosphate-borate buffer + 0.3 mM SDS	65	90	Cohen *et al.* (1987b)
Mixture of nucleosides and nucleotides[a]	pH 7 phosphate buffer + 0.1 M SDS	50	480	Kasper *et al.* (1988)
Deoxynucleosides and their derivatives[a]	pH 7.1 phosphate buffer	70	408	Lahey & St. Clair (1990)
Mixture of five deoxynucleosides and anti-AIDS drugs[b]	pH 6.5 phosphate buffer + 40 mM SDS	75	384	Singhal *et al.* (1992)

cont'd.

Table 1 (cont'd.)

Sample	Buffer composition	Capillary length (cm)	V/cm	Reference
Mixture of 12 deoxynucleotide mono-, di- and triphosphates	pH 6.5 phosphate buffer + 5% Ficoll (Pharmacia)	75	385	Singhal *et al.*, unpublished data
Mixture of mono-, di- and trinucleosides[b]	pH 7.0 phosphate buffer + 0.5% ethylene glycol	42	150	Tsuda *et al.* (1988)
Inosine, hypoxanthine and inosine monophosphate[a]	pH 11, 0.1 M CAPS buffer	22 or 55	416	Nguyen *et al.* (1990)
Mixture of seven common and modified mono- and diphosphates[b]	pH 9 tricine buffer	50	328	Camilleri & Okafo (1991)
Mixture of ribo- and deoxyribonucleotides[b]	pH 8.7 phosphate buffer (50 mM) + 2 mM EDTA + ACN	95	158	Li *et al.* (1992)
Ribonucleoside mono-, di- and triphosphates in leukemic cells[a]	pH 9.4 borate buffer	57	281	Ng *et al.* (1992)
Mixture of nucleosides, mono- and dinucleotides[c]	pH 3.5 salicylate buffer	70	428	Kuhr & Yeung (1988)
Mixture of mono-, di- and trinucleosides[b]	pH 4.0 glutamate-aminobutyrate buffer with polymer-coated capillary	60	675	Dolnik *et al.* (1989)
Mixture of 12 deoxynucleoside mono-, di- and triphosphates[d]	pH 3.5 salicylate buffer	70	440	Gross & Yeung (1989)
Ribo- and deoxyribonucleoside triphosphates[d]	pH 2.5 phosphate buffer + EDTA with polymer-coated capillaries	70	318	Takigiku & Schneider (1991)

[a] Separated by MEKC.
[b] Separated by CZE with EOF.
[c] Separated by CZE without EOF.
[d] Separated by electrophoretic migration.

6.2. *Capillary Zone Electrophoresis with Reduced Electroosmotic Flow*

In CZE, the separations are based purely on the electrophoretic mobility. Acidic buffers (pH 2.7–4.3) have been employed to suppress ionization of silanol groups on the silica surface, significantly reducing the EOF (Kuhr and Yeung, 1988; Dolnik *et al.*, 1989; Gross and Yeung, 1989; Takigiku and Schneider, 1991). The electroosmotic flow can also be suppressed by coating the silica surface (Takigiku and Schneider, 1991). Reversed polarity is used, and anions migrate from the cathode to the anode (Takigiku and Schneider, 1991). Under these conditions, the order of migration is tri-, di-, and then monophosphates (Dolnik *et al.*, 1989). A series of capillary surface modifications consisting of multilayered coatings were evaluated by Smith and El Rassi (1993). These modifications resulted in capillaries with zero or constant electroosmotic flow, and permitted rapid, efficient separations of all nucleotides. O'Neill *et al.* (1994) used a neutral hydrophilic column coating (μ_{com}) to reduce adsorption and EOF for the analysis of nucleotides.

6.3. *Micellar Electrokinetic Chromatography*

In MEKC, a surfactant is added to the CE run buffer at a concentration above that of the critical micelle concentration. In this case, because electroosmotic flow prevails over electrophoretic mobility, the micelles migrate with the electroosmotic flow along with the bulk solution (see Chapter 3). Row *et al.* (1987) resolved mixtures of deoxynucleosides and deoxynucleotides by MEKC using 75 mM SDS in the buffer. Several investigators have employed higher concentrations of SDS (0.1–0.3 M) for the separation of nucleobases and deoxynucleosides (Cohen *et al.*, 1987b; Row *et al.*, 1987; Kasper *et al.*, 1988). Solutes such as purine, which are more hydrophobic, exhibit larger partition coefficients and, therefore, longer migration times. Nucleobases emerged in the following order: U, C, T, G, and then A. Comparisons between ion-pair chromatography and MEKC indicate that MEKC yielded a better separation for a mixture of fourteen nucleosides and nucleotides (Lahey and St. Claire, 1990).

A separation of a mixture of anti-AIDS drugs by MEKC is shown in Fig. 3. These compounds and their derivatives could not be separated by conventional CZE because there is little difference in their net charge. Several surfactants were examined as possible additives, including tetrabutylammonium dihydrogen phosphate (TBAP) and dodecyltrimethylammonium bromide (DTAB). Satisfactory results were obtained by using SDS.

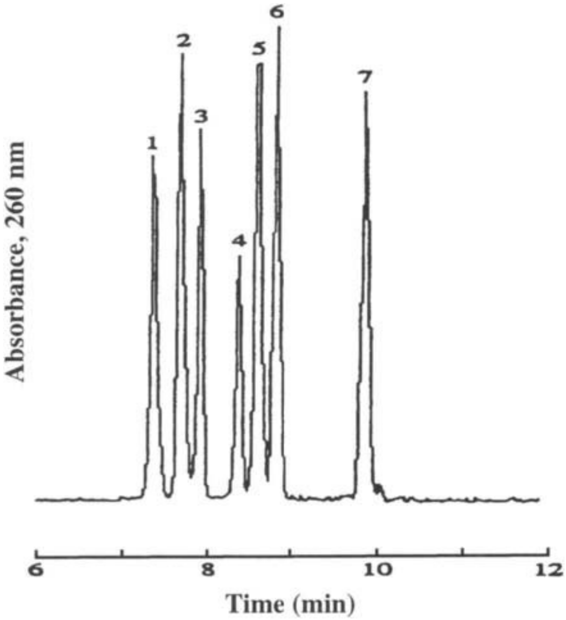

Fig. 3. The separation of a mixture of five antiviral dideoxynucleosides and Glo-azT derivative by MEKC. The mixture was applied to a fused silica capillary column (52 cm × 75 mm) and the electrophoresis carried out in a 50 mM phosphate buffer containing 40 mM SDS, pH 6.5. The separations were performed with 20 kV (116 mA) at 21° (±0.5°) and the liquid flow was monitored at 260 nm. Peaks: 1, impurities present in the synthetic ddI sample. 2, d4T, 2',3'-dideoxy-2',3'-didehydrothymidine. 3, ddC, 2',3'-dideoxycytidine. 4, ddI, 2',3'-dideoxyinosine. 5, azT, 3'-azido-3' deoxythymidine. 6, ddA, 2',3'dideoxyadenosine. 7, Glo-azT, 5'-*O*-glucuronide-3'-azido-3'-deoxythymidine. (Adapted from Singhal *et al.*, 1992.)

6.4. Separations using Soluble Polymers

The use of soluble polymers in conjunction with MEKC has been investigated for the separation of nucleosides and nucleotides. A mixture of deoxynucleosides and deoxynucleotides was resolved by MEKC (Singhal and Xian, 1992) using a sucrose polymer as a run buffer additive (Fig. 4). Twelve nucleoside mono-, di-, and triphosphates were resolved, but the six neutral nucleosides migrated together. The migration order is affected slightly when polysucrose is added to

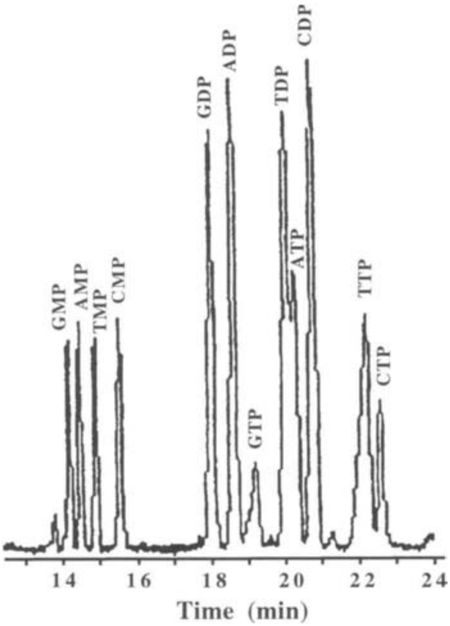

Fig. 4. The separation of nucleoside mono-, di-, and tri-phosphates by polymer CZE. A polysucrose polymer (7.5% Ficoll) was added to the buffer, which had no SDS (see legend for Fig. 3 for experimental details) (Singhal and Xian, 1992).

the run buffer (GTP and ATP appeared among the dinucleotides). The use of polyethylene glycol (0.2 kD) improved the resolution compared to sucrose, but the column efficiency was lowered. Polyethylene glycol suppressed electroosmotic flow and reversed the order of migration. The migration behavior of these analytes can be attributed to simple partition of the solute molecules within the network, but not to sieving effects. An increase in the viscosity of the run buffer plus chemical interactions with the branched polymer may be responsible for differential migration of the solutes.

Globular and highly branched polymers such as polysucrose allow for partitioning, while separations using linear polymers such as hydroxyethylcellulose are based only on entanglement. Under MEKC conditions, a polydextran (dextran T70) and a polysucrose (Ficoll 400) of the same viscosity produced nucleotide separations with different orders of elution. These results seem to confirm that chemical interaction with the polymer is the most important parameter in these separations.

7. Separation of Oligonucleotides

CE has been employed for the separation of nucleic acids ranging from small oligonucleotides to DNAs (linear and circular) of greater than 2000 bp (Kasper *et al.,* 1988). The separation of oligonucleotides by CE has been successfully accomplished with continuous non-partitioning buffers as well as with buffers of complex compositions. Oligonucleotide separations by CGE and PS-CZE have been described earlier in this chapter and are summarized in Table 2.

7.1. Metal-SDS Micellar Surface Interactions

Oligonucleotides cannot be resolved by MEKC alone because both SDS and the solute molecules are negatively charged. Ligand exchange has been successfully employed for CZE separations of oligonucleotides. Metal cations such as Cu^{2+}, Zn^{2+} and Mg^{2+} have been used in combination with MEKC for the separation of oligonucleotides composed of 9 to 12 bases. These ions adhere electrostatically to the surface of anionic micelles and offer a positively charged surface for complexation with oligonucleotides (Cohen *et al.*, 1987b). Metal ions also change the magnitude of the EOF due to their adsorption interaction with the ionized silanol groups in the capillary. The type of metal ion influences the selectivity of the MEKC separation (Cohen *et al.*, 1987b), with Zn^{2+} giving the best separation.

The combination of MEKC and metal complexation yields separations of very high efficiency. A separation of 14 of 18 oligooctamers is shown in Fig. 5 (Cohen *et al.*, 1987b). This high efficiency is a result of the rapid complexation kinetics in concert with partitioning of the molecules with the micelles. Migration times were found to be very reproducible (1% relative standard deviation) (Cohen *et al.*, 1987a) using this technique. The use of mixtures of surfactants may allow further refinement of the micelle surface and could be useful in controlling the distance between ligand-exchange centers. Oligonucleotides of varying sizes (Paulus and Ohms, 1990; Bruin *et al.*, 1991; Demorest and Dubrow, 1991; Baba *et al.*, 1992) have been separated with buffers composed of SDS, divalent cations and urea (Cohen *et al.,* 1987a).

Table 2. Separation of Oligonucleotides and Synthetic Homo- and Heteropolymers

Sample	Buffer composition	Capillary length (cm)	V/cm	Reference
Mixture of five nucleotides[a,b]	pH 7.4 Tris-phosphate buffer (5 mM) + urea + SDS	65	308	Cohen *et al.* (1987a)
Mixture of crude and pure 20-mer oligonucleotides[a]	pH 8.6 Tris-phosphate buffer + urea	30	500	Cohen *et al.* (1987a)
Mixture of 12 polythymidines[a,b]	pH 7 Tris-phosphate buffer (0.5 mM) + urea + SDS	65	308	Cohen *et al.* (1987b)
Mixture of oligonucleotide + 8 bases[a]	pH 7 Tris-phosphate buffer + urea + SDS	60 or 80	258–308	Cohen *et al.* (1987b)
Mixture of $p(dA)_{40-60}$[d]	pH 8.3 Tris-borate buffer + urea + SDS	27	400	Cohen *et al.* (1988a)
Crude 70-mer, purified 90-mer oligodeoxynucleotide[c]	pH 8.3 + urea	30	330	Cohen *et al.* (1988a)
Mixture of $p(dT)_{4-22}$[b,c]	Phosphate buffer with or without 5% ethylene glycol	50	320–400	Kasper *et al.* (1988)
Mixture of 10 $p(dC)_n$[a,b,c]	pH 8.1 Tris-glutamic acid buffer (2:1) + SDS or spermine	50	417	Dolnik *et al.* (1989)
Mixture of $p(dA)_{40-60}$ or $p(dT)_{20-160}$[c]	pH 7.6 Tris-borate buffer + urea	35 or 100	100–300	Guttman *et al.* (1990)
Mixture of $p(dA)_{20}$ and $p(dA)_{40-60}$[d]	pH 8.3 Tris-borate buffer + urea + EDTA	60	308	Heiger *et al.* (1990)
Homo-oligonucleotides $p(dA)_{40-60}$ and synthetic hetero-oligonucleotides[c]	pH 8.6 Tris-borate buffer + urea + EDTA	60	250	Paulus *et al.* (1990)
Mixture of $p(dT)_n$ (up to 160 bases)[c]	pH 8.6 Tris-borate buffer + urea + EDTA	75	400	Paulus *et al.* (1990)

cont'd.

Table 2 (cont'd.)

Sample	Buffer composition	Capillary length (cm)	V/cm	Reference
Standard p(dT)13–50 or crude synthetic oligonucleotides[c,e]	Tris-borate buffer + urea	40	375	Paulus & Ohms (1990)
Mixture of p(A) digested by nuclease P1 and standard $p(dA)_{12-18}$, $p(dA)_{40-60}$[c,f]	pH 8.8 Tris-borate buffer + urea or Tris-HCl buffer	50	200	Baba *et al.* (1991a)
Mixture of $p(dA)_{40-60}$[c]	pH 8.85, Tris-borate buffer + 7 M urea using gel, 5% T, 5% C (25–200 μm cap. ID)	not given	not given	Bruin *et al.* (1991)
Standard mixture of $p(dA)_{14-30}$ and $p(dA)_{40-60}$[c,f]	Tris-borate buffer	42	225	Baba *et al.* (1991b)
Mixture of $p(dA)_{12,17,18}$ and $d(AGTC)_{3,6,9}$[c]	Tris-phosphate buffer with methanol, formamide, urea or ethylene glycol	51	100–400	Demorest & Dubrow (1988)
Mixture of $p(dA)_{20-150}$[c]	Tris-borate buffer + urea	75	240	Turner (1991)
Mixture of $p(dA)_{15-80}$[c]	pH 8.7 Tris-borate buffer + urea + EDTA	30	173	Wang *et al.* (1991)
Partial hydrolysates of $p(dA)_{1-40}$; $p(dA)_{40-60}$; $p(dA)_{40-90}$[c,f]	pH 8.8, Tris-borate-EDTA buffer + 7 M urea using gel, 5 or 7% T; 5% C (100 μm capillary ED)	50	200	Baba *et al.* (1991a)
Mixture of p(A) digested by nuclease P1; standard $p(dA)_{40-60}$[c]	pH 8.6 Tris-borate buffer + urea	50	200	Baba *et al.* (1992)

[a] Separated by MEKC
[b] Separated by CZE with EOF
[c] Separated by CGE
[d] Separated by CZE without EOF
[e] Separated by CGE, slab GE
[f] Separated by LC

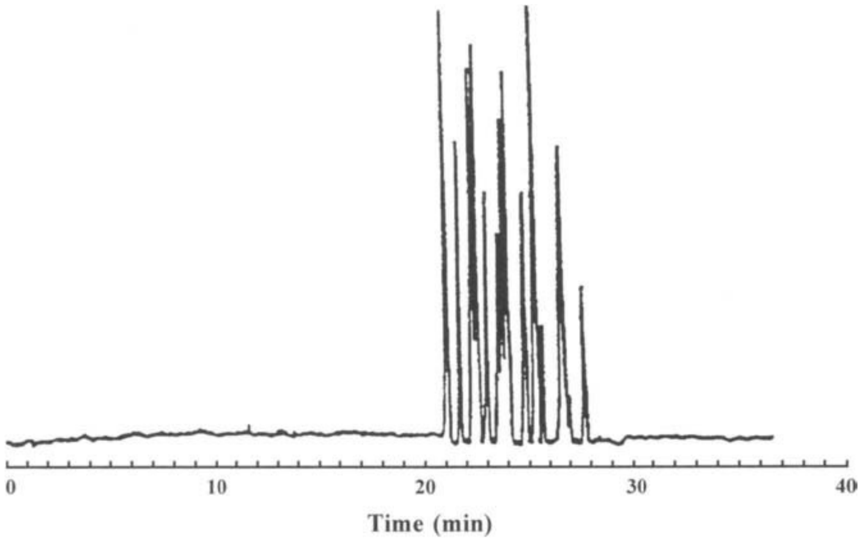

Fig. 5. The separation of 18 oligonucleotides (each containing eight nucleotides) involving MEKC and metal ion complexing. The separation is carried out in a 85-cm capillary with 22 kV/cm and in a Tris-phosphate buffer with 7 M urea, 50 mM SDS, and 3 mM Zn^{2+}, pH 7 and monitored at 260 nm (adapted with permission from Cohen *et al.*, 1987b).

7.2. Capillary Zone Electrophoresis

The effect of the composition of the run buffer on the separation of oligonucleotides by CZE has been investigated (Dolnik *et al.*, 1989). Under acidic buffer conditions, electroosmotic flow is reduced in untreated capillaries; this causes shorter oligomers to migrate toward the cathode and longer ones to migrate toward the anode (Dolnik *et al.*, 1989). The effective mobility of the oligomers is reduced as the ionic strength of the buffer increased. Additives such as spermine and SDS can improve a separation. The spermine molecule functions as an ion-pair reagent and neutralizes the negative charge of the oligonucleotide, reducing its overall electrophoretic mobility. This causes a significant decrease in the effective mobility of the larger oligomers and a reversal of migration order at a spermine concentration of near 1 mM. The SDS will increase electroosmotic flow and, in combination with spermine, result in improved resolution.

8. DNA Fragments Derived from Restriction Endonucleases

Large DNA fragments can be characterized by restriction endonucleases. These enzymes cleave double-stranded DNA with a high degree of specificity. Analysis of the resulting fragments provides information about the overall structure of the macromolecule. Slab-gel electrophoresis is usually employed for the separation of these fragments. However, while it has been used successfully for small fragments, satisfactory results have not been obtained for large fragments because low cross-linked gels are difficult to manipulate. Three different modes of CE have been employed for restriction mapping. MEKC has been used, but with only limited success. Investigators have also employed CGE and CZE with polymer additives. A brief account of each procedure is given below. Specific conditions employed for different applications are compiled in Table 3.

8.1. Micellar Electrokinetic Chromatography

The tertiary structure of DNA results in large differences in the Stokes' radius and is responsible for the frictional drag. The order of increasing "drag" is linear > open or circular > super coiled structures. Thus, differences in frictional forces in combination with small differences in charge and mass permit the separation of these molecules.

Surfactants such as dodecyl sulfate, sodium salt (SDS) or cetyltrimethylammonium bromide (CTAB) are traditionally used for MEKC separations. Baseline separation of DNA fragments is difficult to achieve, and plate numbers are generally low using these additives. Direct injection of untreated restriction fragments yields poor separation efficiencies. This can be attributed to the aggregation of sticky DNA ends. For successful separation of DNA fragments, injection of a preheated sample and rapid application of the electric field are required. It is generally believed that the differences in electrophoretic mobilities in free solution will be independent of the molecular weight of the restriction fragment.

The separation of DNA fragments has been attempted by MEKC using viscous solutions (*e.g.*, 5% ethylene glycol, 5% glycerol, Ficoll–a polysucrose–or polyvinyl pyrrolidone). However, problems with artifacts occurred as well as precipitation of buffer and subsequent loss of current. In addition, separations efficiency was poor. Attempts to separate large linear DNA by this method have failed (Kasper *et al.*, 1988).

Table 3. Separation of DNA Fragments Derived from Restriction Endonuclease Action

Sample	Buffer composition	Capillary length (cm)	V/cm	Reference
Mixture of λ DNA-Hind III and ϕ X174 DNA-Hae III	pH 8.1, 0.1 M Tris-borate buffer, 2.5 mM EDTA + 0.1% SDS	30	500	Cohen *et al.* (1988b)
Pretreated mixture of λ DNA-Hind III and ϕ X174 DNA-Hae III	pH 8.1, 0.1 M Tris-borate buffer, 2.5 mM EDTA + 0.1% SDS	30	500	Cohen *et al.* (1988b)
Pretreated mixture of λ DNA-Hind III and ϕ X174 DNA-Hind II	pH 8.1, 0.1 M Tris-borate buffer, 2.5 mM EDTA + 0.1% SDS	50	300	Cohen *et al.* (1988b)
Pretreated ϕ X174 DNA-Hind II	pH 8.1, 0.1 M Tris-borate buffer, 2.5 mM EDTA + 0.1% SDS	50	300	Cohen *et al.* (1988b)
SV40 RF DNA	pH 8.0, 9 mM Tris-borate buffer, 2.5 mM EDTA + 100 mM ethidium bromide	50	600	Kasper *et al.* (1988)
λ DNA-Hae III	pH 8.0, 9 mM Tris-borate buffer, 2.5 mM EDTA + 10 mM ethidium bromide	20	500	Kasper *et al.* (1988)
λ DNA-Hae III	pH 7.0, 10 mM NaH_2PO_4, 6 mM $Na_2B_4O_7$, 4 M urea + 10 mM CTAB[b]	40	375	Kasper *et al.* (1988)
DNA standards, 88–1746 or 123–4128 bp	TBE[a] buffer + 7 M urea	50	160	Zhu *et al.* (1989)
DNA ladder; various fragments	pH 8.3 TBE buffer + 2 mM EDTA	40	250	Heiger *et al.* (1990)
Mixture of pBR322 DNA-EcoR I/Ava II fragments	TBE buffer + EDTA	50	160	Chen *et al.* (1988)

cont'd.

Table 3 (cont'd.)

Sample	Buffer composition	Capillary length (cm)	V/cm	Reference
DNA ladder; various fragments	TBE buffer	42	140	Chin (1990)
ϕX174 DNA-Hae III fragments	TBE buffer	42	140	Baba *et al.* (1991b)
ϕX174 DNA-Hae III fragments	pH 8.5, 89 mM Tris-borate, 2 mM EDTA + hydroxymethylpropyl-cellulose + ethidium bromide	57	175	Turner (1991)
1 k bp ladder	pH 8.1, 0.1 M Tris-borate, 2.5 mM EDTA + 0.1% SDS	50	400	Strege & Lagu (1991)
1 k bp ladder	SepraGene 5000 buffer (Applied Biosystems)	50	200	Strege & Lagu (1991)
1 k bp ladder	pH 8.0, 0.1 M Tris-borate, 2.5 mM EDTA + 0.5% methylcellulose	50	200	Strege & Lagu (1991)
λ DNA-Hind III fragments	pH 8.0, 0.1 M Tris-borate, 2.5 mM EDTA + 0.5% methylcellulose	50	200	Strege & Lagu (1991)
ϕX174 DNA-Hae III fragments	pH 8.5, 0.1 M Tris-borate, 2 mM EDTA	27	varied	Guttman & Cooke (1991a)
ϕX174 DNA-Hae III fragments	pH 8.5, 0.1 M Tris-borate, 2 mM EDTA + ethidium bromide	47	varied	Guttman & Cooke (1991b)
pBR322 DNA-MspI digest fragments	pH 8.5, 0.1 M Tris-borate, 2 mM EDTA + ethidium bromide	27	100–200	Guttman & Cooke (1991b)
123 bp DNA ladder	pH 8.5, 0.1 M Tris-borate, 2 mM EDTA + ethidium bromide	27	200–300	Guttman & Cooke (1991b)
ϕX174 DNA-Hae III fragments	TBE buffer + 0.125 mM ethidium bromide	25	200	Demana *et al.* (1991)

cont'd.

Table 3 (cont'd.)

Sample	Buffer composition	Capillary length (cm)	V/cm	Reference
ϕ X174 DNA-Hae III fragments; DNA	pH 8.5, 89 mM Tris-borate, 2 mM EDTA + 0.5% hydroxypropyl-methylcellulose-4000	57	175	Schwartz *et al.* (1991)
ϕ X174 DNA-Hae III fragments	TBE buffer + 0.25% hydroxyethylcellulose, EDTA	50	301	Grossman & Soane (1991)
DNA molecules	TBE buffer with or without urea	40–60	300	Chen *et al.* (1991)
ϕ X174 DNA-Hae III fragments. 1 k bp ladder of double-stranded DNA	TBE buffer + 1.7–2.6% agarose	27	185	Bocek & Chrambach (1992)
Standard DNA fragments	TBE buffer + 0.5% hydroxyethylcellulose, EDTA	72	208	Nathakarn-kitkool *et al.* (1992)
PCR and pBR322 DNA fragments (88–1746 bp)	TBE buffer + 2 mM EDTA	35	286	Rodriguez *et al.* (1992)
PCR-amplified DNA of HIV-1 sample	pH 7.2, 8 mM NaB_4O_7, + CTAB[b] + 3.2 mM urea	60	167	Brownlee *et al.* (1990)
1 kb ladder of ϕ X174 DNA-Hae III	TBE buffer, 89 mM Tris-borate, 2 mM EDTA	30	185	Bocek & Chrambach (1992)

[a] TBE, Tris-borate-EDTA

[b] CTAB, cetyltrimethylammonium bromide

8.2. Polymer-solution Capillary Zone Electrophoresis

The best resolution of oligonucleotides has been obtained using Tris-borate buffers. Previously, this was attributed to the formation of borate complexes with the sugar moiety, but this has proven to be impossible since a cis diol is required to form this complex and deoxyribose has no such functional group (Singhal and DeSilva, 1992). In addition, complex formation will simply reduce one cationic phosphate charge by its anionic character. Furthermore, solutions of pH >9 are required in order to form a borate complex (Singhal *et al.*, 1991).

Many cations such as Mg^{2+} and R_4N^+ bind strongly with DNA and their components. CTAB has been added to the buffer to create a system similar to ion-pair LC (Kasper *et al.*, 1988). CTAB was used as a cationic surfactant for separation of AIDS DNA (HIV-1 pro-viral) isolated from blood (Brownlee *et al.*, 1990). Using the polymerase chain reaction and DNA probes labeled with a fluorescent tag provided the basis for amplification and specific detection of the DNA. Using this method, HIV-1 DNA was detected in infected human blood and in cell culture.

Figure 6 shows the separation of a complex mixture of pBR322 plasmid fragments obtained using a run buffer of 0.5% hydroxyethylcellulose in Tris-borate. An increase in resolution is observed with the addition of ethidium bromide. DNA ragments differing by only a few base pairs could be resolved by this approach (Nathakarnkitkool *et al.*, 1992).

8.3. Capillary Gel Electrophoresis

The separation of DNA fragments by CGE employs a different set of conditions than those needed for the oligonucleotides. This is because the oligonucleotides are relatively small with a reasonably simple secondary and tertiary structure, while the DNA fragments are much larger and are very rich in 2° and 3° structures. The size selectivity in CGE is a function of polymer concentration (Heiger *et al.*, 1990). The separation of DNA fragments requires the use of a large pore size in the gel. This can be achieved by reducing the cross-linking for a given polymer concentration (%T). A shorter column can be used if the polymer content is increased. However, a longer column is needed at lower polymer concentrations in order to compensate for the reduced sieving power. Thus, the column length and polymer concentration can be optimized to obtain the best separation of the DNA fragments. In addition, the use of pulsed-field electrophoresis rather than a fixed voltage can be used to further improve resolution (Heiger *et al.*, 1990) (see section 4 for details).

Harke *et al.* (1992) studied the effect of acrylamide content on the resolution of short DNA fragments (25–250 bp) by CGE. Polyacrylamide capillary gels were prepared with constant cross-linking (5% C) and acrylamide content between 2.5 and 6.0% T. Resolution of the fragments increased with acrylamide content. The intercept of the plot of migration time *vs.* fragment length was independent of the %T. The polyacrylamide pore size ranged from 2.5 to 3.5 nm in these

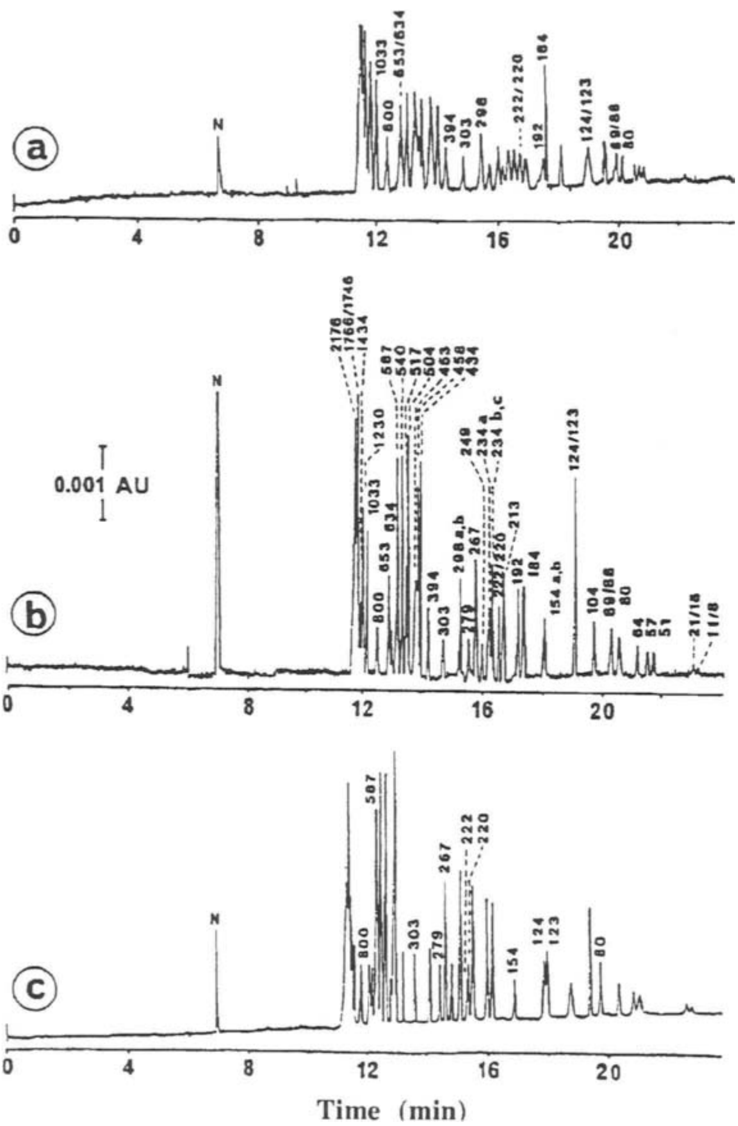

Fig. 6. Effect of ethidium bromide on the separation of DNA restriction fragments by polymer solution CE in a Tris-borate-EDTA buffer containing 0.5% hydroxyethylcellulose. (a) No ethidium bromide; (b) 1.27 μM and (c) 1.27 μM ethidium bromide, respectively. DNA fragments of pBR322 were derived from a mixture of restriction endonucleases, *Ava*II/*Eco*RI, *Hae*III and *Bgl*I/*Hinf*I. (Adapted with permission from Nathakarnkitkool *et al.*, 1992.)

gels, and the number of theoretical plates was independent of total acrylamide concentration. Plate numbers were limited by longitudinal diffusion, but not by thermal gradients. The authors presented a model that predicts retention time, plate numbers, and resolution for these short DNA fragments using acrylamide gels of this type. This model is limited to short fragments and may not be applicable to the separation of larger fragments and other gel compositions.

Guttman and Cooke (1991b) investigated the effect of ethidium bromide on the separation of DNA fragments by CGE. DNA fragments over the range of 20 to 2,000 bp, which could not be resolved in the absence of ethidium bromide, were separated in the presence of this compound. Only double-stranded DNA fragments were affected by ethidium bromide; the resolution of single-stranded homopolymers such as poly dA was not influenced.

The feasibility of performing Southern blots with on-line detection was examined in order to investigate hybridization of the DNA molecule in solution (Chen *et al.*, 1991a). An oligonucleotide with a fluorescent tag (probe) was hybridized with the desired complementary DNA segment prior to electrophoresis. The hybrid DNA migrated more slowly than the single-stranded oligonucleotides. This has been attributed to the single-stranded overhangs on the hybrid DNAs. Generally, double-stranded DNAs will migrate faster than single-stranded oligonucleotides of the same molecular size.

9. DNA Sequence Analysis by Capillary Electrophoresis

The development of rapid, accurate, and efficient DNA sequencing technology is a major challenge of the human genome project. Theoretically, automated DNA sequencers utilizing laser-based fluorescence detection in conjunction with conventional slab-gel electrophoresis can analyze 600,000 bp per day (Smith, 1991). However, the major limitation of the current DNA sequencing technology is the relatively long times required to perform the gel electrophoretic separations of DNA fragments produced in the sequencing reactions. In an attempt to overcome this problem, Lu *et al.* (1994) have constructed a simple, low-cost CGE instrument for high-speed and high-accuracy DNA sequencing. Over 500 bases of sequence were generated in 50 min. Work involving DNA sequencing by CE methods is summarized in Table 4.

Table 4. DNA Sequence Determination by Capillary Electrophoresis[a]

Sample	Buffer composition	Capillary length (cm)	V/cm	Reference
Fluorescein-labeled T or G sequencing mixture of M13mp18	pH 8.3, 90 mM Tris-borate, 0.2 mM EDTA	50	200	Drossman *et al.* (1990)
Chain-termination sequencing reaction products Primer JOE-PRM 18.1 and different templates	pH 7.6, 0.1 M Tris-borate, 2.5 mM EDTA, 7 M urea	75–90	300	Cohen *et al.* (1990)
Four fluorescein-labeled (FAM, JOE, TAMRA, ROX) sequencing reaction mixture of M13mp18 (Smith method)	TBE[b]	70	428	Luckey *et al.* (1990)
Chain-termination sequencing reaction with ddCTP; primer, fluorescein-labeled M-13; template, M13mp18	89 mM Tris-borate, 2 mM EDTA	50	180	Swerdlow & Gesteland (1990)
Chain-termination sequencing reaction mixture with ddCTP; primer, fluorescein-labeled M-13; template, M13mp18	89 mM Tris-borate, 2 mM EDTA	50	200	Swerdlow *et al.* (1990)
Chain-termination sequencing reaction mixture of M13mp18 (Richardson-Tabor-Ansorge method)	Tris-borate + SDS	48	625	Chen *et al.* (1991)
Four fluorescein-labeled (FAM, JOE, TAMRA, ROX) sequencing reaction mixture of M13mp18 (Smith method)	89 mM Tris-borate, 2 mM EDTA	41	150	Swerdlow *et al.* (1991)
Chain-termination sequencing reaction mixture of M13mp18 (Prober method)	Tris-borate EDTA, pH 8.0 in gel, 4% T and 5% C	34	465	Swerdlow *et al.* (1991)

cont'd.

Table 4 (cont'd.)

Sample	Buffer composition	Capillary length (cm)	V/cm	Reference
Chain-termination sequencing reaction mixture of M13mp18 (Richardson-Tabor-Ansorge method)	Tris-borate EDTA, pH 8.0 in gel, 6% T, 5% C, 30% formamide and 7 M urea	37	465	Swerdlow *et al.* (1991)
Chain-termination sequencing reaction mixture of M13mp18 (Prober method) with 17-mer M13 primer	89 mM Tris-borate, 2 mM EDTA	34	465	Zhang *et al.* (1991)
DNA sequencing	Tris-borate EDTA buffer, pH 8.3 + 7 M urea using capillary array with two-color fluorescence detection	25	225	Huang *et al.* (1992b)
G and T fragments from chain-termination sequencing reaction of M13mp18 with fluorescein-tagged primer	Tris-borate	40	240	Huang *et al.* (1992a,b)

[a]Effluents were monitored by fluorescence in all cases.
[b]Tris-borate buffer.

9.1. DNA Sequence Reactions and Separation of DNA Fragments

DNA sequencing relies on the separation and identification of DNA fragments which differ in chain length by a single nucleotide residue. These fragments are generated by base-specific chemical cleavages or, more commonly, by the chain-termination method. The relationship between the base-specific cleavage (or chain termination) and the chain length determines the identity of the terminal residue and, consequently, the order of the residues in the DNA fragment.

A direct comparison of automated slab-gel electrophoresis with CGE has been performed using the enzymatic dideoxynucleotide chain termination reaction (Swerdlow and Gesteland, 1990) . Fluorescently tagged oligonucleotide primers and laser-based on-column detection (limit of detection is 6,000 molecules per peak) were employed. It was shown that CGE separations occurred three times faster with better resolution and higher separation efficiency than the conventional slab-gel sequencing instrument. Agreement of measured and theoretical values for velocity, resolution, and separation efficiency indicates that further improvements might be obtained by increasing the field strength.

The separation by CGE of sequence reaction products derived from single-stranded DNA (M13mp18 phage template) allowed baseline resolution of fragments, differing in chain length by a single nucleotide and in the range of 18 to >330 residues in chain length (Cohen *et al.*, 1990). For example, the time required for the separation of a group of DNA fragments was reduced by a factor of 25 when CGE rather than the conventional slab-gel electrophoresis was used (Drossman *et al.*, 1990). An instrument has been developed for automated DNA sequencing using CGE, which takes advantage of this increase in sequence analysis throughput. This instrument uses ultra-thin capillary gels (Luckey *et al.*, 1990), and the oligonucleotide primer is covalently attached to one of four different fluorophores. Each of the four reactions is performed using a different fluorophore-conjugated primer, and the reaction mixtures are combined and loaded together.

Several goals for DNA sequence analysis have been proposed (Luckey *et al.*, 1990). First, to significantly increase the throughput by developing methods for the simultaneous analysis of multiple samples. The most obvious approach to this is employing multiple capillaries in parallel. Second, to develop CE methodologies to obtain better base resolution. Finally, to develop methodology for the analysis of very small samples. This would make it possible to eliminate the entire step of template amplification, whether *in vivo* (by bacterial growth in culture) or *in vitro* (by the polymerase chain reaction), and instead to determine the sequence directly from DNA molecules isolated from individual plaques or colonies. Progress has been made on all of these fronts (Rocheleau *et al.*, 1992; Smith, 1993; Luckey, 1994).

While CGE offers speed, efficiency and high sensitivity, separations by CZE with polymer additives (either electroosmotic or electrophoretic mode) cause the experimental error (0.2 % RSD) to exceed the increment from one base to the next (Cohen *et al.*, 1990), thus yielding irreproducible results.

9.2. Detection of DNA Fragments

The combination of CGE and laser-induced fluorescence detection offers an analytical tool with unprecedented possibilities. DNA fragments derived from sequencing reactions and labeled with a fluorescent tag can be detected at the attomole level in gel-filled capillaries. Separations with more than a million theoretical plates and detection limits of a few hundred analyte molecules have been reported. Several laser-induced fluorescence (LIF) detectors offering high-sensitivity for CGE have been reported (Swerdlow *et al.*, 1990; Zhang *et al.*, 1991; Yeung, 1993).

Three methods of detection have been developed for DNA sequencing by CGE. One method is based on a set of four primers labeled with fluorescent tags (Applied Biosystems sequencer), employing different dyes and a four-spectral channel sequencing approach. On-column detection yields detection limits of 200 amol for each primer. Sequencing rates of 300 bases/h for fragments of up to 550 bases in length have been obtained (Chen *et al.*, 1991). The second method is based on a set of four dideoxynucleotides labeled with a fluorescent tag (DuPont sequencer) and uses a two-spectral-channel sequencing approach with dyes having closely spaced absorption and emission bands. A sheath-flow cuvette postcolumn detector is used, and a detection limit of 20 amol for labeled ddNTP has been obtained. Sequencing rates of 1000 bases/h have been reported. The third detector is based on the Richardson-Tabor-Ansorge sequencing method (Chen *et al.*, 1991b). This approach uses a single fluorophore and relies on uniform labeling of the reaction product (Ansorge *et al.*, 1990; Tabor and Richardson, 1990). The fluorescein-labeled primer can be detected using LIF detection with an argon ion laser at a wavelength of 488 nm in a single spectral band. The identity of the terminal dideoxynucleotide residue is determined by the peak height. This is accomplished by varying the ratio of dideoxynucleotide triphosphates in the chain-extension reaction.

In order to enhance the speed of DNA sequencing by CGE, gels with greater stability are needed to reduce the time associated with capillary replacement. It will be necessary to operate several capillaries simultaneously to increase the speed for large-scale sequencing projects. Swerdlow *et al.* (1991) proposed the development of a 32-capillary instrument to produce sequencing rates approaching 64,000 bases/h.

Huang *et al.* (1992a,b) have shown that it is possible to perform high-sensitivity fluorescence detection of capillary arrays using a confocal fluorescence scanner. The most important advantage of this approach is that multiple samples can be easily loaded, rapidly separated in parallel, and detected with high sensitivity. The use of capillary arrays potentially resolves the throughput problems that limit the present utility of CE in DNA sequencing. Finally, capillary array electrophoresis provides an opportunity for the large-scale optimization of analytical separations. Commercially made capillary arrays could be constructed for larger-scale parallel sample analysis.

Therefore, this array method can be a valuable new technique for rapid and parallel separations (Huang *et al.*, 1992a,b).

10. References

Ansorge, W, J. Zimmermann, C. Schwager, J. Stegemann, H. Erfle and H. Voss (1990). *Nucleic Acids Res., 18*, 3419–3420.

Baba, Y., T. Matsuura, K. Wakamoto, and M. Tsuhako (1991a). *J. Chromatogr.*, 558, 273–284.

Baba, Y., M. Tsuhako, S. Enomoto, A. M. Chin, and R. S. Dubrow (1991b). *J. High Resolut. Chromatogr., 14*, 204–206.

Baba, Y., T. Matsuura, K. Wakamoto, Y. Morita, Y. Nishitsu, and M. Tsuhako (1992). *Anal. Chem., 64*, 1221–1225.

Barron, A. E., H. W. Blanch and D. S. Soane (1994). *Electrophoresis, 15,* 597–615.

Bello, M. S. and P. G. Righetti (1992). *J. Chromatogr., 606*, 103–111.

Bocek, P. and A. Chrambach (1991). *Electrophoresis, 12*, 1059–1061.

Bocek, P. and A. Chrambach (1992). *Electrophoresis, 13*, 31–34.

Bode, H. J. (1977a). *Anal. Biochem., 83*, 204–210.

Bode, H. J. (1977b). *Anal. Biochem., 83*, 364–371.

Bourque, A. J. and A. S. Cohen (1994). *J. Chromatogr. B, 662,* 343–349.

Brownlee, R. G., F. J. Sunzeri and M. P. Busch (1990). *J. Chromatogr., 533,* 87–96.

Bruin, G. J. M. and A. Paulus (1995). *Anal. Meth. Instrum., 2*, 3–26.

Bruin, G. J. M., G. Stegeman, A. C. Van Asten, X. Xu, J. C. Kraak and H. Poppe (1991). *J. Chromatogr., 559*, 163–181.

Camilleri, P. and G. Okafo (1991). *J. Chromatogr., 541*, 489–495.

Carle, G. F. (1992). In: *Pulse-Field Gel Electrophoresis* (M. Burnmeister and L. Ulanovsky, eds.), pp. 3–18. Humana Press, Clifton, NJ.

Chen, A. J. C., M. Zhu, D. Hansen and S. Burd (1988). *J. Cell Biol., 107,* 241a.

Chen, J., A. S. Cohen and B. L. Karger (1991a). *J. Chromatogr., 559,* 295–305.

Chen, D., H. P. Swerdlow, H. R. Harke, J. Zhang and N. J. Dovichi (1991b). *J. Chromatogr., 559*, 237–246.

Cheng, J. and K. R. Mitchelson (1994). *Anal. Chem., 66*, 4210–4214.

Chien, R. L. and D. S. Burgi (1992). *Anal. Chem., 64,* 1046–1060.

Chin, A. M. (1990). *Research News of Applied Biosystems #2*, 13–16.

Cobb, K. A., V. Dolnik and M. Novotny (1990). *Anal. Chem., 62*, 2478–2483.

Chu, G., D. Vollrath and R. W. Davis (1986). *Science, 234*, 1582–1585.

Cohen, A. S., A. Paulus and B. L. Karger (1987a). *Chromatographia, 24*, 15–24.

Cohen, A. S., S. Terabe, J. A. Smith and B. L. Karger (1987b). *Anal. Chem., 59*, 1021–1027.

Cohen, A. S., D. R. Najarian, A. Paulus, A. Guttman, J. A. Smith and B. L. Karger (1988a). *Proc. Natl. Acad. Sci. USA, 85*, 9660–9663.

Cohen, A. S., D. R. Najarian, J. A. Smith and B. L. Karger (1988b). *J. Chromatogr.*, 458, 323–333.

Cohen, A. S., D. R. Najarian and B. L. Karger (1990). *J. Chromatogr., 516*, 49–60.

Cohen, A. S., D. L. Smisek and P. Keohavong (1993) *Trends Anal. Chem., 12*, 195–202.

Demana, T., M. Lanan and M. D. Morris (1991). *Anal. Chem., 63*, 2795–2797.

Demorest, D. and R. Dubrow (1991). *J. Chromatogr., 559*, 43–56.

Dolnik, V., J. Liu, J. F. Banks Jr., M. V. Novotny and P. Bocek (1989). *J. Chromatogr., 480*, 321–330.

Drossman, H., J. A. Luckey, A. J. Kostichka, J. D'Cunha and L. M. Smith (1990). *Anal. Chem., 62*, 900–903.

Effenhauser, C. S., A. Paulus, A. Manz and H. M. Widmer (1994). *Anal. Chem., 66*, 2949–2953.

Gross, L. and E. S. Yeung (1989). *J. Chromatogr., 480*, 169–178.

Grossman, P. D. and D. S. Soane (1991). *J. Chromatogr., 559,* 1233–1236.

Grune, T., G. A. Ross, H. Schmidt, W. Siems and D. Perrett (1993). *J. Chromatogr., 636,* 101–111.

Guttman, A. (1992). In: *Handbook of Capillary Electrophoresis* (J. P. Landers, ed.) pp. 129–146. CRC Press, Boca Raton, FL.

Guttman, A. and N. Cooke (1991a). *J. Chromatogr., 559*, 285–294.

Guttman, A. and N. Cooke (1991b). *Anal. Chem., 63*, 2038–2042.

Guttman, A., A. S. Cohen, D. N. Heiger and B. L. Karger (1990). *Anal. Chem., 62*, 137–141.

Guttman, A., A. Arai and K. Magyar (1992). *J. Chromatogr., 608*, 175–179.

Harke, H. R., S. Bay, J. Z. Zhang, M. J. Rocheleau and N. J. Dovichi (1992). *J. Chromatogr., 608*, 143–150.

Heiger, D. N., A. S. Cohen and B. L. Karger (1990). *J. Chromatogr., 516*, 33–48.

Hernandez, L. B. G. Hoebel and N. A. Guzman (1990). *Amer. Chem. Soc. Symp. Ser. 434* (*Anal. Biotech.: Capillary Electrophor. Chromatogr.*) 50–59.

Hjertén, S. J. (1985). *J. Chromatogr., 347*, 191–198.

Huang, X., W. F. Coleman and R. N. Zare (1989). *J. Chromatogr., 480,* 95.

Huang, X. C., M. A. Quesada and R. A. Mathies (1992a). *Anal. Chem.*, *64*, 967–972.

Huang, X. C., M. A. Quesada and R. A. Mathies (1992b). *Anal. Chem.*, *64*, 2149–2154.

Kansal, A. K., W. R. Parkhurst and R. P. Singhal (1991). *J. Liq. Chromatogr.*, *14*, 97–114.

Karger, B. L., A. S. Cohen and A. Guttman (1989). *J. Chromatogr.*, *492*, 585–614.

Kasper, T. J., M. Melera, P. Gozel and R. G. Brownlee (1988). *J. Chromatogr.*, *458*, 303–312.

Kuhr, W. G. and E. S. Yeung (1988). *Anal. Chem.*, *60*, 2642–2646.

Lahey, A. and R. L. St. Claire III (1990). *Amer. Lab.*, *22*, 68–70.

Lai, E., B. W. Birren, S. M. Clark, M. I. Simon and L. Hood (1989). *BioTechniques*, *7*, 34–42.

Langevin, D. and F. Rondelez (1978). *Polymer*, *19*, 875–882.

Li, S. F. Y. (1992). In: *Capillary Electrophoresis: Principles, Practice and Applications*, pp. 423–437. Elsevier, Amsterdam.

Li, W., A. Moussa and R. W. Giese (1992). *J. Chromatogr.*, *608*, 171–174.

Lu, H., E. Arriaga, D. Y. Chen and N. J. Dovichi (1994). *J. Chromatogr. A*, *680*, 497–501.

Luckey, J. A., H. Drossman, A. J. Kostichka, D. A. Mead, J. D'Cunha, T. B. Norris and L. M. Smith (1990). *Nucleic Acids Res.*, *18*, 4417–4421.

Luckey, J. A., H. Drossman, T. Kostichka, L. M. Smith (1993). *Meth. Enzymol.*, *218*, 154–172.

Manabe, T., N. Chen, S. Terabe, M. Yohda and I. Endo (1994). *Anal. Chem.*, *66*, 4243–4252.

Maxam, A. and W. Gilbert (1977). *Proc. Natl. Acad. Sci. USA*, *74*, 560–564.

Mullis, K. and F. A. Falcona (1981). *Meth. Enzymol.*, *155*, 335–350.

Nathakarnkitkool, S., P. J. Oefner, G. Bartsch, M. A. Chin and G. K. Bonn (1992). *Electrophoresis*, *13*, 18–31.

Nelson, R. J., A. Paulus, A. S. Cohen, A. Guttman and B. L. Karger (1989). *J. Chromatogr.*, *480*, 111–127.

Ng, M., T. F. Blaschke, A. A. Arias and R. N. Zare (1992). *Anal. Chem.*, *64*, 1682–1684.

Nguyen, A.-L., J. H. T. Luong and C. Masson (1990). *Anal. Chem.*, *62*, 2490–2493.

O'Neill, K., X. Shao, Z. Zhao, A. Malik and M. L. Lee (1994). *Anal. Biochem.*, *222*, 185–189.

Pariat, Y. F., J. Berka, D. N. Heiger, T. Schmitt, M. Vilenchik, A. S. Cohen, F. Foret, and B. L. Karger (1993). *J. Chromatogr. A*, *652*, 57–66.

Paulus, A. and J. I. Ohms (1990). *J. Chromatogr.*, *507*, 113–123.

Paulus, A., E. Gassmann and M. J. Field (1990). *Electrophoresis, 11*, 702–708.

Paulus, A. and D. Husken (1993). *Electrophoresis, 14*, 27–35.

Pentoney, S. L., Jr., R. N. Zare and J. F. Quint (1989). *Anal. Chem., 61*, 1642–1647.

Pentoney, S. L., Jr., K. D. Konrad and W. Kaye (1992). *Electrophoresis, 13*, 467–474.

Poli, J. B. and M. R. Schure (1992). *Anal. Chem., 64*, 896–904.

Rocheleau, M. J., M. Josee and N. J. Dovichi (1992). *J. Microcol. Sep., 4*, 449–453.

Rodriguez, R., M. Zhu and T. Wehr (1992). *Amer. Biotech. Lab.*, 21–22.

Row, K. H., W. H. Griest and M. P. Maskarinec (1987). *J. Chromatogr., 409*, 193–203.

Ruiz-Martinez, M. C., J. Berka, A. Belenkii, F. Foret, A. W. Miller and B. L. Karger (1993). *Anal. Chem., 65*, 2851–2859.

Sanger, F., S. Nicklen and A. R. Coulson (1977). *Proc. Natl. Acad. Sci.* USA, *74*, 5463–5467.

Schomburg, G. (1993). In: *Capillary Electrophoresis, Theory and Practice* (P. Camilleri, ed.), pp. 255–310. CRC Press, Boca Raton, FL.

Schwartz, D. C. and C. R. Cantor (1984). *Cell, 37*, 6775.

Schwartz, H. E., K. Ulfelder, F. J. Sunzeri, M. P. Busch and R. G. Brownlee (1991). *J. Chromatogr., 559*, 267–283.

Singhal, R. P., G. D. Griffin and G. D. Novelli (1976). *Biochemistry, 15*, 5083–5087.

Singhal, R. P. and S. S. M. DeSilva (1992). In: *Advances in Chromatography* (J. C. Giddings, E. Grushka, and P. R. Brown, eds.), Vol. 31, pp. 293–335. Dekker, New York.

Singhal, R. P. and J. Xian (1992). 27th Midwest Regional ACS Meeting, Lawrence, KS, November 4–6, 1992.

Singhal, R. P., B. Ramamurthy, N. Govindraj and Y. Sarwar (1991). *J. Chromatogr., 543*, 17–38.

Singhal, R. P., D. Hughbanks and J. Xian (1992). *J. Chromatogr., 609*, 147–161.

Slater, G. W. and J. Noolandi (1989). *Biopolymers, 28*, 1781–1791.

Smith, L. M. (1991). *Nature 349*, 812–813.

Smith, L. M. (1993). *Curr. Opinions Biotech., 4*, 37–40.

Smith, J. T. and Z. El Rassi (1993). *Electrophoresis, 14*, 396–406.

Srivatsa, G. S., M. Batt, J. Schuette, R. H. Carlson, J. Fitchett, C. Lee and D. L. Cole (1994). *J. Chromatogr. A., 680*, 469–477.

Strege, M. and Lagu, A. (1991). *Anal. Chem., 63*, 1233–1236.

Swerdlow H. and R. Gesteland (1990). *Nucleic Acids Res., 18*, 1415–1419.

Swerdlow, H., S. Wu, H. Harke and N. J. Dovichi (1990). *J. Chromatogr., 516*, 61–67.

Swerdlow, H., J. Z. Zhang, D. Y. Chen, H. R. Harke, R. Grey, S.. Wu, N. J. Dovichi and C. Fuller (1991). *Anal. Chem., 63*, 2835–2841.

Tabor, S. and C. C. Richardson (1990). *J. Biol. Chem., 265*, 8322–8328.

Takigiku, R. and R. E. Schneider (1991). *J. Chromatogr., 559*, 247–256.

Tietz, D., M. H. Gotlieb, J. S. Fawcett and A. Chrambach (1986). *Electrophoresis, 7*, 217–220.

Towns, J. K. and F. E. Regnier (1990). *J. Chromatogr., 516*, 69–78.

Tsuda, T., G. Nakagawa, M. Sato and K. Yagi (1983). *J. Appl. Biochem., 5*, 330–336.

Tsuda, T., K. Takagi, T. Watanabe and T. Satake (1988). *J. High Res. Chromatogr. Chromatogr. Comm., 11*, 721–723.

Turner, K. A. (1991). *LC-GC 9,* 350–356.

van de Nessa, R. J., N. H. Velthorst, U. A. Th. Brinkman and C. Gooijer (1995). *J. Chromatogr., 704*, 1–25.

Vilenchik, M., A. Belenky and A. S. Cohen (1994) *J. Chromatogr., 663*, 105–193.

Wang, T., G. J. Bruin, J. C. Kraak and H. Poppe (1991). *Anal. Chem., 63*, 2207–2208.

Xian, J. (1994). *Application of Capillary Electrophoresis for the Separation of Biomolecules*, Ph.D. dissertation, Wichita State University.

Yeung, E. S. (1993). In: *Capillary Electrophoresis Technology* (N. Guzman, ed.), p. 587. Dekker, New York.

Zagursky, R. J. and R. M. McCormick (1990). *BioTechniques 9*, 74–79.

Zhang, J., D. Chen, S. Wu, H. R. Harke and N. J. Dovichi (1991). *Clinical Chem., 37*, 1492–1496.

Zhu, M., D. L. Hansen, S. Burd and F. Gannon (1989). *J. Chromatogr., 480*, 311–319.

CHAPTER 11

The Use of Capillary Electrophoresis in Pharmaceutical Development

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1. Introduction

Having read the other chapters in this volume, one may ask, "Why a separate treatment on the use of capillary electrophoresis in pharmaceutical development?" In fact, every chapter in this book addresses some aspect of how and when capillary electrophoresis (CE) in its many forms can be applied to both pharmaceutical and biomedical problems. A number of excellent review articles (Nishi and Terabe, 1990; Deyl *et al.*, 1994; Rabel and Stobaugh, 1994, Smith and Evans, 1994) and book chapters (Demarest *et al.*, 1992; Perrett, 1994) have addressed applications of capillary electrophoresis and its uses for problem-solving in the pharmaceutical sciences. It is certainly our hope that the readers of this book will continue to make use of the current literature, as this is the only way for scientists to remain current on any of the "state-of-the-art" applications of capillary electrophoresis or any other analytical technique. However, in this chapter, we will evaluate capillary electrophoresis from the view of the industrial pharmaceutical development scientist.

We have attempted in this volume to provide the reader with information that will not necessarily change significantly with time. As has been mentioned throughout, capillary electrophoresis is an exciting technique with advantages and disadvantages. Advantages of an analytical technique tend to be exploited, while disadvantages tend to be overcome. Although we hope that the information presented here will be useful years from now, we know as scientists that even as this chapter goes to press much of the information is becoming obsolete. We hope to inform and bring an understanding of this technique in the context of the pharmaceutical development process. We will also explore how capillary electrophoresis could impact the development of a pharmaceutical compound and/or dosage form. Perhaps the information presented here will generate new ideas and provide some insight into how an analytical technique being developed is adopted for use by members of the pharmaceutical industry.

1.1. Chemical Entity to Marketed Product: The Development of a New Pharmaceutical

The analytical chemist in the pharmaceutical industry is part of a development process. This process moves a chemical substance (small molecule, peptide, protein, etc.) that is known to modify a biological activity or the course of a disease state to a commercial product that is suitable for human consumption. Once a drug substance candidate has been identified, it must be manipulated into a formulation that can effectively deliver the drug to the requisite site in the body. This formulation is the drug product. The ultimate objective is a drug product that is safe and demonstrates efficacy in humans, and can be produced at a reasonable cost for the consumer.

1.2. Evaluation of the Drug Substance and Drug Product

In the early stages of research, the drug substance must be evaluated to demonstrate a) that the chemical compound in question may be reliably synthesized in the same way and b) that it has the chemical structure it is purported to have. This is not necessarily an easy task, but it is absolutely critical. Analytical characterization completed in the early development stages on a drug substance candidate will provide the information that is required to make choices on how to proceed with the development of the compound. Early elimination of chemical substances that are unsafe or inactive can be very cost effective. As mentioned above, the most important early experiments are a) pharmacological, demonstrating that a drug has positive biological activity, and b) toxicological, demonstrating that the drug is safe. The toxicological experiments must show that, while the drug has biological activity, any apparent toxicity does not outweigh the benefits expected to be seen in humans.

It is necessary that the early analytical methods and results comply with the following requirements: 1) the analytical methods used provide reliable results with a fast turnaround time; 2) the results provided will remain consistent throughout the development cycle of the drug product; and 3) if possible, these methods are transferable to laboratories doing more repetitive testing and will be amenable to automation. However, often at this stage only a very small amount of the active chemical entity is available for analysis. The bulk of the synthesis material available at this time will be needed for pharmacological and toxicological trials. It is up to the analytical chemist to provide the maximum information while using the minimum amount of material possible. Therefore, the cost savings provided by a particular technique may not be as important as issues of reliability and speed. For instance, while NMR can provide information on the levels of residual solvents in a drug substance using a small amount of material, it is not necessarily as cost effective as gas chromatography for repetitive measurements.

The drug substance candidate in question may be a small organic molecule, a higher molecular weight substance such as a peptide (see Fig. 1), or an extremely complex molecule such as a protein or an antibody. After the identification process is complete, the most important questions are, “How pure is it?” and “What are the impurities?” To assess purity attributes on a routine basis, a reference standard must first be prepared and characterized. A reference

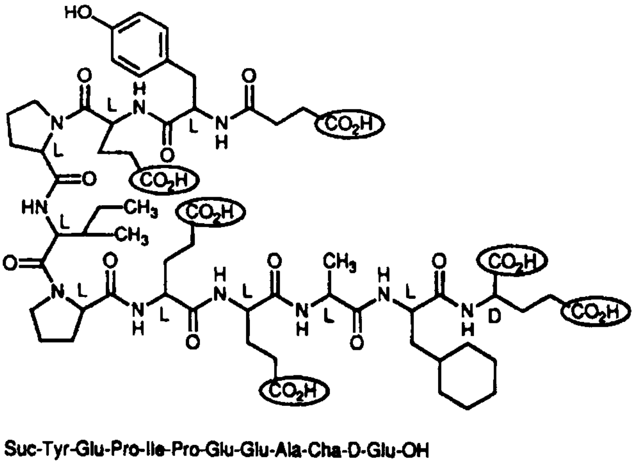

Fig. 1. Chemical structure of MDL 28,050. (Reproduced with permission from Chen *et al.*, 1990).

standard is generally defined as a substance of the highest purity reasonably attainable (Food and Drug Administration, February, 1987). Appropriate testing for a reference standard may include the following: elemental analysis, various separation methods (chromatography, electrophoresis), infrared and ultraviolet spectra, nuclear magnetic resonance spectra, mass spectrometry, specific rotation, X-ray crystallography, phase solubility analysis, thermal analysis methods, radioimmunoassay, and bioassay procedures. The information obtained must be evaluated in its entirety to assign a purity for the reference material. The importance of the purity assignment for the reference standard should not be underestimated, as this value will define the purity of the drug substance and drug products used in releasing materials to be used for toxicology and clinical testing.

Once a reference material is available, the purity of the drug substance candidate may be evaluated with some certainty. These purity determinations may include some or all of the items listed in Table 1; it is not meant to be inclusive as each new chemical entity must be evaluated on a case-by-case basis.

Table 1. Analytical Considerations for Defining the Purity of a Drug Substance Candidate

Analytical Test
• Determination of residual solvents
• Determination of heavy metals
• Chromatographic and/or electrophoretic evaluation: determination of synthesis-related Impurities
• Chromatographic and/or electrophoretic evaluation: determination of degradation impurities
• Determination of residual ash on ignition
• Determination of moisture/water content
• Potentiometric titration
• Determination of residual catalysts used in the synthesis.
• Determination of microbial contamination
• Determination of enantiomeric purity
• Assurance of morphology

After the basic structural attributes have been defined, it is necessary to characterize all chemical attributes of the drug substance. These may include pK_a values, solubility characteristics, partition coefficients, and preliminary stability characteristics. If a new chemical entity shows biological activity but undergoes rapid degradation, it will be difficult to provide to consumers; thus, stability properties must be evaluated as well. These data are necessary to understand the potential *in vivo* properties of the drug substance and will also provide the necessary preformulation information for the pharmaceutical scientist to create an adequate dosage form for humans.

Many of the positive attributes of CE have sparked interest among those in the pharmaceutical industry. Although there are still issues concerning the concentration detection limits, elegant separations may be accomplished with a minute amount of material. In addition, since CE provides separations with high efficiencies, the ability to evaluate purity can be quite powerful. For instance, in the synthesis of the molecule shown in Fig. 1, capillary electrophoresis may be used to evaluate the presence of possible deletion products that may result. In early research stages the time needed to complete the separation (over two hours) is not as important as adequate detection of possibly harmful byproducts (Fig. 2).

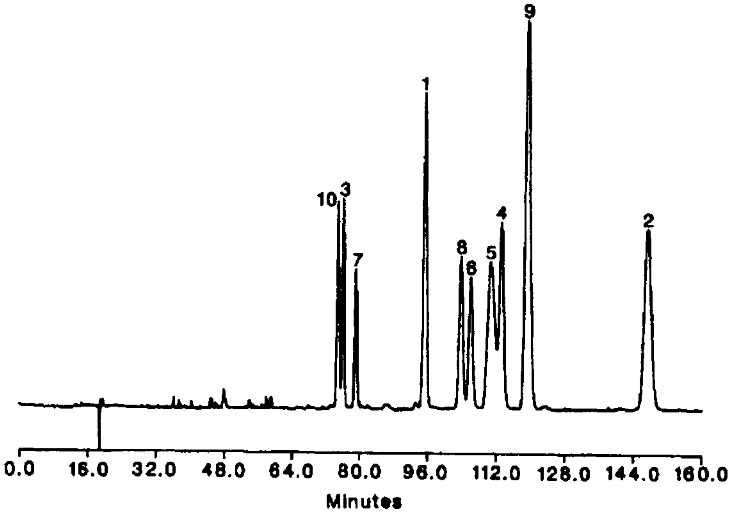

Fig. 2. CZE separation of MDL 28,050 from its deletion byproducts. (Reproduced with permission from Chen *et al.*, 1990).

As the development proceeds, the production techniques will be refined to create a commercially viable process that produces drug substance of high quality in a reproducible manner. During that same period of time, the analytical methodologies to evaluate the drug substance will also be refined, utilizing the best techniques with an eye toward information content and speed of analysis

Capillary electrophoresis techniques may also be applied to the study of drug products. In the drug product, one finds that in most cases the once "pure" drug substance is now modified with other known chemical entities (excipients) to create a dosage form. This modification must be completed in order to allow delivery of the drug and to ensure its reproducible absorption in the body. Analytical methodologies are developed so as to ensure the specific determination of the active chemical entity in the presence of the excipients (Table 2). The goal is to develop analytical methods for the dosage form that will adequately separate the active compound from related substances and excipients in a reproducible manner.

Table 2. Some Analytical Tests for Defining the Acceptability of a Drug Dosage Form

Analytical Test
• Determination of potency
• Determination of residual solvents
• Chromatographic and/or electrophoretic evaluation: determination of synthesis related impurities
• Chromatographic and/or electrophoretic evaluation: determination of degradation impurities
• Determination of individual dosage form content. (content uniformity)
• Determination of moisture/water content
• Surrogate drug release tests (dissolution, disintegration)
• Determination of preservatives
• Determination of microbial integrity (sterility)
• Assurance of morphology of the drug substance

Once the original purity evaluations are completed, they will be refined and adapted throughout the drug development process to complete routine purity evaluations. Among the more important purity evaluations is the testing of the stability of the drug substance and drug product. It is the responsibility of the drug manufacturer to ensure the quality of the drug product over its approved shelf-life. The ultimate products that are being utilized contain chemical substances that must be carefully evaluated for possible interactions over the short and long term.

Generally, in the development of a new chemical entity, the chemist will continue to refine analytical methods to the point where testing methods include high efficiency techniques that provide high quality results in the shortest amount of time. During the development process, one must always take into account the final customer. The chemist must ensure that the drug will be safe and efficacious while still remembering that cost controls are very important to the consumer. In the industrial laboratory, the most valued chemist can provide high quality analytical methods in a timely manner to ensure that new treatments are provided to the public.

1.3. Bioanalytical Applications in the Drug Development Process.

The evaluation of drug substances and drug products is only one part of the analytical development process. Analytical methodology must be developed for the biological studies that will be completed with the drug substance and drug product. A significant number of studies must take place prior to a drug being given to humans. These preclinical studies provide assurance that a drug will be safe. The pharmaceutical scientist needs to determine insofar as possible the effects of a drug *in vivo* prior to human trials.

The determination of drugs in biological fluids and tissue can be very challenging, and analytical methods must be developed for several different types of studies. Initial pharmacology studies are necessary to determine the bioavailability of the drug substance. This bioavailability is important as it is necessary to develop drug dosage formulations and to make initial estimates for the dosage level to be provided to humans. In these studies, blood levels must be determined. It is also necessary to know at what sites in the body the drug will absorb. These same tests might be used in the future to determine blood levels in humans during important clinical studies.

Since there can be a significant amount of biotransformation, it is also important to have analytical methods to define the rate and type of metabolism the drug will undergo. Initially, these studies may be completed using preparations that will mimic the *in vivo* activity. These studies will then move to *in vivo* observations. This work is necessary to ensure that the metabolites of drug transformation are also safe during the use of a drug product.

While early studies may involve a limited number of samples, as a drug moves into larger studies to determine efficacy in humans, the number of samples that need to be evaluated can become quite numerous. Since the results of these studies have a significant impact on evaluating the results of a drug study in humans, the analytical methodology used to evaluate the samples must meet defined criteria. The first criterion is that the method be specific for the analytes of interest, whether it is the new chemical entity or an important metabolite. Second, the detection must be such that minute quantities can be reproducibly detected in the biological matrices. Finally, the speed of the technique is important, since the number of samples and the demand for the results will both be great.

2. Considerations for CE Instrumentation

The use and maintenance of analytical instrumentation in a pharmaceutical laboratory is very important. In general, the pharmaceutical development laboratory is regulated as are all other aspects of drug development. This regulation is in place to ensure a safe and efficacious drug product for public consumption (Shulman *et al.*, 1995). In the development process, the level of regulation and the specific guidelines for the maintenance and care of instrumentation become more rigorous as the drug gets closer to commercialization. However, most regulations and guidelines tend to make sense from the operative point of view for doing analytical chemistry. In-house built equipment may be used in the research environment. However, as the analytical testing moves from research into development and finally into laboratories providing analytical services for commercial production, the need for control of instrumentation becomes more rigorous. This includes requirements for adequate technology transfer, criteria to define proper training, and systems to validate and define operational qualification for the instrument.

Most major manufacturers of liquid chromatography (LC) instrumentation are actively developing capillary electrophoresis instrumentation. They have readily taken advantage of what was learned during the development of the liquid chromatography instrumentation utilized by the pharmaceutical industry to supply instrumentation that meets applicable regulatory requirements, in addition to the necessity for automation. CE systems are sold by several major manufacturers of analytical instrumentation, including Perkin-Elmer (Applied Biosystems), Hewlett-Packard, Beckman, Dionex, Bio-Rad, Waters, Thermoseparation Systems, ISCO and ATI/Unicam. This list is by no means complete, and the technology is changing quite rapidly. In this chapter we will discuss currently available instrumentation from a pharmaceutical development viewpoint.

The necessary components for instrumentation specifically required by the pharmaceutical industrial laboratory are illustrate in Fig. 3. Other chapters in this book have highlighted specific needs for instruments that are used for research applications of capillary electrophoresis. Choosing instrumentation for the industrial pharmaceutical analytical laboratory requires the evaluation of many aspects of CE instrumentation (see Table 3). In the early stages of drug development, the chemist will be evaluating instrumentation based upon the need to get a required answer in a timely manner. Once a product becomes commercially available, ease of use and maintenance needs, as well as the necessity of meeting regulatory requirements, become more important in a list of considerations.

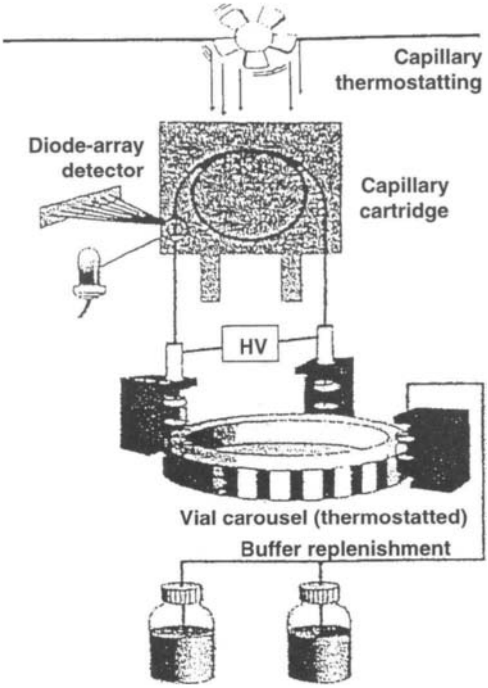

Fig. 3. Schematic of a typical automated capillary electrophoresis system that would have utility in the industrial pharmaceutical laboratory. (Reproduced with permission from Heiger, 1992.)

2.1. System Design

In considering system design, we will address requirements for the instrumentation necessary for a typical development or commercial analytical services laboratory. The type of instrument purchased will be determined by the requirements of the particular laboratory. In some cases, versatility will yield to automation, while all laboratories are looking for systems that can be easily upgraded as this technique matures. In the industrial environment, everyone is looking for the most instrument for their investment.

Table 3. Considerations for the Evaluation of Capillary Electrophoresis Instrumentation for the Pharmaceutical Laboratory.

- Ease of use
- Software integration development
- Ease of maintenance
- Detectors available
- Instrument qualification/validation
- Operator training and qualifications
- Operating qualification guidelines

Systems may contain space for anywhere from 20 to 80 samples. A trade-off may have to be made depending on usage criteria. If a system holds a minimum number of vials yet provides excellent capabilities for method development, it probably could meet the needs of the development laboratory. For the support of products created for widespread commercial use, sample throughput is generally more important. Some instruments have sampling systems that support method development experiments, in that the sample holder area permits the use of one set of vials from each vial holder for purging the capillary and run buffers. This makes it very suitable for method development because a set of runs may be automated. Such properties as the pH of the buffers or solvent modifications can be varied through the use of the solution vial pairs. This can be thought of as somewhat analogous to solvent programming for LC. Designs for sample placement tend not to be specific to instrumentation. This will be a recurring theme as other aspects of system design are discussed. Much of the automated LC equipment available today is somewhat modular, allowing for upgrades and evening the swapping of instrumentation from system to system. Automated capillary electrophoresis systems tend to be built as integrated systems.

Capillary placement and temperature control are unique considerations for CE system evaluation. Capillaries used for separation in commercial systems range from the very straightforward (cut the silica capillary, place into system) to very high-tech cartridges and cassettes. Again, the type of usage for the system will be important. Simple systems in which capillaries may be rapidly exchanged can be quite useful in the early stages of method development. Once a method is developed, cartridge holders offer advantages in capillary placement. They range from the very simple to those with bar-coding that is read by the instrument each time the cartridge is inserted into the instrument. This allows tracking of the number of injections and the instrument conditions that were used with that particular capillary. It provides a history of the capillary and is good documentation for Good Manufacturing Practices (laboratory) in the pharmaceutical industry.

Capillaries may be purchased with many types of configurations (cartridges, built-in bubble cells for detection, etc.) and chemistries (internal wall derivatized or coated, gels, etc.). Many analysts invest the effort in preparing their own capillaries (with and without cartridges) for separation. As with the early development of LC columns (when laboratories packed their own columns), many capillaries are developed and prepared in-house. As techniques for capillary manufacture develop, it will probably become more cost effective for scientists in the industrial pharmaceutical laboratory to purchase commercial capillaries to ensure reproducibility and ease of use. Monetary investment versus time investment will control the development and use of commercial capillaries in the industrial laboratory.

An important aspect of CE system design is the method used to maintain the capillary temperature. This is much more important in capillary electrophoresis than in LC. Cooling may be accomplished by forcing air into a defined and insulated space in which the capillary is placed. There are systems with refrigeration units and those that have the temperature control system built around the capillary itself to ensure very controlled temperature (and capillary purchase). While system temperatures may be controlled anywhere from subambient to 60°C, the type of system required will depend upon laboratory needs; however, the better the system for temperature control, the better the method reproducibility will be.

All injection modes (vacuum, gravity, pressure and electrokinetic) are available in most of the commercially available instrumentation. As reproducibility is of the highest importance in the development and validation of analytical methods, vacuum and pressure injection, which can be precisely controlled by system electronics, should be available for the system chosen.

2.2. Detectors

Detection is one of the many challenges in designing a integrated, commercially available capillary electrophoresis system. Most systems feature single or multi-wavelength UV/Vis detectors. Some systems offer rapid scanning and UV/Vis diode-array detectors. There may be a trade-off between the higher sensitivity and quieter baseline of a multiwavelength unit and the information content of the diode array. Most commercial manufacturers are working on ways to increase sensitivity and improve traceability of lamp use. These include such ideas as remote location of the lamp from the capillary to help decrease localized heating due to the UV lamp source, "Z cells" and bubble type cells to increase detector pathlengths, and self-calibrating UV detectors with energy readouts so that the lamp life can be tracked. The issues with the use of UV-Vis detectors for CE in the pharmaceutical laboratory are the same as those found for LC. While there is broad use of UV detection in pharmaceutical laboratories for such functions as drug substance purity evaluation, this technique does not tend to have the requisite sensitivity or selectivity for determination of the same substances in biological matrices.

While much research work has been completed, mass spectrometric, laser fluoresence, and conductivity detectors at this point are available for very few commercial instruments. A commercial electrochemical detector is not available as of the publication of this chapter. As the technique matures and demand increases, so will options for commercial instruments.

2.3. Automation and Data Manipulation

Commercial suppliers have taken advantage of what has been previously developed for liquid chromatography systems and have applied this knowledge to the development of computer interfaces to control CE instrumentation and evaluate data. The user does not necessarily have to take advantage of the automation and data manipulation packages available for the systems (and these tend to be changing as fast or faster than the computers themselves). In the industrial environment, the cost of the computer systems is generally considered to be outweighed by the time savings, as well as the need for consistent operator training.

At this time, CE-specific software for commercial instrumentation is still being developed. Since most computer systems were adapted from systems currently used for LC, some training may be required to ensure that the laboratory analyst does not confuse the two separation techniques. CE-specific software that utilizes CE terms such as migration time versus retention time should be considered. This helps the analyst to think about CE as a separation method different from liquid chromatography. This is particularly helpful for the novice user, and will more than likely be addressed as CE matures as a technique. As concepts and applications are still being developed at a rapid rate, there is a need to determine what the user actually wants or needs a system to do versus what is available.

Manufacturers of capillary electrophoresis systems are creating niche markets in sample separation and method development. This promotes consumer loyalty and can make certain techniques available for the novice user who may not have a significant background in the field. Currently available kits include premade kits for chiral, anion and cation separations. Prepackaged systems for certain biological marker determinations are also available.

2.4. *Instrument Validation and Qualification*

When evaluating an instrument manufacturer, one must consider what they can provide in terms of instrument validation/qualification for their product. In addition, if the user does not have an intimate understanding of the instrumentation and the CE technique, software and hardware support should be carefully assessed. It is often as useful to research the background of the instrument manufacturer (technical support, ISO 9000 certification, training, history and future financial viability of the organization) as the properties of the instrument being evaluated for purchase.

Validation and qualification of instrumentation begin prior to shipment from the factory. One should consider that it is better to actually be built into the development and manufacture of the instrument. Documentation of each step for development and manufacture is crucial. This should include testing of the hardware and software to current standards.

At installation, it is necessary to ensure that the system is being set up according to specific standard operating procedures. The instrument manufacturer should provide detailed information concerning site requirements for the installation of the instrument. Purchasing an instrument has become a partnership with the manufacturer; the instrument should not just show up in a box. It is generally useful to integrate the system into the laboratory environment.

The validation program should begin with the development of maintenance logs for the documentation of installation, preventive maintenance, repairs and recertification performed on the equipment. Software validation requires traceability and security of data, plus documentation of actual analysis parameters and errors.

On-site revalidation ensures both regulatory compliance and continued adequate system performance. Under routine operation, equipment should be revalidated at least annually and perhaps more often. In integrating the system into the laboratory environment, its level of use and history of maintenance should be seriously evaluated. Revalidation should also be performed after maintenance or repair, or as appropriate for a particular laboratory protocol. All equipment should be revalidated to original factory specifications using certified testing procedures. These tests should include wavelength accuracy, baseline stability, linearity for detectors, etc. In today's pharmaceutical laboratory you are not necessarily purchasing an instrument—you are purchasing methods to ensure laboratory compliance for that instrument.

3. Method Validation

Validation of an analytical method is a process through which one demonstrates that an analytical method is suitable for its intended purpose. It was introduced as a regulatory requirement in the United States in 1978 (Federal Register, 1978) and has since become a part of registration applications for pharmaceuticals in many other countries. Any analytical procedure used to assess the quality of a pharmaceutical intended for human or animal consumption is subject to validation. The process includes the preparation of a validation protocol, testing, and documentation. Although currently there are differences in the technical requirements among various regulatory agencies, a guideline entitled "Validation of Analytical Procedures" prepared by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) outlines the parameters that should be considered during the validation exercise. These parameters are selectivity/specificity, accuracy, linearity, range, sensitivity, precision, and robustness (ruggedness).

Capillary electrophoresis has been regarded as an alternative and complementary technique to LC for the analysis of small molecule pharmaceuticals (McLaughlin *et al.*, 1992). Demonstrated applications have included identification of components (Altria *et al.*, 1993), quantitation of the active moiety (Thomas *et al.*, 1994) and determination of impurities (Altria *et al.*, 1993), as well as chiral separations (Novotny *et al.*, 1994). When a CE method, typically in a capillary zone electrophoresis (CZE) or a micellar electrokinetic (MEKC) mode, is employed for quality control of pharmaceuticals, its performance characteristics fall into the same category as those of LC and GC techniques. Thus, the validation principles for chromatographic methods also apply to CE methods. Among those validation parameters described in the ICH guideline, sensitivity and precision are sometimes more difficult to validate for CE methods than for chromatographic methods. This is due mainly to the intrinsic limitations of the CE technique, such as nanoliter sample load, less precise injection mechanisms, and short pathlength for UV detection. Finally, the establishment of adequate system suitability tests that define the minimum performance criteria that a system must meet prior to usage (United States Pharmacopoeia, USP, XXIII, 1995) may be more complicated for the CE system.

The preparation of a validation protocol for a CE method may follow the ICH guideline. Each validation parameter described in the guideline could require the evaluation of several different parameters, and each may need to be addressed in a different manner, depending on the intended application of the method. Validation parameters pertinent to CE methods are discussed below.

3.1 Specificity/Selectivity

Specificity is the ability to assess unequivocally the analyte of interest in the presence of those components which may be expected to be present in the sample matrix. Specificity is the most critical parameter of any analytical method used for stability and assay determinations for new pharmaceuticals. It is in general the first validation parameter addressed in the establishment of analytical methods for the determination of assay. The U.S. Pharmacopoeia defines the specificity of an analytical method as its ability to measure accurately and specifically the analyte in the presence of components that may be expected in the sample matrix (USP XXIII).

Selectivity is generally applicable to all chromatographic techniques. Since the performance characteristics of CE as a separation method resemble those of an LC method, the term selectivity is addressed in this discussion. The selectivity of a CE method is typically validated by demonstrating that the separation of analytes is free of interferences under nominal method conditions. The source of interferences could be impurities, degradants, and sample matrix. It is generally desirable that the resolution between the analyte and its nearest peak be greater than 2. One of the advantages of CE is its high efficiency. Separation of several analytes may be achieved within a short period of time frame. Therefore, the repeatability of migration time for each analyte is very important. For practical routine applications, efficiency must be accompanied by a suitable precision. The migration time window for peak identification should be established based on the instrument repeatability and should be free of interferences. Factors affecting the selectivity need to be identified. An allowable range for variation of each factor must be investigated and established as part of the validation protocol. Selectivity is an important criterion for the evaluation of method robustness.

3.2 Accuracy

All quantitative test methods must be validated for accuracy. In the ICH guideline, accuracy is defined as "the closeness of agreement between the value which is accepted as a conventional true value or an accepted reference value and the value found." It is a process of measuring the bias of the analytical method. In the analysis of small pharmaceuticals using CE, the sample is typically quantitated against a reference standard. The procedure gives a purity value relative to the standard rather than an absolute true value. Bias usually occurs when the sample and standard matrices are different. For bulk drug substance assay, the sample matrix is essentially the same as the standard matrix. The accuracy of the assay test may be measured by spiking the sample with impurities. Depending on the method precision and the specification limits for assay test, spiking impurities at several levels may be necessary to demonstrate the accuracy. For example, for a method that determines drug substance assay within 97–103% with less than 1% precision (repeatability), the accuracy can be evaluated by testing samples spiked with 2%, 3%, and 4% of impurities. The bias for a drug substance assay should not be greater than 1% (Canada, Drugs Directorate Guidelines, 1992).

For drug products, the sample matrix contains excipients from the formulation and can sometimes be quite different than the standard matrix. The differences between sample and standard matrices may affect the CE performance and cause bias in the method accuracy. The normal approach to validate the accuracy of drug product analysis is to analyze samples of placebo excipients spiked with known amounts of active. The spiking range of at least 80% to 120% of the label claim is appropriate. A bias of less than 2% (Canada, Drugs Directorate Guidelines, 1992) is usually acceptable for drug product assay.

If the quantitation of impurities, metabolites or degradation products is done by an external standard method, then the validation of accuracy is similar to those for drug substance and drug product assays. Depending on the method sensitivity, accuracy should be demonstrated from the limit of quantitation to about 200% of the specification limit. A bias of less than 5% may be considered acceptable for impurity analysis.

The results of accuracy studies on spiked samples can also be used to demonstrate the linearity and repeatability. However, the minimum amount of data required for each parameter is different. Data from triplicate determinations at each of at least three concentration levels spaced equidistantly over the spiking range are necessary for accuracy and repeatability validations. For linearity validation, at least five concentration levels with duplicate determinations at each level are required.

Accuracy of the method is indicated by the closeness of 100% recovery. The bias and the repeatability of the method can be derived from the mean and the standard deviation of the percent recovery data. For linearity, the data should be analyzed by linear regression analysis. Then the correlation coefficient (r) provides the measurements of the linearity, the y-intercept is a measure of the potential method bias, and the slope represents the sensitivity of the analyte response. All of these results should meet the criteria set forth for each parameter.

When the peak area percent method is used for the quantitation, such as for impurity or enantiomeric purity assessments, bias of peak area measurements due to analyte mobility differences should be evaluated and addressed. Correction of peak area with migration time has been shown to improve the accuracy (Altria, 1993).

3.3. Linearity

Linearity is a validation parameter for quantitative tests. The linearity of a method is its ability to achieve test results that are directly proportional to the concentration of analyte in the sample. Linearity should be established across the concentrations that encompass the range of the method. The linearity range may vary depending on the application. Typically for assay tests of drug substance and drug product, linearity in the range of 50–150% of the nominal sample concentration should be demonstrated. For impurity determination, the linearity is validated in the range from the limit of quantitation to about 200% of the specified limit. When the peak area percent method is used to assess the impurity, the linearity of both the impurity and the reference compound should be established.

For the establishment of linearity, data from at least five concentration levels equidistant over the range are necessary. Results from the accuracy study may be used here if there are enough data points. Test samples can be serially diluted solutions containing the analyte at proper concentrations or solutions of synthetic mixtures with proper amounts of analyte added.

Data are to be analyzed by linear regression analysis. The linearity is measured by the correlation coefficient (r), which should be greater than 0.999. The y-intercept indicates the potential bias of the method. The 95% confidence intervals of the y-intercept should include the method bias determined under *Accuracy*.

3.4. Range

The range of the method is the interval between the upper and lower concentrations of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity. All quantitative tests need to specify the range. The range specification must be based on the results of linearity, accuracy, and repeatability studies.

3.5. Sensitivity

3.5.1. Limit of Detection

Limit of detection (LOD) is a validation parameter for limit tests. It is the lowest concentration of analyte in a sample that can be reliably detected. For a CE method, the LOD of an analyte can be defined as the concentration at which the analyte peak is approximately two to three times the noise (USP XXIII).

3.5.2. Limit of Quantitation

Limit of quantitation (LOQ) is a validation parameter required for quantitative measurement of impurities. It is the lowest concentration of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. For a CE method, the limit of quantitation can be defined as the concentration at which the analyte gives a signal-to-noise ratio of approximately 10:1, or the concentration at which the analyte can be measured with a relative standard deviation of approximately 10% (Canada, Drugs Directorate Guidelines, 1992), whichever is lower.

3.6. Precision

Precision is the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. It may be a measure of the degree of repeatability, intermediate precision, or reproducibility. Precision is represented by the values of the standard deviation or the relative standard deviation (RSD) of the replicate analyses. The precision requirement of a CE method depends on its intended usage. For drug substance assay determination, typical acceptable repeatability is less than 1% RSD. Typical acceptable repeatability for drug product assay is less than 2% RSD, and for impurity determination is less than 5% RSD (Canada, Drugs Directorate Guidelines, 1992). For a CE method, the precision can be improved by using internal standards and by correcting the peak area values with migration times.

3.7. Repeatability

Repeatability is the precision of the method within a laboratory under the same operating conditions (same analyst, same instrument) over a short interval of time. It can be assessed by using the results of the accuracy study, percent recovery data of at least nine determinations over the range tested, or at least six determinations of a sample at 100% of the nominal concentration. Standard deviation and relative standard deviation of the multiple determinations are calculated.

3.8. Intermediate precision

Intermediate precision expresses the within-laboratories variations due to different analysts, different days, and different instruments. This can be measured by duplicating the repeatability study using a different analyst on a different day with a different instrument. A pooled standard deviation and relative standard deviation indicate the intermediate precision.

3.9. Reproducibility

Reproducibility is the measure of the variations between laboratories. It can be assessed by pooling the repeatability or intermediate precision results from different laboratories.

3.10. Robustness (Ruggedness)

Robustness is a measure of the capacity of the method to remain unaffected by small, but deliberate variations in method parameters, and provides an indication of its reliability during normal usage. Typically, method performance parameters such as specificity/ selectivity, accuracy, sensitivity, and precision are used to assess robustness. For a CE method, robustness may be evaluated by varying the following method parameters:

- pH, ionic strength, and the modifiers of the running buffer
- Voltage
- Capillary oven temperature
- Different capillaries
- Capillary flushing procedures
- Injection modes
- Detection
- Sample preparation procedures
- Sample solution stability

Both the main effect of each individual method parameter and the interaction effects among method parameters should be investigated. An experimental design approach can be very helpful in identifying the critical parameters and assessing their impact on method performance. Allowable variations of each method parameter should be specified in the method.

3.11. System suitability tests

The establishment of a system suitability test (United States Pharmacopoeia, USP, XXIII, 1995) is also important for a CE method. System suitability tests define the minimum performance criteria that a CE system must meet for the intended usage. For the day-to-day laboratory operations, system suitability tests are performed prior to analysis to demonstrate adequate CE system performance and to ensure the validity of the test results.

System suitability tests are an integral part of the CE method. Their purpose is to verify the adequacy of the integrated system, including sample, equipment, and operations for the intended analysis. They may include tests for resolution, peak tailing, repeatability, sensitivity, and any performance characteristics that are sensitive to slight variations of the system. The tests are to be established based on the results of the validation studies, especially the robustness study. The minimum performance criteria that a CE system must meet for the intended application must be specified in the method. In essence, the operator must determine that every portion of the system is in compliance with the parameters in the method developed. This includes not just software and instrument hardware but, for CE, the capillary characteristics as well. As shown in Fig. 4, a method that can provide excellent resolution of two enantiomers can be adversely affected by the shape of the capillary inlet. If system suitability (evaluating the resolution of enantiomer standards) had not

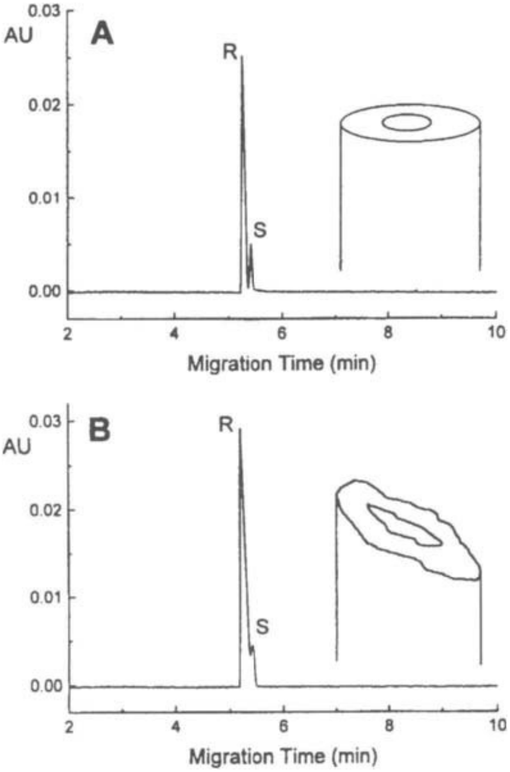

Fig. 4. Effect of the physical shape of the inlet of a gel-filled capillary on the resolution of naproxen enantiomers. Test solute: 0.1 mg/mL (R)-naproxen and 0.01 mg/mL (S)-naproxen in water. (A) Properly cut (perpendicular to the axis of the capillary) column inlet and (B) oblique (45°) cut column inlet. (Reproduced with permission from Guttman and Schwartz, 1995).

been performed prior to a sample analysis in this case, the contaminating isomer might have gone undetected. By evaluating the system suitability prior to beginning an analysis, one can be certain of all analytical results obtained from a method.

4. The Use of CE to Address Challenges in the Development of Pharmaceuticals

4.1. Preclinical Studies and the Use of CE

As mentioned in the overview of the drug development process, it is desirable to determine the physical and chemical characteristics of a drug candidate as early as possible in this process. Knowledge of these properties is often invaluable in preformulation and formulation work, yet physicochemical characterization in early development is often incomplete due to the scarcity of material dedicated to this objective early on (especially for proteins) or not predictive due to the limited relevance of the applied characterization techniques to real-life conditions.

The pharmaceutical analytical community first became interested in CE to assist in the important separations necessary for the evaluation of proteins and peptides of biochemical interest (Nielsen *et al.*, 1989). In the early development of recombinant proteins, very little material was available for study, and available chromatographic methodology could require the complete supply for a single analysis and still not provide the needed resolution to determine closely related synthetic impurities. Figure 5 depicts analytical results from a pharmaceutical laboratory where capillary electrophoresis was applied to this type of problem and offered a significant amount of information quite rapidly (less than 10 minutes).

Several of the inherent features of capillary electrophoresis offer advantages for physicochemical determinations compared with classical or other chromatographic methods. First, the small sample amounts required by CE allow a limited quantity of sample to be apportioned for a wider variety of determinations. Second, CE can be performed at extremes of pH and can therefore be used to characterize the behavior of strongly acidic or basic analytes. This contrasts with LC characterization, which is typically limited to pH 2–8 due to degradation of the stationary phase silica backbone outside this range. (Although there are LC stationary phases resistant to degradation, they often do not possess the favorable efficiency and peak symmetry found in silica-based stationary phases.) Third (and this is also an advantage over LC), the typical absence of organic modifiers in CE

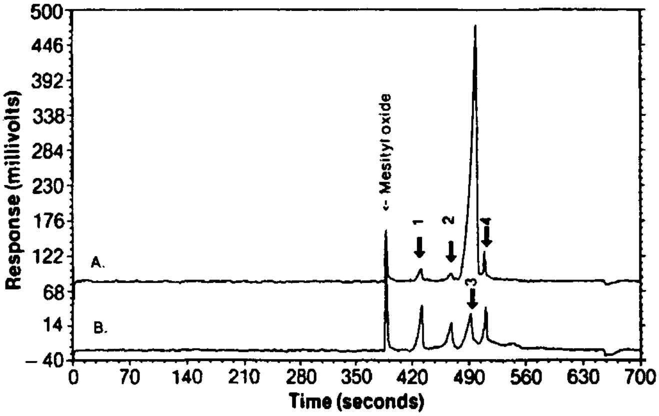

Fig. 5. Biosynthetic human insulin (BHI) and various derivatives. CZE separation of BHI from derivatives which could accompany crude production lots of BHI before preparative isolation is completed. (A) BHI mixed with a small amount of four BHI derivatives. (B) The BHI derivative mixture alone. Peaks: (1) [diarginyl-B31-B32] BHI; (2) [arginyl-A0] BHI; (3) [des-alanine-B30} porcine insulin; and (4) [desamido-A21] BHI; 500 mV = 0.01 AU. (Reproduced with permission from Nielsen *et al.*, 1989).

electrolyte buffers allows examination of analyte behavior in an environment that more closely resembles a physiological system.

A number of applications of CE to the determination of ionization constants (pK_a) have been documented (Beckers *et al.*, 1991; Cleveland *et al.*, 1993,1994; Gluck and Cleveland, 1994a,b). This approach is predicated on the conversion of the concentration terms in the Henderson-Hasselbach equation to electrophoretic mobilities. CE measurements of mobility as a function of electrolyte buffer pH give sigmoidal plots from which the ionization constants can be estimated (Fig. 6). Results correlate well with those determined by other methods, such as spectrophotometry.

An analogous approach is used to determine the isoelectric points (pI) of acidic and basic proteins (Yao *et al.*, 1994). Here a plot of mobility as a function of electrolyte buffer pH is used to determine the pH at which the mobility is zero, which by definition is the isoelectric point. Interference caused by non-specific protein adsorption with capillary silanol groups when pH < pI is eliminated by coating the capillary with a polycationic reagent. The results are comparable with

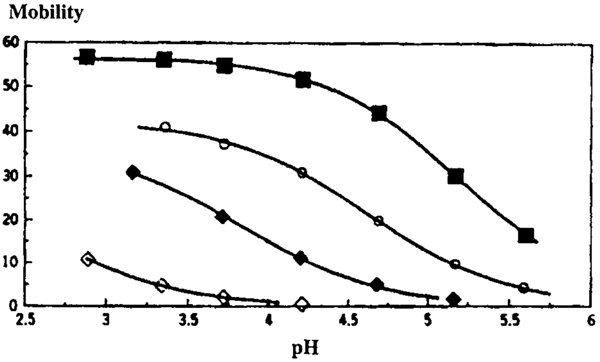

Fig. 6. Plots of mobility ($m_e \times 10^5$ cm^2 V s) vs. pH for bases with superimposed curve fits. ■ = Pyridine; ○ = aniline; ◆ = *p*-bromoaniline; ◇ = *o*-bromoaniline. (Reproduced with permission from Cleveland *et al.*, 1993).

those obtained by isoelectric focusing, but do not require the time and effort expended on manipulating gels in the older technique.

MEKC shows utility for estimation of *n*-octanol-water partition coefficients (Herbert and Dorsey, 1995). Correlation is demonstrated between the MEKC capacity factor (k') at pH 7.0 and partition coefficients (K_{ow}) taken from the literature. Examination of the k'-K_{ow} relationship for subgroups divided according to ionizability at pH 7.0, ability to hydrogen bond, and functional class shows the distinct influences of these properties.

An unusual application of CE is the measurement of the viscosity of liquids and solutions (Bello *et al.*, 1994). This is based on measuring the time it takes for the boundary between two liquids, one of known viscosity and the other the analyte of interest, to migrate through the capillary from injector to detector. The viscosity calculation is derived by application of the Poiseulle equation to both liquids in the capillary.

4.2. Separation of Enantiomers—Chiral Separations

Regulatory interest in chiral drugs is due to the known differences found in pharmacodynamic, pharmacokinetic and toxicologic properties of drug enantiomers. Traditional approaches for measuring enantiomeric excess involve direct (such as polarimetry) and indirect (derivatization of a mixture with an optically active reagent, which is then separated into the resulting diastereomers) methods. These techniques seemed to provide sufficient information. However, in

1992, the FDA announced the availability of a policy statement on the development of stereoisomeric drugs (De Camp, 1993). This statement had been in preparation for many years. In fact, other countries had previously made similar statements about the issues raised when a stereoisomer is being developed as a pharmaceutical. What was becoming apparent was that these regulatory statements would have a significant impact on how pharmaceutical analytical chemists would perform their work. It became necessary to develop analytical methodologies for chiral drug substances and products that would be equivalent in accuracy and precision to all other methods for the determination of related substances. These methods would have to be validated and would need to be run on a routine basis. These tests are needed to evaluate the drug substance and the finished dosage form, both on initial release and over their labeled shelf-life. In addition, regulatory agencies were concerned about the fate of chiral drugs *in vivo*. Was the chemical entity stereochemically stable *in vivo*? Would a chirally pure enantiomer racemize? If so, what would be the extent of the racemization? These issues required the analytical chemist to develop methodologies that could more readily address these critical questions.

Although indirect derivatization procedures are applicable to these types of studies, there are several drawbacks. This technique is somewhat time-consuming and not always reproducible; in addition, the methods require validation of the derivatization times and the purity of the derivatization reagent as well as all other parameters. To address these issues, a significant amount of effort has been directed toward the development of direct liquid chromatographic methods for chiral resolution. This direct separation mode depends on the formation of labile diastereomers (performed via hydrogen bonding, dipole-dipole, π–π, and/or hydrophobic interactions) between the enantiomers and the chiral environment with which they interact. One way of accomplishing this is by adding reagents capable of these interactions to the LC mobile phase. While this was shown to work, the quantities of reagents required generally made the use of this technique prohibitive on a routine basis. A significant advance from this came in the design of stationary phases that resulted in on-column transient interactions that allowed for separation. These "chiral" columns provided the tool needed to evaluate enantiomeric purity on a routine basis. While all of these methods provide the technology to answer the questions described above, there are still several obstacles to be overcome. These include the time required for preparation and separation, the resolution that the systems are capable of, and the cost of the "chiral" columns. While preparing bonded chiral stationary phases eliminates the need for the quantities of reagents required for use in a mobile phase, the materials used are quite expensive. In addition, the columns can be very sensitive to minor changes in mobile phase content, and do not necessarily have a long lifetime.

It has been demonstrated that capillary electrophoresis techniques may be able to overcome some of the disadvantages found when using direct methods for enantiomer measurement with liquid chromatography. The method in which CE and chromatography are combined is known as electrokinetic chromatography (EKC). This technique is discussed in detail by Foley and Ahuja in Chapter 3. Among the several modes of EKC, micellar EKC (MEKC) permits the separation of neutral or nonionic compounds. As MEKC involves the use of a pseudostationary phase, direct chiral separations may be performed. Since the volume of solution required for MEKC is very small, adding special chiral recognition agents directly to the carrier electrolyte is not costly. Optically active bile salts, cyclodextrins, crown ethers, and chiral molecular micelles are added to the mobile phase, and enantiomeric resolution by stereoselective interactions with the solute occurs. This has been one of the most important research areas in capillary electrophoresis (Nishi and Terabe, 1993).

Many different reagents that have been investigated have provided chiral recognition during MEKC (see Table 4). A significant amount of work has been done, and to cite each work here would be impractical, so a single example is provided for each. The mechanisms by which these reagents work can be placed in many different categories. These include inclusion complexes (I), host-guest complexion complexes (HG), chiral surfactant (CS), complexation agent (CA), indirect derivatization agents (IDA) and ionic and hydrophobic interactions (IHI). It should be noted that there are a significant number of reagents that have been investigated and not all mechanisms of action are well defined.

As can be seen in Table 5, perhaps the most popular chiral recognition agents are cyclodextrins and modified cyclodextrins. These cyclic oligosaccharides consist of either 6, 7 or 8 (α, β, γ) interior glucose units, which form a cone containing hydroxyl units at the entrance of the cavity and with an interior which is relatively hydrophobic. When an analyte forms inclusion complexes in aqueous solution differential hydrogen bonding of an analyte with the hydroxyl units at the outer rim of the cyclodextrin allows for resolution of enantiomers. Often, the quality of a resolution will be determined by the concentration of the cyclodextrin in the electrolyte solution. This is demonstrated in Fig. 7, which illustrates the chiral resolution of aminoglutethimide. Without the addition of cyclodextrins, no separation is seen. When using α-cyclodextrin some resolution is obtained, but the concentration required for a baseline separation is twice that required when using γ-cyclodextrin (Anigbogu *et al.*, 1995). Since the

Table 4. Some Reagents used for Chiral Separations by Capillary Electrophoresis.

Reagent	Reference
β-Cyclodextrin (I)	Otsuka and Terabe (1993)
γ-Cyclodextrin (I)	Pruñonosa *et al.* (1992)
Carboxymethyl-β-cyclodextrin (I)	Anigbogu *et al.* (1995)
Hydroxypropyl-β-cyclodextrin (I)	Noroski *et al.* (1995)
Methyl-β-cyclodextrin, Heptakis(2,3-di-O-acetyl)-β-cyclodextrin (I)	Matchett *et al.* (1995)
Cyclodextrins in liquid gels (I)	Cruzado and Vigh (1992)
Copper (II)-aspartame (IDA)	Gozel *et al.* (1987)
Copper (II)-L-histidine (IDA)	Gassmann *et al.* (1985)
DL-Marfey's Reagent (IDA)	Tran *et al.* (1990)
N-Dodecanoyl-L-amino acids (CS)	Dobashi *et al.* (1989)
Heptakis(2,6-O-methyl)-β-cyclodextrin (I)	Branch *et al.* (1994)
Bile salts (CS)	Nishi *et al.* (1990b).
10% mylose (I)	D'Hulst and Verbeke (1992)
Crown ethers (HGC)	Kuhn *et al.* (1992)
Mucopolysaccharides	Nishi *et al.* (1990)

most basic forms of cyclodextrins (α, β, γ) have limited solubilities, derivatized cyclodextrins have become quite popular and are very useful. The derivatized cyclodextrins are much more soluble in aqueous and organically modified electrolyte solutions and have demonstrated interesting EKC selectivities.

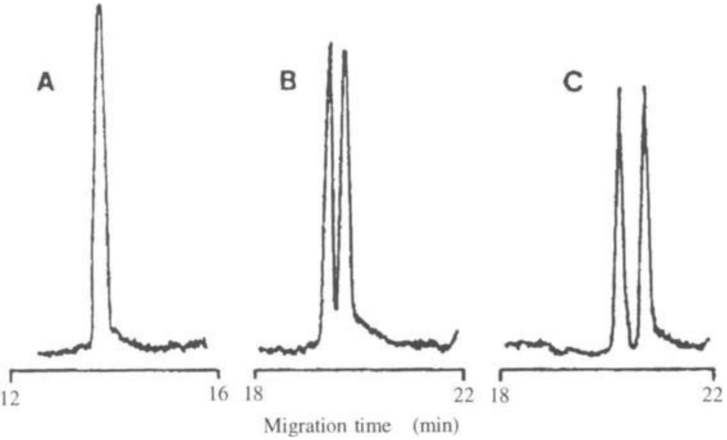

Fig. 7. Neutral cyclodextrin-modified CE (CD-CE) separations of the enantiomers of aminoglutethimide. Running buffer: NaH_2PO_4-$Na_2B_4O_7$ (pH 3) at 15 kV applied voltage. (A) no cyclodextrins added; (B) conditions the same as in (A) with 10 mM α-cyclodextrin; and (C) conditions the same as (A) with 5 mM γ-cyclodextrin. (Reproduced with permission from Anigbogu *et al.*, 1995.)

Using chondroitin sulfate (sodium salt) and heparin (sodium salt), which are both mucopolysaccharides, Nishi *et al.* (1995) have demonstrated that the use of ionic interactions and hydrophobic interactions can lead to very elegant chiral separations when used in conjunction with electrokinetic chromatography. As shown in Fig. 8, these species are charged, linear, sulfated polysaccharides having very large masses. When used with capillary electrophoresis in the EKC mode, several chemical entities (neutral and basic) with pharmacologic activities were shown to be enantioseparated. Critical factors for achieving separation included concentration and pH of the electrolyte. In addition, this separation technique was applied to the "real-world" situation by evaluating the determination of contaminating enantiomers in optically pure drug substances. Figure 9 shows the resolution of 0.2% of the RR-form of diltiazem from the active SS-form. Although this can also be accomplished using direct chiral liquid chromatography, this example is one of many that demonstrates the utility of chiral separations using capillary electrophoresis. When using liquid chromatography, the column must be dedicated to enantiomeric separations. When using EKC to perform chiral separations, the capillary may cleaned and reused for other applications.

For a more detailed discussion of the separation mechanisms used for optical resolution by micellar EKC, we recommend any of several

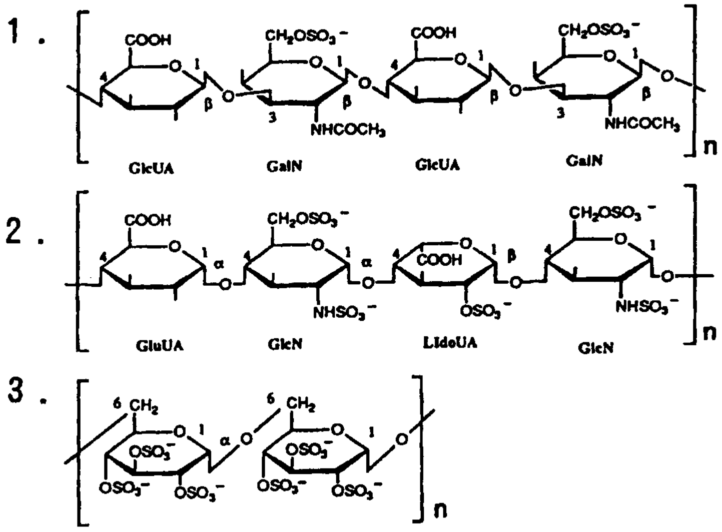

Fig. 8. Unit structure of anionic polysaccharides: (1) chondroitin sulfate C; (2) heparin; (3) dextran sulfate. (Reproduced with permission from Nishi *et al.*, 1995.)

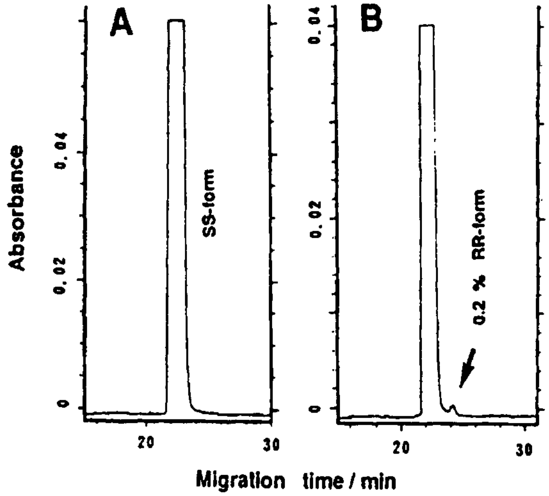

Fig. 9. Optical purity testing of diltiazem hydrochloride: (A) standard diltiazem hydrochloride (SS-Form) and (B) 0.2% RR-form spiked into standard diltiazem. Conditions: 3% chondroitin sulfate C in 20 mM phosphate-borate buffer (pH 2.4); separation capillary 75 µm i.d. × 57 cm (effective length 50 cm); applied voltage, 20 kV; detection 235 nm; temperature 23°C. Injection times of standard solutions are 6 s. (Reproduced with permission from Nishi *et al.*, 1995.)

excellent review articles that have previously been published (Issaq, 1994; Rogan *et al.,* 1994; Nishi and Terabe, 1995). It should be noted that because enantiomeric separations using CE is one of the most dynamic areas of research in separation science, one should continue to refer to the current literature for recent developments.

4.3. Determination of Analytes in Biological Fluids.

There is considerable interest in the analysis of drugs, metabolites, proteins, and other constituents of biological samples (urine, serum, etc.) from research, diagnostic, therapeutic, and forensic perspectives (Thormann *et al.*, 1993; Deyl *et al.*, 1994). However, there are some practical difficulties associated with obtaining such information. Very often large numbers of clinical or forensic samples need to be processed with reasonably rapid turnaround. Sample volumes are typically quite limited and contain very low levels of the analytes of interest. Furthermore, biological samples are by nature complex matrices, and there is significant potential for assay interference.

Although other techniques (LC, GC-MS, immunoassay) for analyzing biological samples exist, CE possesses several advantages, some unique and some shared with other approaches. CE is amenable to automation, high sample throughput, and multiple detection modes. The low sample volume requirements meet the need for a technique that works with little available sample. The very low flow rates used and the consequent minimal dilution of the sample in the CE electrolyte buffer provide high mass sensitivity and allow the detection of drugs and metabolites at very low levels. This is in contrast to LC, where a few microliters of injected sample are diluted in a relatively large volume of flowing mobile phase and the mass sensitivity is considerably lower. (The concentration sensitivities of the two techniques are approximately equivalent because LC detection flow cells are much larger than the optically transparent capillary section serving the same purpose in CE. Samples with concentration levels low enough to require sample preconcentration for LC typically also require it for CE.) Femtomole mass (ng/mL concentration) limits of detection are achievable in biological samples for analytes (or their derivatives) sensitive to appropriate detection techniques, such as laser-induced fluorescence (LIF) (Soini *et al.*, 1992; Gonzalez *et al.*, 1993). (See Chapter 4 for a detailed discussion of LIF in CE.)

Possibly the most important advantage of CE in the analysis of biological samples is its very high efficiency and resultant separating power. With capillaries containing several hundred thousand or more theoretical plates, separations of the analytes of interest from a complex matrix, or of closely related analytes from each other, can be effected. The separating power of CE often simplifies and sometimes eliminates sample preparation before analysis. This can be a substantial benefit when isolation of analyte from a complex matrix is complicated by limited sample volume. CE with limited or no sample preparation is also an attractive alternative to analytical techniques requiring complex sample preparation before analysis. This is evident from examples where both reversed-phase LC and MEKC were used to determine analytes in the same samples (Wolfisberg *et al.*, 1993; Caslavska *et al.*, 1994). In one case, LC sample preparation included liquid-liquid extraction, centrifugation, evaporation to dryness, and reconstitution of the residue. For the MEKC analysis, samples were either injected directly or diluted with water before injection. In another case, a CE method using sample preparation requiring deproteinization and centrifugation steps was clearly preferable to either of two LC methods. The first LC method has a sample preparation step involving a triple extraction before analysis while the second involves no sample preparation but column life is limited to fifty injections (Garcia and Shihabi, 1993b).

The analyst must also address the requirements for sample throughput. Generally, it is expected that a large number of samples will be required for the demonstration of efficacy in humans of any biologically active compound. The faster that samples may be evaluated for the levels of active drug and metabolites, the faster results of clinical trials may be understood. Examples, exist in the literature (Perret and Ross, 1995) where total analysis times available using capillary electrophoresis have been reduced to less than 1.5 minutes in human saliva (Fig. 10).

In addition, the bioanalytical chemistry may be used to evaluate the level of inorganic ions (Wildman *et al.*, 1991), organic acids (Shirao *et al.*, 1994) and other smaller molecules. These are species which, while not man-made, could be a significant indication of the bioanalytical effects of man-made chemical entities used for their pharmacologic actions (Fig. 11).

Although the examples above are clear-cut and by no means unique, it is an oversimplification to assume that sample preparation for CE can be totally dispensed with. The same procedures (extraction, protein precipitation, ultrafiltration, etc.) used to prepare biological samples for other analytical techniques yield the same advantages when used with CE. These would include simpler CE method conditions, shorter run times, and decreased chances of capillary contamination. Sample matrix effects, which can affect analyte migration times and quantitation relative to standards and cause electrophoretic interferences, are also reduced. For example, ultrafiltration of serum and saliva samples eliminates electrophoretic interferences from proteins in MEKC of some drugs, but is also used to obtain cleaner and

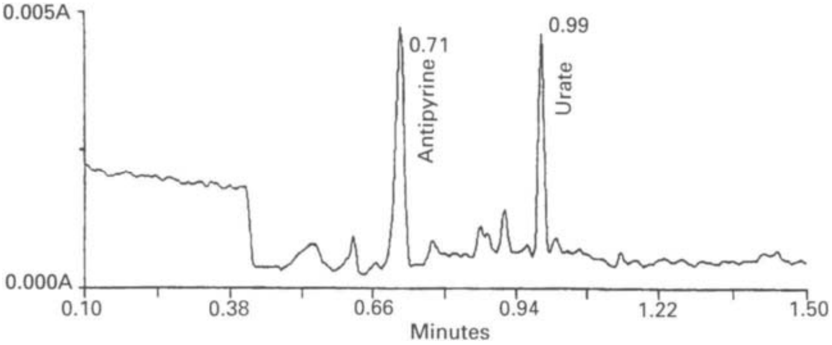

Fig. 10. Fast CE analysis of antipyrine in saliva. Conditions: buffer 25 mM SDS pH 9.6; capillary 30 cm (23 cm to detector) × 50 μm i.d., bare fused silica; detection UV 200–300 nm; load 10 s hydrodynamic; run 25 kV; temperature 15°C. (Reproduced with permission from Perrett and Ross, 1995).

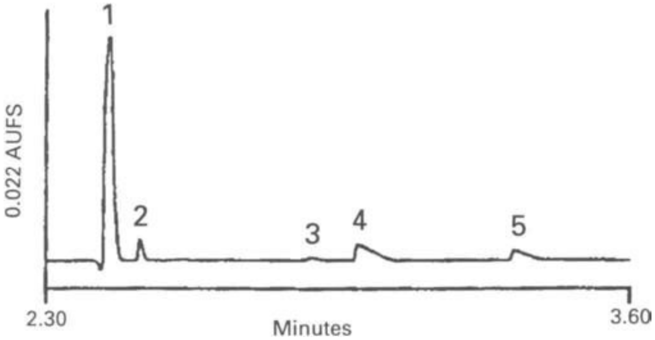

Fig. 11. Electropherogram of inorganic analytes determined in human urine. Conditions: capillary, 60 cm × 50 μm i.d. fused silica; electrolyte, chromate at pH 8.0 with NICE-Pak OFM Anion-BT (patent applied for); injection, hydrostatic for 60 s; detection, indirect UV at 254 nm; potential, 20 kV. Dilute urine (50 X in deionized water; solutes: 1 = chloride (109 ppm); 2 = sulfate (7.9 ppm); 3 = citrate (3.2 ppm); 4 = phosphate (16.2 ppm); 5 = carbonate (12.2 ppm). (Reproduced with permission from Wildman *et al.*, 1991.)

more consistent electropherograms even in those cases where the protein does not interfere with detection and quantitation of the drug (Caslavska *et al.*, 1993; Thormann *et al.*, 1993; Deyl *et al.*, 1994). A more detailed discussion on sample preparation can be found in Chapter 7.

Ruggedness is a desirable characteristic for analytical methods. For CE of biological samples, ruggedness is critical in two senses. The term implies not just the optimization of the separation, but also the degree of resistance to sample matrix effects. CZE and MEKC are the two CE modes most widely used and preferred for biological samples, although other modes, such as capillary isotachophoresis, have also been investigated for this purpose (Caslavska *et al.*, 1993). In CZE, selectivity is achieved mainly through manipulation of injection parameters (time and electrolyte content), applied voltage, and electrolyte buffer type, concentration, and pH. These conditions require careful optimization during method development (Garcia and Shihabi, 1993a,b; Shihabi, 1993) to both optimize the separation and to insure that sample matrix effects on analyte migration time and quantitation are minimized. The major disadvantage of CZE for analysis of biological samples is the tendency of proteins in the sample to adhere to the walls of the silica capillary and affect subsequent injections, causing problems with later electropherograms and requiring frequent capillary washes.

MEKC is typically more amenable than CZE to direct injection of biological samples with little or no sample preparation. This is due to solubilization of proteins by micelles, which eliminates protein-capillary interactions and improves the consistency of analyte migration time (Nishi and Terabe, 1990; Nishi *et al.*, 1990). MEKC also offers a wider variety of operating parameters to use in method optimization, since separation depends on both analyte ionic character and hydrophobicity. In MEKC, the analyst manipulates all of the parameters listed previously for CZE, and can also optimize the type and concentration of both the micelle and, if used, additives such as organic modifiers and salts (Lee *et al.*, 1993). It is more of a challenge to balance these additional parameters, but MEKC broadens the variety of analytes amenable to CE to include nonionic species. In cases where difficulties are encountered with both CZE and MEKC, polymeric modifiers, cyclodextrin-polyethylene oxide-polydextran) can be added to the electrolyte buffer to accomplish separations (Soini *et al.,* 1994). An alternative to such rigorous method development is to attempt the cancellation of matrix effects by preparing standards in the biological fluid matrix, but this is useful only when the matrix does not cause electrophoretic interference.

The majority of the CE literature of biological samples encompasses work with serum, plasma, saliva, and urine. Forensic, diagnostic, or therapeutic investigations of hair (Tagliaro *et al.*, 1993), cerebrospinal fluid (Hiraoka *et al.*, 1994), renal tissue (Fig. 12) (Phillips - and Kimmel, 1994), tumor cells (Shao *et al.*, 1994), brain tissue (Yoo *et al.*, 1993; Schmerr *et al.*, 1994) (Figs. 13 and 14), and drain fluid from the lungs (Wolfisberg *et al.*, 1993) have also been reported.

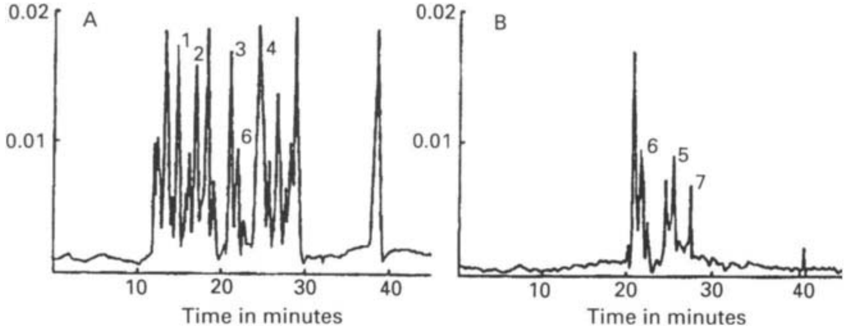

Fig. 12. Capillary electrophoresis of microdissected renal glomerular tissue extracts following filtration through (A) an M_r 30,000 cut-off membrane filter and (B) an M_r 10,000 cut-off membrane filter. Separation performed in a 75 cm (60 cm to detector) × 75 μm i.d. polyimide-coated capillary at 15°C. Voltage: 27 kV; buffer: 0.1M phosphate, pH 7.0; detection at 200 nm, 0.02 AU. Peaks: 1 = IL-6; 2 = γIFN; 3 = TNFα; 4 = IL-1; 5 = IL-8; 6 = RANTES (Regulated on Activation, Normal T-cell Expressed and Secreted); 7 = MCP-1 (monocyte chemotactic protein-1). (Reproduced with permission from Phillips and Kimmel, 1994).

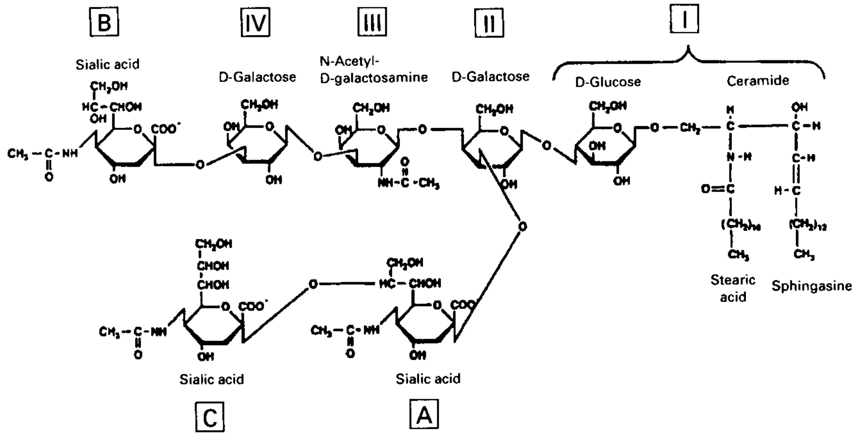

Fig. 13. Structures of some representative brain gangliosides. $\mathrm{G_{M1}}$: I, II, III, IV, A. $\mathrm{G_{D1a}}$: I, II, III, IV, A, B. $\mathrm{G_{D1b}}$: I, II, III, IV, A, C. $\mathrm{G_{T1b}}$: I, II, III, IV, A, B, C. (Reproduced with permission from Yoo *et al.*, 1993).

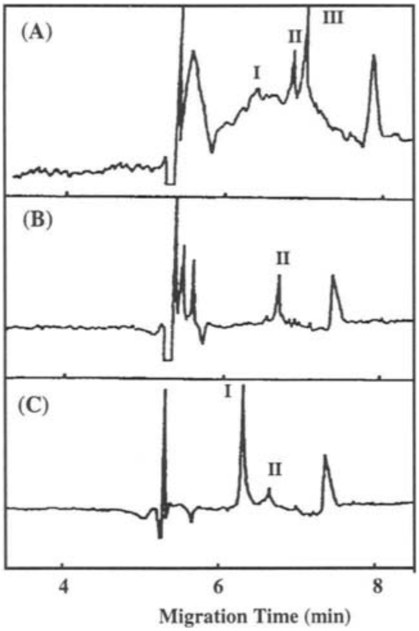

Fig 14. Electropherograms of gangliosides in biological matrices: (A) deer antler; (B) apricot seed; (C) rat brain. Conditions: . Peaks: I = G_{M1}; II =G_{D1a}; III = G_{D1b}. (Reproduced with permission from Yoo, *et al.*, 1993).

4.4. Routine Applications in Pharmaceutical Analytical Laboratories

Since capillary electrophoresis in its many forms is essentially a rather young technique, any applications involving analytes of pharmaceutical interest have generally been considered sufficiently interesting for publication. However, it has not necessarily become clear exactly in what ways CE will displace LC and/or become favored as the separation method of choice in the pharmaceutical laboratory. The technique certainly has many possibilities, but LC is an established technique (as outlined in Table 5) while CE is relatively new. Whether the advantages will be seen to provide benefits of significant quality to warrant replacing current techniques remains to be seen.

Table 5. Capillary Electrophoresis Techniques Compared to those of Liquid Chromatography in the Pharmaceutical Industry.

Rapid sample throughput, speed of analysis (CE+)
Amount of sample required for analysis (CE+)
Amount of reagent required to complete analysis (CE+)
Improved automation (LC+)
Ease of training in use of technique (LC = CE)
Reproducibility of technique (instrument-to-instrument, capillary-to-capillary) (LC+)
Ease of transfer of the technology from the development laboratory into the commercial support laboratory (LC+)
Ease of maintenance of instrumentation (CE = LC)
General applicability to many products (LC+)
Regulatory acceptance (LC+)

An important aspect will be the acceptance in full by worldwide regulatory agencies and pharmacopoeias. This is not necessarily as straightforward as it might sound. It should be noted that FDA laboratories are publishing their observations on the use of the technique (Flurer, 1995). This study illustrated the use of CE for the identification and quantification of selected aminoglycoside antibiotics and suggested it as a potential alternative method to the assays given by the U.S. Pharmacopoeia. This trend can be considered an important step forward in CE methodology acceptance. Numerous papers have been published on other applications. To find the application for a specific drug type or particular condition, we suggest a search of the current literature. Table 6 provides some examples of other pharmaceutical analytical challenges that have been addressed using various forms of capillary electrophoresis.

4.5. Future Trends.

The use of capillary electrophoresis in the pharmaceutical industrial laboratory will expand as research and development laboratories continue to demonstrate applications. As with most techniques in an industrial setting, it will be necessary to prove that capillary electrophoresis provides an adequate competitive advantage for the development of new chemical entities. Since the major analytical technique in the laboratory is liquid chromatography in its various forms, it is in response to the disadvantages of this technique that

Table 6. Additional Examples of Capillary Electrophoresis in Pharmaceutical Development

Application in Pharmaceutical Development	Reference
Methods to Evaluate and/or Improve the Accuracy of CE for the Quantitative Analysis of Pharmaceuticals and Related Substances	1. Altria (1993a) 2. Jumppanen and Riekkola (1995) 3. Hettiarachchi and Cheung (1993) 4. Taylor and Reid (1994) 5. Bechet *et al.* (1995)
Development and Validation of Methods for Determination of Ions as Counter-ions and Impurities in Drug Substances, in Drug Products, and in Biological Fluids	1. Swartz (1993) 2. Wu *et al.* (1993) 3. Janini *et al.* (1994) 4. Altria *et al.* (1994) 5. Nair and Izzo (1993) 6. Harrold *et al.* (1993)
Comparison of Liquid Chromatography and Capillary Electrophoresis Applied to Pharmaceutical Analysis	1. Cheung *et al.* (1993) 2. Lozano *et al.* (1995) 3. Williams *et al.* (1993) 4. Corran and Sutcliffe (1993)
Determination of Impurities in Drug Substance	1. Dawson *et al.* (1995) 2. Altria (1993b) 3. Hsieh *et al.* (1994) 4. Shah and Quinones 1995) 5. Qin *et al.* (1992)
Applications to Dosage Forms, including Excipients and Preservatives	1. Dang *et al.* (1993) 2. Tsai *et al.* (1992) 3. Ling *et al.* (1992) 4. Fanali *et al.* (1992) 5. Bullock (1993)
Applications to Pharmaceutical/Biotechnology Projects	1. Watson and Yao (1993) 2. Langenhuizen and Janssen (1993) 3. Hughes and Richberg (1993)

capillary electrophoresis will compete. As establishment of capillary electrophoresis in the pharmaceutical laboratory will require a significant monetary investment, it would be most useful to demonstrate saving of analysis time and resources. In addition, there can tend to be a "follow-the-leader" mentality in this industry, so it will be necessary to demonstrate that the technique is sanctioned by regulatory authorities worldwide as an acceptable analytical method. In certain applications, such as those described in above, this has been successfully accomplished. For CE to expand its usage and become as ubiquitous in the pharmaceutical analytical laboratory as liquid chromatography, a significant amount of communication concerning the technique and definitive proof that this is indeed a better mousetrap will be required.

5. References

Altria, K. D. and D. C. M. Luscombe (1993). *J. Pharm. Biomed. Anal., 11,* 415–420.

Altria, K. D. and Y. L. Chanter (1993). *J. Chromatogr. A, 652,* 415–420.

Altria, K. D., T. Wood, R. Kitscha. and A. Roberts-McIntosh (1994). *J. Pharmaceutical and Biomedical Analysis, 13* , 33–38.

Altria, K. D. (1993a). *J. Chromatogr., 646,* 245–257.

Altria, K. D. (1993b). *J. Chromatogr., 634,* 323–328.

Anigbogu, V. C., C. L. Copper and M. J. Sepaniak (1995). *J. Chromatogr. A, 705,* 343–349.

Bechet, I., M. Fillet, Ph. Hubert and J. Crommen (1995). *J. Pharm. Biomedical Analysis, 13 (4–5),* 497–503.

Beckers, J. L., F. M. Everaerts and M. T. Ackermans (1991). *J. Chromatogr., 537*, 407–428.

Bello, M. S., R. Rezzonico and P. G. Righetti (1994). *J. Chromatogr. A, 659,* 199–204.

Branch, S. K., U. Holzgrabe, T. M. Jefferies, H. Mallwitz and M. W. Matchett (1994). *J. Pharm.. Biomed. Anal., 12* , 1507–1517.

Bullock, J. (1993). *J. Chromatogr., 645,* 169–177.

Caslavska, J., E. Hufschmid, R. Theurillat, C. Desiderio, H. Wolfisberg and W. Thormann (1994). *J. Chromatogr. B, Biomed. Appl., 656,* 219–231.

Caslavska, J., S. Lienhard and W. Thormann (1993). *J. Chromatogr., 638,* 335–342.

Chen, T-M., R. C. George and M. H. Payne (1990). *J. High Res. Chromatogr.,* 782–784.

Cheung, A. P., T. Nguyenle and K. Hettiarachchi (1993). *J. Pharm. Biomed. Anal. 11* , 1261–1267.

Cleveland, J. A., Jr., M. H. Benko, S. J. Gluck and L. M. Walbroehl (1993). *J. Chromatogr. A, 652,* 301–308.

Cleveland, J. A., Jr., C. L, Martin and S. J. Gluck (1994a). *J. Chromatogr. A, 679,* 167–171.

Corran, P. H. and N. Sutcliffe (1993). *J. Chromatogr., 636,* 87–94.

Cruzado, I. D. and G. Vigh, (1992). *J. Chromatogr., 608,* 421–425.

Dang, Q-X, L-X. Yan, Z.-P. Sun and D.-K. Ling (1993). *J. Chromatogr., 630,* 363–369.

Dawson, J. R., S. C. Nichols and G. E. Taylor (1995). *J. Chromatogr. A., 700,* 163–172

De Camp, W. H.(1993). *J. Pharm . Biomed. Anal., 11,* 1167–1172.

Demarest, C. W., E. A. Monnot-Chase, J. Jiu and R. Weinberger (1992). Separation of small molecules by high performance capillary electrophoresis. In *Capillary Electrophoresis–Theory and Practice*, (P. D. Grossman and J. C. Colburn, eds.), pp. 301–341. Academic Press, San Diego, CA .

Deyl, Z., F. Tagliaro and I. Miksík (1994). *J. Chromatogr. B, Biomed. Appl., 656,* 3–27.

D'Hulst, A. and N. Verbeke (1992). *J. Chromatogr., 608,* 275–287.

Fanali, S., M. Cristalli, A. Nardi and M. G. Quaglia (1992). *Il Farmaco, 47,* 711–722.

Dobashi, A., T. Ono, S. Hara and J. Yamaguchi, (1989). *Anal. Chem., 61,* 1984–1986.

Federal Register, 43, 45077, September 29, 1978.

Federal Register, 60, 1257, Wednesday, March 1, 1995/Notices.

Food and Drug Administration, Guideline for Submitting Supporting Documentation in Drug Applications for the Manufacture of Drug Substances, February, 1987.

Flurer, C. L. (1995). *J. Pharmaceut. & Biomed. Anal., 13 (7),* 809–816.

Garcia, L. L. and Z. K. Shihabi (1993a). *J. Chromatogr. A, 652,* 465–469.

Garcia, L. L. and Z. K. Shihabi (1993b). *J. Liq. Chromatogr., 16(9–10),* 2049–2057.

Gassmann, E., J. E. Kuo and R. N. Zare (1985). *Science (Washington D.C., 230,* 813–814.

Gluck, S. J. and J. A. Cleveland, Jr. (1994b). *J. Chromatogr. A, 680,* 43–48.

Gluck, S. J. and J. A. Cleveland, Jr. (1994c). *J. Chromatogr. A, 680,* 49–56.

Gonzalez, E., R. Montes and J. J. Laserna (1993). *Anal. Chim. Acta, 282,* 687–693.

Gozel, P., E. Gassmann, H. Michelsen and R. N. Zare (1987). *Anal. Chem., 59,* 44–49.

Guttman, A. and H. E. Schwartz (1995). *Anal. Chem., 67,* 2279–2283.

Harrold, M. P., M. J. Wojtusik, J. Riviello and P. Henson (1993). *J. Chromatogr., 640,* 463–471.

Heiger, D. N. High Performance Capillary Electrophoresis—An Introduction (1992). Hewlett–Packard Company Publication Number 12–5091–6199E , p. 78.

Herbert, B. J. and J. G. Dorsey, (1995). *Anal. Chem., 59,* 744–49.

Hettiarachchi, K. and A. P. Cheung (1993). *J. Pharm. Biomed. Anal., 11*, 1251–1259.

Hiraoka, A., J. Akai, I. Tominaga, M. Hattori, H. Sasaki and T. Arato (1994). *J. Chromatogr. A, 680,* 243–246.

Hsieh, F. Y. L., J. Cai and J. Henion (1994). *J. Chromatogr. A, 679,* 206–211.

Hughes, D. E. and P. Richberg (1993). *J. Chromatogr., 635,* 313–318.

Issaq, H. J. (1994) *Instrum. Science Technol., 22,* 110–149.

Janini, G. M., K. C. Chan, G. M. Muschik and H. J. Issaq (1994). *J. Chromatogr. B: Biomed. Applications, 657,* 419–423.

Jumppanen, J. H. and M.-L. Riekkola (1995). *Anal. Chem., 67,* 1060–1066.

Kuhn, R., F. Stoecklin and F. Erni (1992). *Chromatographia, 33,* 33–36.

International Conference on Harmonization; *Guideline on Validation of Analytical Procedures*; Draft 5, April 1995.

Langenhuizen, M. H. J. M. and P. S. L. Janssen. (1993). *J. Chromatogr., 638,* 311–318.

Lee, K.-J., J. Lee and D. Moon (1993). *J. Chromatogr., Biomed. Appl., 616,* 135–143.

Ling, B. L., W. R. G. Baeyens, P. Van Acker and C. Dewaele (1992). *J. Pharm. Biomed. Anal. 10*, 717–721.

Lozano, R., F. V. Warren Jr., S. Perlman. and J. M. Joseph (1995) *J. Pharm. Biomed. Anal. ,13* , 139–148.

McLaughlin, G. M., J. A. Nolan, J. L. Lindahl, R. H. Palmieri, K. W. Anderson, S. C. Morris, J. A., Morrison and T. J. Bronzert (1992). *J. Liq. Chromatogr., 15,* 961–1021.

Matchett, M. W., S. K. Branch, S. K. and T. M. Jefferies (1995). *J. Chromatogr. A 705,* 351–361.

Minister of National Health and Welfare, Health Protection Branch, Health and Welfare Canada (1992). *Drugs Directorate Guidelines, Acceptable Methods, (draft).*

Nair, J. B. and C. G. Izzo, (1993). *J. Chromatogr., 640,* 445–461.

Nielsen, R. G., G. S. Sittampalam and E. C. Rickard (1989). *Anal. Biochem., 177,* 20–26.

Nishi, H. and S. Terabe (1990). *Electrophoresis, 11,* 691–701.

Nishi, H. and S. Terabe (1993). *J. Pharm. Biomedical Anal. 11,* 1277–1287.

Nishi, H. and S. Terabe (1995) *J. Chromatogr. A., 694,* 245–276.

Nishi, H., T. Fukuyama and M. Matsuo (1990). *J. Chromatogr., 515,* 245–255.

Nishi, H., T. Fukyama, M. Matsuo. and S. Terabe (1990b). *J. Chromatogr., 515,* 233–243.

Nishi, H., K. Nakamura, H. Nakai and T. Sato (1995). *Anal. Chem., 67,* 2334–2341.

Noroski, J. E., D. J. Mayo and M. Moran (1995). *J. Pharm. Biomed. Anal. 13* , 45–52.

Novotny, M., H. Soini and M. Stefansson (1994). *Anal. Chem., 66,* 646A–655A.

Otsuka, K. and Terabe, S. (1993). *J. Liq. Chromatogr., 16* , 945–953.

Perrett, D., *Capillary Electrophoresis in Biomedical and Pharmaceutical Research, in Capillary Electrophoresis: Theory and Practice,* pp. 371–407.

Perret, D. and G. A. Ross, (1995). *J. Chromatogr., 700,* 179–186.

Phillips, T. M. and P. L. Kimmel (1994). *J. Chromatogr. B, Biomed. Appl., 656,* 259–266.

Pruñonosa, J., R. Obach, A. Diezcascon and L. Gouesclou (1992). *J. Chromatogr. B, Biomed. Appl., 574,* 127–132.

Qin, X.-Z, D. P. Ip and E. W. Tsai (1992). *J. Chromatogr., 626,* 251–258.

Rabel, S. R. and J. F. Stobaugh (1993). *Pharm. Res., 10,* 171- 186.

Rogan, M. M., K. D. Altria and D. M. Goodall (1994) *Chirality, 6,* 25–40.

Schmerr, M. J., K. R. Goodwin and R. C. Cutlip (1994). *J. Chromatogr. A, 680,* 447–453.

Shah, P. A., and R. Quinones, (1995). *J. Liq. Chromatogr, 18,* 1349–1362.

Shao, X., K. O'Neill, Z. Zhao, S. Andersen, A. Malik and M. Lee (1994). *J. Chromatogr. A, 680,* 463–468.

Shihabi, Z. K. (1993). *J. Liq. Chromatogr., 16(9–10),* 2059–2068.

Shirao, M., R. Furuta, S. Suzuki, H. Nakazawa, S. Fujita and T. Maruyama (1994). *J. Chromatogr. A, 680,* 247–251.

Shulman, S. R., P. Hewitt and M. Manocchia (1995). *Drug Information Journal, 29,* 385–413.

Smith, N. W. and M. B. Evans (1994). *J. Pharm. Biomed. Anal. 12,* 579–611.

Soini, H., M. V. Novotny and M.-L. Riekkola (1992). *J. Microcol. Sep., 4,* 313–318.

Soini, H., M.-L. Riekkola and M. V. Novotny (1994). *J. Chromatogr. A, 680,* 623–634.

Swartz, M. E. (1993). *J. Chromatogr., 640,* 441–444.

Tagliaro, F., C. Poiesi, R. Aiello, R. Dorizzi, S. Ghielmi and M. Marigo (1993). *J. Chromatogr., 638,* 303–309.

Taylor, R. B. and R. G. Reid (1994). *J. Pharm. Biomed. Anal., 13,* 21–26.

The Gold Sheet, F-D-C Reports, Vol. 29, No. 6, 1995.

Thomas, B. R., X. G. Fang, X. Chen, R. J. Tyrrel and S. Ghodbane (1994). *J. Chromatogr. B, 657,* 383–394.

Thormann, W., S. Lienhard and P. Wernly (1993). *J. Chromatogr., 636,* 137–148.

Tran, A. D., T. Blanc and E. J. Leopold (1990). *J. Chromatogr., 516,* 241–249.

Tsai, E. W., M. M. Singh, H. H. Lu, D. P. Ip and M. A. Brooks (1992). *J. Chromatogr., 626,* 245–250.

United States Pharmacopeia (USP). XXIII (1995). Physical Tests <621>, 1776.

United States Pharmacopeia (USP). XXIII (1995). General Information <1225>, 1982.

Watson, E. and F. Yao, (1993). *Anal. Biochem., 210,* 389–393.

Wildman, B. J., P. E. Jackson, W. R. Jones and P. G. Alden (1991). *J. Chromatogr. 546,* 459–466.

Williams, S. J., D. M. Goodall and K. P. Evans (1993). *J. Chromatogr. 629,* 379–384.

Wolfisberg, H., A. Schmutz, R. Stotzerand W. Thormann (1993). *J. Chromatogr. A, 652,* 407–416.

Wu, N., W. J. Horvath, P. Sun and C. W. Huie (1993). *J. Chromatogr., 635,* 307–312.

Yao, Y. J., K. S. Khoo, M. C. M. Chung and S. F. Y. Li (1994). *J. Chromatogr. A,* 431–435.

Yoo, Y., Y. Kim, G.-J. Jhon and J. Park (1993). *J. Chromatogr. A, 652,* 431–439.

CHAPTER 12

Capillary Electrophoresis-based Methods for *in vivo* Analysis: Single Cell Analysis and Microdialysis Sampling

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1. Introduction to *in vivo* Analysis

The use of capillary electrophoresis (CE) to monitor biological events is an expanding area. CE has been used for monitoring drugs and their metabolites in tissue as well as endogenous compounds in single cells. Recently, CE has been employed for both on-line and off-line analysis of microdialysis samples. This chapter will provide a discussion of recent advances in the use of CE for *in vivo* analysis.

Many pharmaceutical and biomedical scientists conduct studies using whole animals, tissue homogenates, isolated organs or tissue culture. Each level of biological organization offers differing types of *in vivo* information. The higher levels provide information on the complex biochemical interactions occurring between cells, tissues and even whole organs. However, such information may be difficult or impossible to interpret fully due to the highly interdependent nature of these interactions. As a result, a biological process under study may be observed or even quantitated in a whole animal, yet fundamental questions regarding the origin or progression of a process may not be easily understood or explained.

Studies at lower levels of biological organization, such as tissue culture, can provide information that is more easily interpreted but does not account for all potential interactions and thus may not necessarily supply a complete model of the actual *in vivo* process.

Obviously, complete knowledge of the chemical and biochemical mechanisms of important biological processes such as genetic control of disease, immune regulation, and the aging process is not achievable. However, obtaining clear cause and effect relationships at the cellular level will provide a great deal of insight into many biological processes. Once cellular data are obtained, more complex interactions such as those between heterogeneous cell types within a tissue, communication among cells and, eventually, interorgan associations could be incorporated into the model describing the biological process. A wide variety of analytical tools are needed to help provide *in vivo* information at different levels of biological organization. These include:

1. tools for the determination of analytes at systemic levels,
2. tools for determination of specific tissue levels of analytes within a living animal, and
3. tools that permit the study of the biotransformation of substances within isolated organs, tissues or cells.

2. Definitions

Pharmacokinetics is the study of the temporal profiles of *in vivo* drug absorption, distribution, metabolism and excretion (Gibaldi and Perrier, 1982). In the study of drug absorption, pharmacokinetics can be utilized as a quantitative tool to improve the design of biological experiments and clinical treatments. This is because pharmacokinetics sheds light upon the relationship between the dosage parameters of a drug and its ultimate efficacy (or toxicity). The clinical application of pharmacokinetics reduces the uncertainty inherent in the dosage/efficacy relationship. Similarly, pharmacokinetic studies can provide useful information concerning the absorption and elimination of a drug. In order to gain access to this important information, however, analytical methodologies capable of accurately determining drug and metabolite species in a biological matrix are required.

Drug metabolism and distribution studies can also benefit from improved methods of *in vivo* analysis. The body chemically modifies foreign compounds (including drugs) in an attempt to enhance the detoxification and elimination of these species. For example, some species that are ultimately carcinogenic are not carcinogenic in the original form in which they enter the body. These species are metabolized by normal "detoxication" pathways to reactive intermediates that are responsible for deleterious effects (Horning, 1983). Toxicity resulting from the formation of reactive intermediates during the metabolic process is of particular interest to the pharmaceutical and biomedical community.

Because much of the neurochemical functioning of the mammalian brain remains an enigma, workers in the neurosciences also benefit from improved methods of *in vivo* analysis. Two very significant areas of neurochemical study currently relate to the basic functioning of the neurotransmission process itself and the relationship between neurotransmission and behavior in an unperturbed animal. Several authors have described the analytical challenges inherent in the study of neurochemical systems (Adams, 1976; Justice, 1987; Wightman *et al.*, 1988).

It is clear that *in vivo* analytical techniques have an impact upon a wide variety of scientific disciplines and that improved *in vivo* analytical methods will provide many benefits to society.

2.1. Goals and Motivations for in vivo Analysis

An ideal *in vivo* monitoring system would incorporate a number of characteristics, such as:

1. Analytes involved in biological processes will be monitored with suitable accuracy and precision.
2. The temporal resolution of the *in vivo* monitoring systems will be significantly greater than the frequency of the biological process.
3. The *in vivo* monitoring system must be applicable to many analytes with suitable sensitivity.
4. The biological system under study must be perturbed only minimally during the experiment.
5. The *in vivo* monitoring system should be automated and should minimize the need for sample preparation.
6. The *in vivo* monitoring system should have the capability to probe multiple physiological sites in an animal simultaneously.
7. The *in vivo* probes should be small enough to provide sufficient spatial resolution to monitor chemical events in heterogeneous tissue (*e.g.*, the brain).

No analytical device currently possesses all of these characteristics, although advances have been made toward the general goal of improved *in vivo* analysis.

2.2. *Classical in vivo Analysis*

The types of analytical techniques utilized for analysis of *in vivo* species vary considerably with the region of the body involved and the compounds being studied. The physiological nature of the biological process of interest also has a bearing upon the technique required. This section will focus on classical methods of obtaining *in vivo* information in neurochemistry and pharmacokinetics/drug metabolism.

2.2.1. *Neurochemistry*

The study of the functioning of the central nervous system poses a number of complex scientific questions, for example: What chemical species are involved in the neurotransmission process? What are the *in vivo* concentrations of these species and in which specific regions of the brain are these species found? At what rates do the processes of neurotransmitter release, uptake and metabolism occur? How do external stimuli and observed behavior correlate with neurotransmitter dynamics, which occur on sub-second time scales? It is not surprising that a single analytical method cannot provide answers to such a wide variety of difficult questions.

Dynamic chemical processes such as neurotransmitter uptake and release are commonly probed via techniques such as *in vivo* voltammetry (Adams, 1976; Wightman *et al.*, 1988) and implanted ion-selective electrodes (Ammann, 1986). These techniques provide sub-second response and analysis times compatible with the transient nature of the neurotransmission process. One drawback of this approach is that any electroactive species within the extracellular fluid (ECF) of the brain can potentially interfere with the determination since no physical separation of analyte from matrix is performed. Several advances have been made using selective membranes and waveforms. However, this chapter will focus not on methods for obtaining highly transient chemical information using single analyte biosensors but on CE-based techniques that provide excellent chemical selectivity with somewhat less rapid temporal resolutions.

Obtaining chemical information with less stringent temporal restrictions, such as determining the identity and average concentration of species present in a specific region of the brain over a given length of time, is generally best addressed via a dialysis or perfusion sampling methodology in conjunction with an analytical separation and detection system. Such methods provide the excellent selectivity necessary for the unambiguous identification of analytes. However, temporal resolutions are limited, and these methods are normally restricted to tracking processes with times scales on the order of five minutes to several days.

Sampling and analyzing compounds from within specific regions of the brain can be an extremely challenging task. Because the brain is quite heterogeneous, neurochemists may wish to sample from very small, deeply buried locations. Early methods of determining the concentration of a given species within a brain region consisted of sacrificing the animal, surgically removing the desired region, homogenizing the tissue, and analyzing the resulting mixture. This approach has clear disadvantages in terms of monitoring dynamic processes. Death and dissection of the animal can affect the observed concentrations of the analytes. It can also be difficult to accurately determine concentration of analytes within discrete brain regions.

In order to circumvent problems with tissue sampling, perfusion and dialysis sampling techniques were developed. One of the earliest perfusion sampling devices was the cortical cup (Dodd and Bradford, 1974). It consisted of a cylinder filled with artificial cerebral spinal fluid (ACSF) firmly positioned against the cerebral cortex of the experimental animal. Analyte species within the cortex diffuse into the ACSF and are then collected for analysis in a periodic or continuous fashion. The technique is relatively convenient and can be used on awake animals. However, due to the design of the apparatus, studies of structures deep within the brain cannot be performed. The temporal resolution of the method is 10–20 min per sample.

An alternate method is push-pull perfusion (Fox and Hilton, 1958; Gaddum, 1961; Delgado, 1962). One common design of the push-pull cannula consists of a concentric arrangement of two tubes, one being somewhat shorter than the other. Two pumps are used to control the flow of ACSF through the cannula. One delivers ACSF into the brain region while another withdraws it at an equivalent rate. This technique can be used to probe deep brain structures in awake animals. However, the turbulence and flow associated with the pumping of liquid directly upon exposed cells increases tissue trauma near the tip of the cannula. The temporal resolution of this method is on the order of 5–20 min per sample.

Dialysis-based sampling techniques were developed to address some of the perceived shortcomings of push-pull perfusion methods. Early dialysis measurements consisted of implanting a macroscopic semi-permeable dialysis sac filled with isotonic saline in the brain of the experimental animal (Bito *et al.*, 1966). Species equilibrated through the dialysis membrane and were collected within the sac. At the desired sampling time, the sac was surgically removed and its contents analyzed. This method is limited to obtaining a single sample per experimental animal and, due to its integrative nature, provides poor temporal resolution. An improved version of this technique is microdialysis, in which a hollow dialysis fiber is implanted within the brain and perfused with ACSF. Species equilibrating across the fiber membrane are collected off-line as discrete volume samples.

2.2.2. *Metabolism and Pharmacokinetics*

Classical metabolism studies of drugs and other xenobiotics have often been conducted in tissue preparations. Samples are analyzed at discrete time intervals to provide information on the formation of metabolic products. However, because cellular compartments are ruptured during the homogenization of the prepared tissue, the drug may interact with enzymes or other species in the tissue preparation that it would not normally have encountered *in vivo*. Such interactions may have a dramatic effect on the outcome of the experiment. Another limitation of tissue preparations is that they cannot account for the many interactions affecting drug metabolism that occur between different tissues and organs.

The pharmacokinetics of a drug are most commonly determined by delivering a known dose of the drug to an experimental animal and subsequently removing blood samples at discrete time intervals. These blood samples are then analyzed for drug content to provide a concentration versus time curve for the drug. Typically, total drug levels rather than the more physiologically relevant free non-protein bound drug levels are determined. Also, enzymatic degradation of the drug can occur during sample collection and storage, complicating the interpretation of the data. A related problem is that in order for samples to be separated by liquid chromatography (LC) or other methods, a protein precipitation step is generally needed prior to analysis.

To investigate tissue levels of a drug as a function of time, the classical approach is the sacrifice of one or more experimental animals at each temporal point upon the concentration-time curve. The tissue is then surgically removed and analyzed for drug content. Clearly, the use of experimental animals should be minimized for financial and ethical reasons. Better precision can also be obtained by carrying out the entire pharmacokinetic experiment in a single animal, since substantial animal-to-animal variation in metabolism of the drug may exist. As seen here, an opportunity exists for improved analytical techniques that can provide *in vivo* analyses for both neurochemical and pharmacokinetics/drug metabolism studies. The use of microdialysis sampling as an *in vivo* analysis method will now be described.

2.3. *Introduction to Microdialysis*

Microdialysis sampling is an *in vivo* perfusion sampling technique useful for monitoring the extracellular chemistry of selected physiological regions. It was initially investigated by Bito *et al.* (1966) and Delgado *et al.* (1972) and developed in its modern form by Ungerstedt *et al.* (1982). A number of excellent reviews detailing the principles and applications of microdialysis have appeared (Ungerstedt and Hallström, 1987; Benveniste, 1989; Benveniste and Hüttemeier, 1990; Lönnroth and Smith, 1990; Hamberger *et al.*, 1991; Lönnroth, 1991; Lunte *et al.*, 1991; Ungerstedt, 1991; Westerink, 1992). To conduct microdialysis sampling, a 1–4 mm length of hollow, semipermeable dialysis tubing (0.3 mm dia.) is implanted in the biological tissue of interest. Capillary tubes are connected to the ends of the hollow membrane and are used to perfuse physiological salt solutions through the tubular membrane. The flow turbulence associated with the perfusate is now completely contained within the dialysis membrane. Thus, there is minimal trauma to the tissue after the initial implantation of the probe. Low molecular weight substances in the extracellular space diffuse into the interior of the probe and are carried away, much as if the dialysis probe were functioning as a capillary blood vessel. In addition, it is possible to deliver a substance to a specific organ or tissue by including the substance of interest in the perfusing fluid. The perfusing fluid is collected externally and the concentration of the species of interest determined. Temporal information is obtained through correlation of the analyte concentration with its collection time.

Microdialysis is carried out by first implanting a dialysis probe within the organ or tissue of interest. A number of different microdialysis probes exist. The most popular design is the concentric probe, shown in Fig. 1. Others include the flexible probe, linear and shunt designs. The probe body is composed of an inert material such as stainless steel or fused silica. Commonly used dialysis membrane materials include polycarbonate, polyacrylonitrile, and regenerated cellulose. Molecular weight cutoffs of 5 to 20 kD are common.

Perfusate compositions that precisely match the composition of the ECF being sampled are generally utilized. It is also possible to include a drug species in the perfusate to achieve local delivery of the species to a specific tissue. Metabolites can then be recovered via the same probe or another probe placed in close proximity to the first. Such an experiment provides information on local metabolism without delivering a systemic dose of the drug.

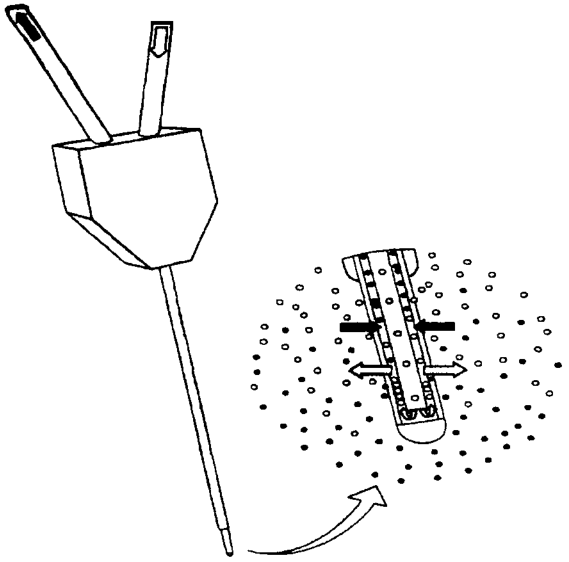

Fig. 1. Design of a concentric microdialysis probe. Species in the extracellular space equilibrate across the dialysis membrane and are collected in the dialysate leaving the probe. (Reproduced with permission from Ungerstedt, 1991.)

Microdialysis experiments require a system for animal restraint and handling (especially for studies with awake animals) and a high-precision syringe pump for delivery of the perfusate. Analysis may be performed off-line or on-line. For off-line analysis, the sample collection system may involve simply capturing dialysate in microvials or may include the use of a refrigerated fraction collector. On-line analysis of microdialysis samples requires a valve or interface to couple the microdialysis system directly to the analysis system and will require some form of central control unit, typically a microcomputer. A typical experimental apparatus for microdialysis studies is shown in Fig. 2.

Several important points are relevant to the overall success of the microdialysis experiment. The rate of diffusion of the analyte from the extracellular space into the probe body is dependent upon the flow rate of the perfusate. For this reason, a highly stable (generally low) flow rate must be employed in order to obtain quantitative and reproducible estimates of the concentrations of the analytes within the *in vivo* environment. Well-regulated syringe pumps are thus required.

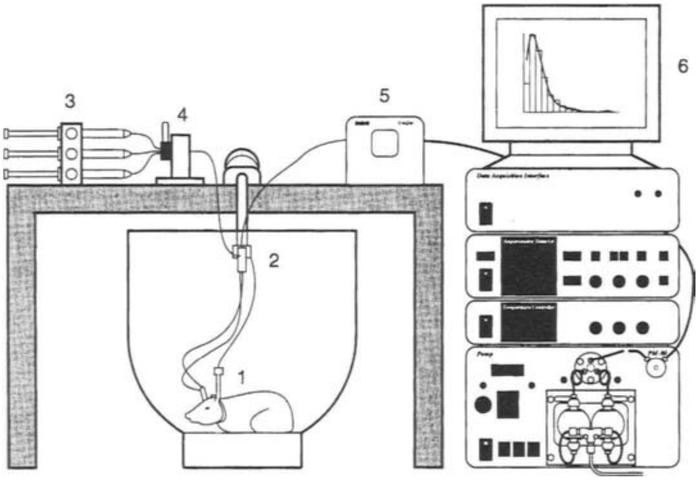

Fig. 2. System for on-line microdialysis sampling and analysis. 1, Dialysis probe; 2, "Bee Keeper" container; 3, syringe pump; 4, switch for changeover to new syringe; 5, on-line injector; 6, presentation of data as a pharmacokinetic curve. (Reproduced with permission from BAS, West Lafayette, IN.)

Studies on awake, freely moving animals require animals to be housed in an awake animal system in order to permit free range of motion while making it impossible for the animal to destroy the implanted probe or its associated tubing. Studies on anesthetized animals are much simpler since the behavior of the animal need not be considered; however, it is known that anesthetics can have significant effects upon pharmacokinetics of drugs or levels of neurotransmitters. Anesthetized animals should be placed on a heating pad or under a heating lamp to help maintain proper body temperature during the experiment.

In order to obtain data that accurately reflect *in vivo* conditions, the microdialysis sampling system should maintain the temporal integrity of the sampled dialysate. Therefore, mixing of the dialysate stream must be minimized and care should be exercised in the selection of the tubing, valves and connecting fittings to minimize extra-column zone broadening and dispersive effects.

One substantial analytical advantage of microdialysis sampling over biosensors is that samples collected off-line may be analyzed by any suitable analytical technique, or even by multiple techniques. A number of separation and detection methods, including LC, CE, mass spectroscopy (MS) (Deterding *et al.*, 1992; Caprioli and Lin, 1994), immunoassay (Maidment *et al.*, 1989) and enzymatic techniques (Roda *et al.*, 1991; Van der Kuil and Korf, 1991; Boutelle *et al.*, 1992 have been used for the analysis of microdialysates. The most commonly employed method is LC with electrochemical or fluorescence detection. It is often used for studies of brain chemistry, for example in the analysis of neurotransmitter species such as dopamine, norepinephrine, γ-amino butyric acid (GABA), aspartate, glutamate, and taurine.

2.4. Calibration

Quantitatively relating the concentrations of species in the dialysate to the actual extracellular concentration in the region sampled is not a trivial matter. Microdialysis does not obtain actual samples of the ECF, so the system must be calibrated to provide accurate absolute *in vivo* concentration values. The challenge involved in determining the actual concentrations of analytes present within the organ of interest lies in the fact that the analytes are collected as they diffuse through a semipermeable membrane due to the influence of a concentration gradient. The dialysate and the extracellular environment are not in complete equilibrium. Recovery of analyte across the probe is customarily not 100%. Therefore, the concentration of analyte in the collected perfusate is generally a fraction of the concentration in the ECF. The final concentration of analytes within the collected dialysate will depend upon a number of physical parameters such as perfusion flow rate; surface area; molecular weight cutoff and composition of the dialysis membrane; the temperature and diffusive characteristics of the tissue in which the probe is implanted; the elapsed time from probe implantation to onset of the experiment; the rate of metabolism and transport of the analyte through the tissue; any "drainage" or depletion of the analyte from within the environment surrounding the probe due to the effects of the probe itself; and the physical and chemical properties of the specific analyte species.

Relative recovery is defined as the ratio of analyte concentration in the collected dialysate to its concentration in the extracellular environment (Zetterström *et al.*, 1982). Absolute recovery is defined as the mass of analyte recovered from the dialysate per unit time. Unless otherwise indicated, the term recovery as used herein will refer to the relative recovery.

The most dramatic and easily controlled experimental parameter that affects recovery is the perfusate flow rate. Recovery is inversely and exponentially related to flow rate, as seen in Fig. 3. The absolute recovery, or the flux across the membrane, does not increase dramatically at flow rates above the 2–3 μl/min region. However, at very high flow rates, there can be a loss of analyte due to ultrafiltration. The relationship of flow rate and recovery is especially important as it affects the temporal resolution of microdialysis. The slower the flow rate, the more concentrated the sample, but also the less sample per unit time. High flow rates provide larger volumes per unit time, but the analyte in the dialysate is less concentrated, putting more stress on the analytical method. Analysts typically must choose between improving temporal resolution through the use of increased perfusate flow rates or improving detectability by lowering perfusate flow rates to increase recovery levels.

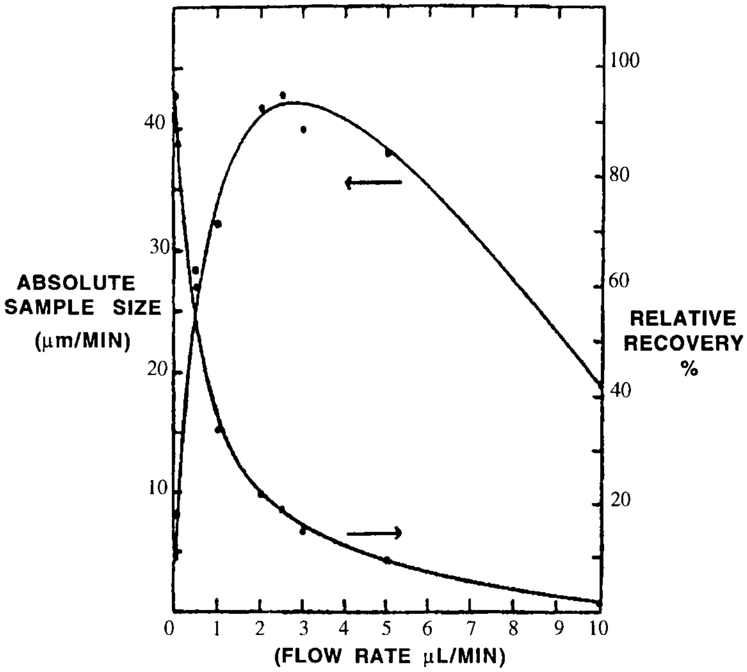

Fig. 3. Relationship between relative recovery and perfusate flow rate. Low perfusate flow rates (roughly 1 μl\min) are required to maintain high probe recoveries. (Reproduced with permission from Wages *et al.*, 1986.)

2.5. Applications of Microdialysis

Many early applications of microdialysis involved neurochemistry. Microdialysis was developed for neurochemical applications for several specific reasons. The cell layer between the interior of the blood vessels within the brain and the brain itself, known as the blood-brain barrier, prevents the rapid transport of most hydrophilic species between the brain interstitial space and the bloodstream. Thus, chemistry in the brain, unlike in other organs, cannot be monitored by collecting blood samples. Another complicating factor in neurochemical analysis is that the brain is known to be very heterogeneous. Each site may control different physiological functions and possess differing extracellular chemical concentrations. Gathering accurate chemical information regarding the extracellular composition of these complex sites requires a method such as microdialysis, which provides good spatial and temporal resolution.

2.6. Off-line Analysis Of Microdialysis Samples by CE

A number of groups have demonstrated the application of capillary electrophoresis (CE) separations to the analysis of collected microdialysis samples. Hernandez *et al.* (1991) evaluated the performance of off-line CE separations for the calibration of microdialysis probes used in the study of the effects of cocaine, lidocaine and procaine delivered locally to the nucleus accumbens of a rat. The effects of the delivered species on dopamine levels were assessed by collecting dialysate samples at 20 min intervals and determining the dopamine by LC with electrochemical detection. Capillary electrophoresis with electrochemical detection (CEEC) has been used for the analysis of i.v. microdialysis samples collected for pharmacokinetic studies of L-dopa in the rat (O'Shea *et al.*, 1992a). The detection limit for L-dopa was 3.9 ng/ml (98 amol). The method exhibited the potential for improved temporal resolution relative to LC analysis, as only nanoliter volumes of sample were injected, though in this study samples were collected at 5 min intervals.

O'Shea *et al.* (1992b) also utilized a CEEC system to determine extracellular levels of aspartate, glutamate, and alanine in microdialysis samples from rat brain. The amino acids were derivatized off-line with naphthalene-2,3-carboxaldehyde. The method was used to monitor changes in levels of excitatory amino acids upon an influx of a high K^+ concentration in the microdialysis perfusate. Temporal resolution was again 5 min per sample. Fig. 4 shows the effectiveness of this technique in providing this information.

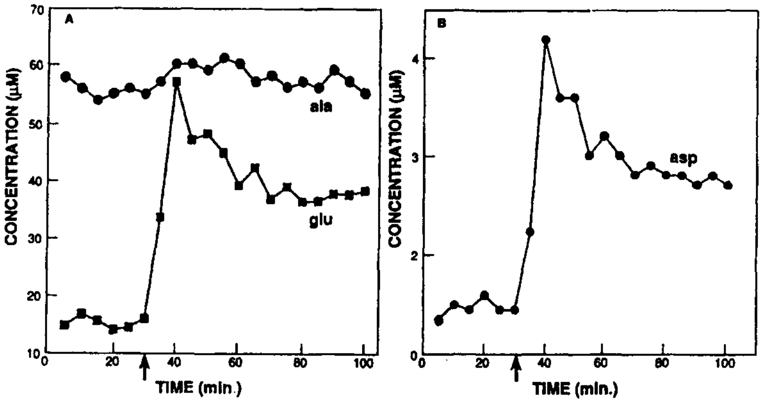

Fig. 4. Concentration-time curves of (A) alanine and glutamate and (B) aspartate in rat brain. Amino acids were derivatized off-line with naphthalenedialdehyde (NDA) and analyzed via CE-EC. The arrow indicates application of potassium stimulation. (Reproduced with permission from O'Shea *et al.*, 1992b.)

Tellez *et al.* (1992) described the use of CE for the off-line analysis of microdialysis samples collected for pharmacokinetic studies of phenobarbital. Probes were implanted in both the blood and brain of a rat, and pharmacokinetic curves were obtained simultaneously. Temporal resolution was 10 min. Hernandez has performed the analysis of aspartate and glutamate in brain microdialysates in less than 2 min (Hernandez *et al.*, 1991).

More recently, Malone *et al.* (1995) reported the use of CEEC to determine the release of kynurenine and tryptophan in rat brain. The application of CEEC as the analysis system permitted the use of slow flow rates and, therefore, higher recoveries in the dialysis probe (72.5% for tryptophan). Temporal resolution was 15 min/sample. Once basal levels were determined, a 100 mg/kg of tryptophan was administered i.p., and the increase of tryptophan and kynurenine in the ECF was monitored. Tryptophan was found to increase three- to fourfold following loading, and a concentration of 2–3 mM (n=3) was reached approximately 90 min after administration.

The use of CE for the off-line analysis of collected dialysate samples does have some advantages over LC. CE separations are typically of higher resolution and are more rapid and CE also requires only nanoliters of injected sample in order to conduct a separation. Therefore, the same 5 μl sample can be analyzed several times without appreciable loss of volume. Due to its small sample volume requirements, CE has the potential to dramatically improve the temporal resolution of microdialysis experiments. However, a system for handling the sub-microliter samples would be necessary. This can be accomplished using on-line microdialysis-CE.

2.7. On-line Analysis Of Microdialysis Samples

Off-line analysis of microdialysis samples is limited by the sample volume requirements of the analytical method. For conventional HPLC, approximately 10 µl of sample is usually needed for analysis. In order to maintain high recoveries of analyte, the perfusate flow rate must be kept low, typically on the order of 1 µl/min. Therefore, if a 10 µl sample is needed for the analyses, the temporal resolution is 10 min.

On-line analysis allows the manipulation of smaller volume samples. In 1986, Wages *et al.* reported using a microbore (1 mm i.d.) LC column for on-line analysis of dialysate samples obtained from rat brain using a push-pull perfusion system. The small sample volume requirements of the system allowed the use of flow rates of 0.1 µl/min. These very low flow rates produced recoveries of dopamine of over 90%. These enhanced recovery levels significantly improved the detection of dopamine, which is present in the extracellular space at the 10–50 nM level. Extensions of this basic on-line microdialysis-micro-LC concept now allow the routine measurement of 20 nM dopamine in freely moving animals at sampling intervals of 5 min (Church and Justice, 1987). Rapid sampling intervals such as these are important for the characterization of transient biological processes, such as the release of neurotransmitters during behavioral experiments. Recently, microdialysis has been coupled to fast microbore LC for pharmacokinetic studies. Separations of 40 s have been reported (Chen and Lunte, 1995). Although a 500 nl sample loop was used, the sample volume required for reproducible injections was 1.5 µl. In this case, the temporal resolution is limited by the separation.

2.8. Analysis of Microdialysis Samples with On-line CE Separations

In order to permit the automated analysis of nanoliter volumes of biological fluids, a on-line microdialysis-CE system is required. The first on-line microdialysis-CE system was reported by Hogan *et al.* (1994). The experimental layout of such a system is shown in Fig. 5. A microdialysis sampling system perfuses the probe with perfusate supplied by pump 1, collects dialysate from the experimental animal

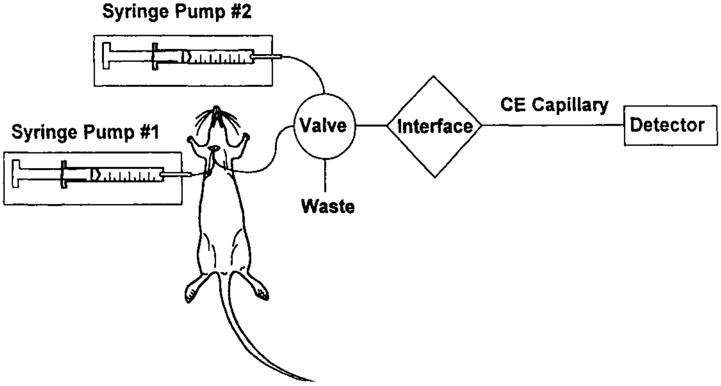

Fig. 5. Experimental arrangement of on-line microdialysis-CE system. Dialysate from the probe fills the valve sample loop, which is flushed by buffer from pump #2 at each sampling interval. Dialysate plug is flushed to the interface where a fraction of the plug is injected onto the CE system for separation and laser based detection. See text for details of equipment. (Reproduced with permission from Hogan *et al.*, 1993.)

and then fills the 60 nl loop of a LC sampling valve with the dialysate. Fresh CE running buffer is continually flushed through the second loop of the valve and through the transfer line leading to the interface by pump 2. At specified periods, the valve is switched and the 60 nl bolus of dialysate is flushed by the flowing CE buffer stream to the interface. An expanded view of the interface is shown in Fig. 6.

The injection interface consists of a reservoir containing the electrophoretic buffer into which the CE capillary and transfer line are placed. The capillaries are aligned so that effluent from the transfer line continuously flushes across the face of the CE capillary. Reproducible alignment of the capillaries in the interface is provided by a set of four guide tubes. The gap between the capillaries is maintained at 50 μm and can be measured directly via optical microscopy. Optimization of interface parameters such as interface geometry, transfer buffer flow rate, and valve switching interval has been accomplished. The valve and interface described allow the efficient sampling of nanoliter volumes of dialysate collected directly from an *in vivo* setting.

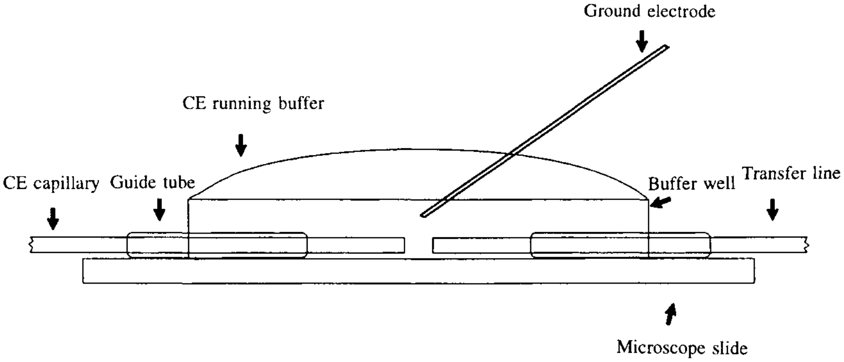

Fig. 6. Expanded view of interface between microdialysis sampling system and CE separation system. Plug of dialysate is flushed across the orifice of CE column where a portion of sample is injected electrophoretically onto the column. Remainder of sample is flushed out of the 50 μm gap between transfer line and CE capillary. (Reproduced with permission from Hogan *et al.*, 1993.)

As the sample exits the transfer line, it is automatically injected electrophoretically onto the CE capillary which is operating continuously at high voltage. The running voltage of the CE system does not necessarily require adjustment during the injection process and is typically held at –25 to –30 kV. The analytes are injected at the grounded electrode of the CE system. Consequently, the injection valve and transfer line are not at high potential and no voltage gradient exists within the transfer line.

On-line microdialysis-CE has been used for the determination of the pharmacokinetics of a novel antineoplastic agent, 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR4233), using laser-induced fluorescence detection. Resolution of SR4233 from its main metabolite, 3-amino-1,2,4-benzotriazine-1-oxide (SR4317), was accomplished in 60 s, with total sampling time and temporal resolution at the 90 s level as shown in Fig. 7. The entire pharmacokinetic curve for the parent drug can be obtained in this fashion, as shown in Fig. 8.

The temporal response of the coupled microdialysis-CE system was tested by the use of rapid *in vitro* concentration shifts at the probe. The system was able to faithfully monitor essentially instantaneous concentration changes at the probe with sampling intervals at the 90 s level. Elapsed time from the occurrence of a specific event within the rat to the monitoring of that event by the microdialysis-CE system is on the order of 5–7 min.

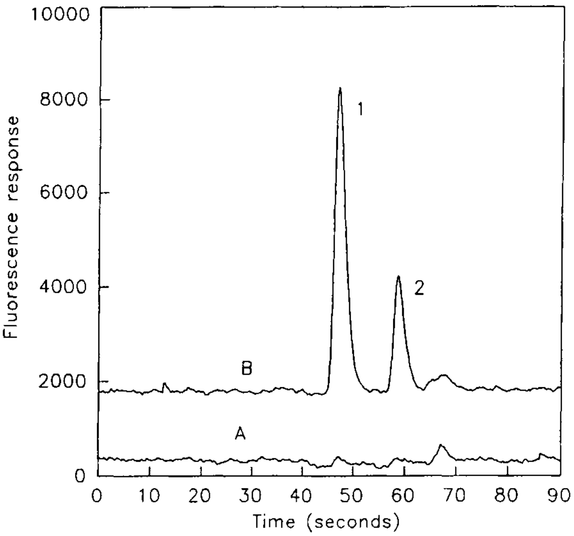

Fig. 7. Typical results from on-line microdialysis-CE system. A, Electropherogram obtained prior to dosing of animal; B, electropherogram obtained after 4 mg/kg dose of SR4233 to animal. Peak 1, SR4233; Peak 2, SR4317. (Reproduced with permission from Hogan *et al.*, 1993.)

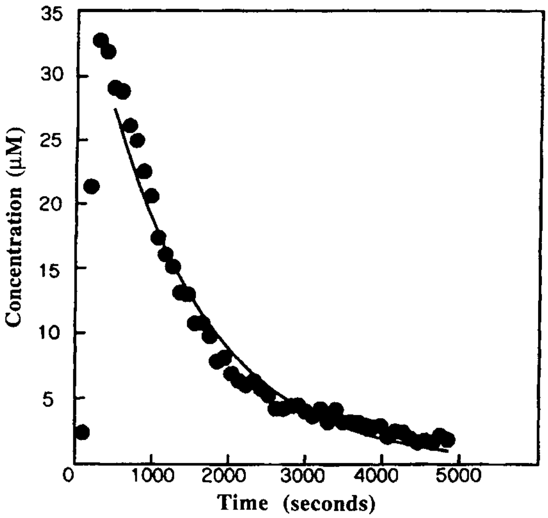

Fig. 8. *in vivo* pharmacokinetic results obtained from on-line microdialysis-CE system. Typical concentration-time curve obtained for a 4 mg/kg dose of SR4233 in the anesthetized rat. (Reproduced with permission from Hogan *et al.*, 1993.)

This work demonstrates physical separation and quantitation of analytes from an *in vivo* setting at sampling rates significantly faster than chromatographic separation methods. Clearly, the potential for substantially improved separation speed exists. With further instrumental improvements, the microdialysis-CE system should be capable of providing total analysis times at the 30–45 s level. This is an important milestone as such an analysis time would then begin to reach the same temporal level as the diffusion process of the analytes through the extracellular space and probe membrane. Further instrumental improvements must be directed toward enhanced selectivity and resolution with maintenance of separation speed rather than a reduction in total retention time.

The applicability of the on-line microdialysis-CE system described above is limited by the number of naturally fluorescent analytes available. The system utilized a He-Cd laser with an excitation wavelength of 442 nm. In order to provide a more generally applicable system, Zhou *et al* (1995) developed a microdialysis-CE system into which on-line derivatization was integrated. This variation adds nonfluorescent primary amines to the list of possible analytes that can be studied by this method. On-line derivatization was achieved by pumping naphthalenedialdehyde (NDA) and cyanide (CN) separately into a premixing tee (Fig. 9). This mixture is then transferred into a mixing cross where the dialysate is also being introduced via sheath flow. A microinjection valve alternately sends the derivatized sample and the CE run buffer to the injection interface and ultimately to the CE separation column for separation and detection. In this study, aspartate and glutamate from rat brain microdialysates were derivatized with NDA/CN and analyzed on-line at two min intervals. Changes in aspartate and glutamate following a 10 min high K^+ stimulation were monitored to evaluate the response time of the system (Fig. 10). Nearly a fivefold increase in the responses for both aspartate and glutamate was observed.

An interface previously developed by Lemmo and Jorgenson (1993) to couple capillary LC and CZE has been modified by Lada *et al* (1995) for use as an interface between microdialysis sampling and CZE. This interface is made from two steel plates separated by a 75-mm thick polytetrafluoroethylene (PTFE) spacer and is held together with screws. A flow channel is created by cutting a slit in the spacer. The outlet of the dialysis probe is positioned directly across from the inlet of the CE separation capillary with the gap distance being defined by the thickness of the spacer. Two additional ports allow flow of the electrophoresis buffer perpendicular to the dialysis and CZE capillaries (Fig. 11). Gating flow is maintained during a CZE run to provide electrophoresis buffer to the separation capillary and to pre-

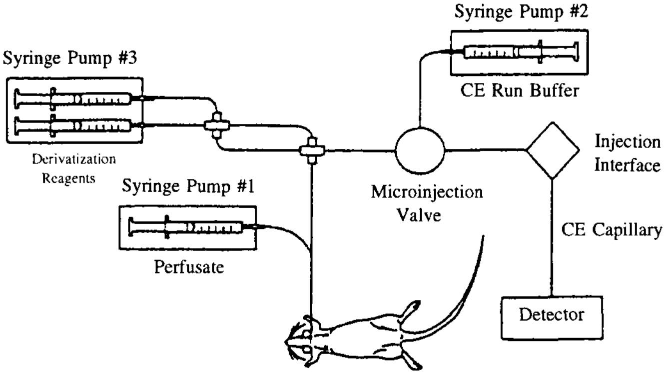

Fig. 9a. Schematic diagram of microdialysis-CE system with on-line derivatization. (Reproduced with permission from Zhou *et al.*, 1995.)

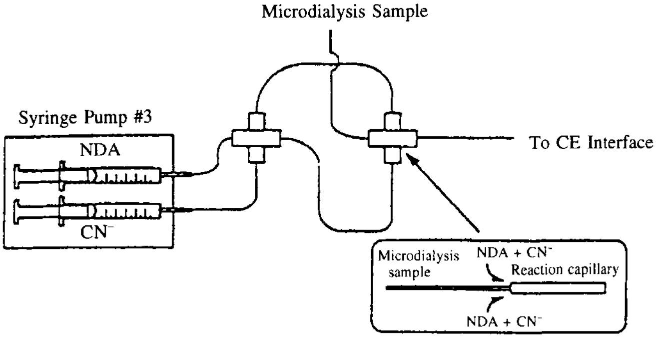

Fig. 9b. Schematic diagram of the on-line derivatization apparatus. Inset: Expanded view of on-line reactor. (Reproduced with permission from Zhou *et al.*, 1995.)

vent the introduction of dialysate. Use of this system was evaluated by monitoring ascorbic acid in rat brain with UV detection at 265 nm. Basal levels were determined to be approximately 50 mM in the ECF.

The two on-line systems described in this section differ mainly in their methods of injection. Injections on gating flow systems are made by stopping the gating flow with a valve and turning the electrophoresis voltage off so that dialysate builds up near the separation capillary (10 s delay). The injection voltage is then applied (+1.0 kV for 10 s) to make an injection, after which the gating flow is resumed and the separation voltage applied. The systems previously described (Hogan *et al.*, 1994; Zhou *et al.*, 1995) utilized a valve to send a plug of

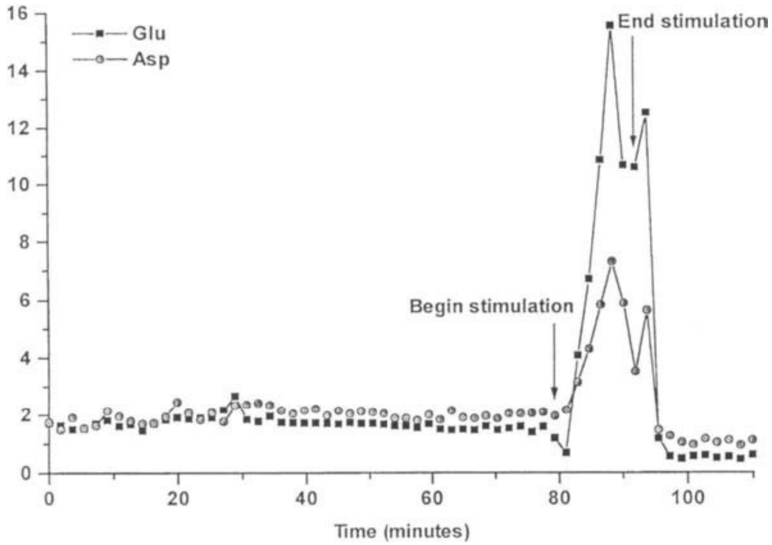

Fig. 10. Plot of aspartate and glutamate release during stimulation of brain by high K$^+$. (Reproduced with permission from Zhou *et al.*, 1995.)

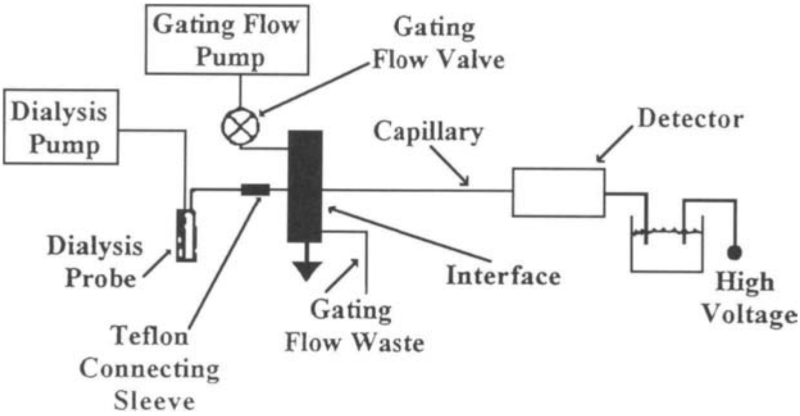

Fig. 11. Block diagram of microdialysis-CZE system with gating interface. (Reproduced with permission from Lada *et al.*, 1995.)

dialysate to the separation capillary where the separation voltage is continually being applied. The gating flow interface has a smaller dead volume than the microinjection valve, making it possible to perfuse the microdialysis probe at very low flow rates to achieve high recoveries (98%) while still having good temporal resolution. The use of these low flow rates eliminates many of the calibration issues.

It is of interest to extend the on-line microdialysis-CE work described here to the study of the correlation between release of neurotransmitters and behavior on rapid time scales. Such neurochemical studies could provide multiparameter information not available from biosensors, which are typically limited to the analysis of a single species. Such experiments require the development of alternate detection modes such as electrochemical and MS to enlarge the number of potential analytes available for analysis.

Application of this type of technology is not limited to study of pharmacokinetics or neurochemistry. The high temporal resolution and rapid feedback of these systems make them interesting candidates for use in monitoring highly transient clinical events, such as those involved in trauma or surgery. Improved temporal resolution for microdialysis methods as discussed in this chapter will expand the scope of biochemical processes amenable to study by this technique. Of course, such instrumentation would also be suitable for the generation of nearly any pharmacokinetic curve, as the elimination half-lives of most drugs would be substantially longer than the roughly 1 min sampling periods discussed here.

3. Use of CE Separation Techniques for the Analysis of Isolated Biological Cells

3.1. Introduction

Analytical methodology has evolved for the study of the chemical composition and functioning of small numbers of identified biological cells or even of single isolated cells. The use of microseparation techniques such as CE for the analysis of the cytoplasmic contents of cells has become extremely important.

One aim of the study of biological processes at the cellular level is to provide a more complete understanding of important and poorly understood cellular mechanisms. These would include aspects of protein synthesis, cell division and embryonic development. A thorough understanding of biochemical cause and effect relationships within the cell and throughout the body will be necessary to properly identify therapeutic targets, optimize drug delivery concepts, and effectively eradicate pathogenic conditions while minimizing undesirable side effects. The study of isolated cells and tissues permits a number of types of investigations critical to our understanding of important cellular functions. Several examples of these types of investigations are given in Table 1. Clearly, in order to expedite the study of complex and unknown cellular processes, uncontrolled cell-to-cell interactions must be eliminated. As fundamental understanding of intracellular processes develops, more complicated cell-to-cell and even inter-organ interactions may eventually be modeled.

Table 1. Investigations Requiring Single Cell Analysis

1. Study of cellular property (composition, function) without interference from or misidentification with other cells.
2. Study of the distribution of a cellular property among the cells of a given cellular population.
3. Study of intracellular events

Upon close inspection, it is clear that unilateral assumptions of biological homogeneity of tissue cannot be made. A high degree of cellular heterogeneity plays an important role in understanding fundamental health-related questions. Other examples can be drawn from the fields of genetics, immunology and neuroscience quite readily. In fact, members of the neuroscience community have been involved in the development of methods capable of probing the chemical basis of neuronal activity at the single cell level for over 50 years.

3.2. Challenges to Cell Analysis

As biological cells vary greatly in size and morphology, the analytical challenges inherent in the analysis of the contents or functioning of isolated biological cells varies greatly. The approximate cell diameters and volumes, where available, of some selected biological cells are given in Table 2.

Obviously, a wide range of cell diameters and volumes exists in nature. Many cells have important, but poorly understood functions that could be better elucidated with improved analysis techniques; however, most mammalian cells are not large enough to permit simple and comprehensive analyses.

Table 2. Cell Diameters and Volumes of Selected Biological Cells

Cell	Length (μm)	Volume (10–15 l)
Invertebrate neuron (*Helix aspersa*) [1]	125	10^6
Adrenomedullary cell [2]	13–16	$1–2 \times 10^3$
Lymphocyte	8–14 [3]	172–204 [4]
Erythrocyte	8–9 [5]	53–87 [6]
E. coli [7]	0.7–1.5	0.5–1.5

[1] Kennedy *et al.* 1987
[2] Cooper *et al.*, 1992
[3] Albritton, 1952
[4] Ben-Sasson *et al.*, 1974
[5] Weinstein, 1974
[6] Lushbaugh *et al.*, 1962
[7] Kubitschek, 1958

An analytical instrument well suited for the analysis of single cells or small numbers of isolated cells would possess a number of characteristics. These would include:

- Non-destructive, with *in vivo* capabilities
- Detection limits compatible with levels of minor components present
- Temporal response compatible with that of cellular events
- Ability to spatially discriminate between analytes present within distinct cellular compartments
- High rate of analysis to allow statistical surveys of cell populations

Few analytical techniques can satisfy more than two of these restrictions, much less the entire group. However, a battery of techniques having a range of useful capabilities in given situations has evolved. It is not possible here to survey all techniques providing intracellular information.

3.3. *Classical Cell Analysis Techniques*

As stated previously, neurochemists are especially interested in probing the biochemical and functional properties of discrete regions of the brain, so a battery of separation techniques for the analysis of single neurons has evolved. However, due to the small cell sizes of most mammalian neurons, it has been necessary to utilize large invertebrate neurons as analogous neural models of much smaller mammalian neurons. Techniques such as micro-thin-layer chromatography (Osborne *et al.*, 1973), LC (Lent *et al.*, 1983), gas chromatography (GC)-MS (McAdoo, 1978) and enzymatic radiolabeling (McCaman *et al.*, 1973) have been developed to provide analytical information at this level. In general, these cell analysis techniques are of limited utility because they a) require several neurons to be pooled for analysis, b) require the analyst to pre-select the compounds to be detected, and c) are difficult to perform.

Analysis techniques which do not incorporate a physical separation of the cellular species can have advantages over separation-based measurements in terms of spatially and temporally resolved cellular measurements. However, a clear demand exists for separation-based methods that can provide multiparameter biochemical information for quantitative mapping and multi-analyte analysis of the contents of isolated cells. Obtaining quantitative chemical information regarding a number of unambiguously identified analytes determined simultaneously is of significant importance, but is difficult to accomplish.

3.4. Use of Microcolumn Separations for the Analysis of Single Cells

The most direct route for obtaining chemical information on the contents of a cell would seemingly be disruption of the cell and analysis of its contents with modern analytical separation instruments in much the same manner that any ordinary sample would be analyzed. This approach, though clearly one with many advantages, is a considerable analytical challenge. The minute cellular volumes (femtoliter to picoliter) and complex biological matrices of mammalian cells lead to extraordinary sample handling, preparation and analysis requirements due to the need for micromanipulation and microanalysis. The motivation for attempting such separation-based analyses is that these methods are capable of providing much more detailed chemical information than are techniques which do not utilize a separation. Physical separations are performed upon the contents of the cells; previous methods relied upon the chemical specificity of a reagent for selectivity. Thus, separation-based methods are expected to supply multiparameter information on specific intracellular species of metabolic and biochemical importance that cannot otherwise be easily obtained. Several reviews have covered the topic of the analysis of cellular samples via microseparation techniques (Kennedy *et al.*, 1989; Ewing *et al.*, 1992; Hogan and Yeung, 1993; Yeung, 1994; Jankowski *et al.*, 1995).

Advances in microcolumn separation techniques have made possible the direct separation and detection of the contents of single cells.

Kennedy *et al.* (1987) analyzed the content of an invertebrate neuron from the snail *Helix aspersa* by open-tubular liquid chromatography (OTLC). The cell was transferred into a glass microvial using a specially designed micropipette. It was then dispersed and centrifuged, and the supernatant was injected onto the OTLC system. The neurotransmitters dopamine and serotonin and their amino acid precursors tyrosine and tryptophan were determined in individual neurons. Voltammetric detection was used to provide qualitative as well as quantitative information on the analytes. The results suggested that dopamine and serotonin coexist in individual neurons at similar concentration levels, which contradicts existing neurobiological theory.

Wallingford and Ewing (1988) employed CE for single cell analysis. In this case, a tapered microinjector was constructed at the injection end of a CE capillary. A schematic diagram of the microinjector, which had been characterized for separation efficiency and contributions to band broadening (Wallingford and Ewing, 1987a), is shown in Fig. 12. It was used to pierce the cell membranes of an intact neuron

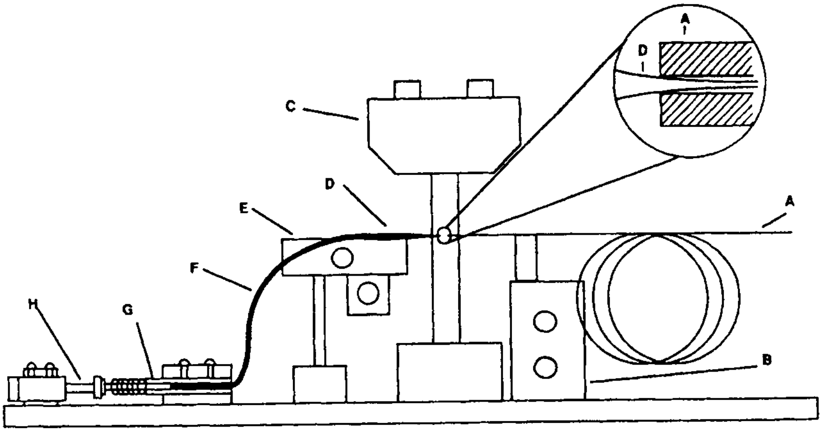

Fig. 12. Schematic diagram of microinjector. The labels correspond to the following parts: *A* is the chromatographic column, *B* is the column positioning stage, *C* is the stereomicroscope, D is the micropipette, E is the micromanipulator, F is the connecting tubing, G is the spring-loaded syringe, and H is the micrometer. The darkened areas are those that are filled with mercury. The inset shows the pipette inserted into the column read for injection. Note that the pipette fits tightly again the column. (Reproduced with permission from Kennedy *et al.*, 1987.)

from the snail *Planorbis corneus*. A sample of cellular cytoplasm was drawn into the capillary and was analyzed by capillary electrophoresis. CEEC was employed for the analysis (Wallingford and Ewing, 1987b). A variety of peaks were obtained from the cytoplasmic analysis, but were not identified.

In 1989, Kennedy *et al.* demonstrated the separation and detection of CBI-derivatized amino acids isolated from neurons of *Helix aspersa* via CE with laser-induced fluorescence detection (Fig. 13). The resulting derivatives are efficient fluorophores whose absorption matches the 442 nm emission of the He-Cd laser. No attempt was made to quantitate the peaks obtained; however, the large signal-to-noise ratios obtained implied that further miniaturization of the technique to mammalian cells may be possible.

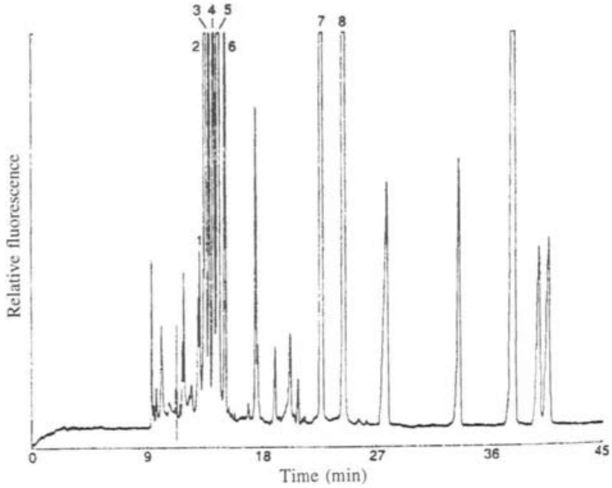

Fig. 13. CZE-LIF run of NDA derivatives of an E4 cell. The numbered peaks correspond to NDA-labeled amino acids as follows: 1, Trp; 2, Gln, His, Ile, Leu, Met, Phe; 3, Asn, Thr, Tyr, Val; 4, Ser; 5, Ala; 6, Gly; 7, Glu; and 8, Asp. Conditions of the run were as follows: capillary had an ID of 25 µm and was 104.5 cm long; detection was done 79.5 cm from the injection end; buffer was 0.01 M borate, 0.04 KCl, pH 9.5; the applied potential was –25 kV (–8 µA). Injection was made at the grounded end of the capillary. (Reproduced with permission from Kennedy *et al.*, 1989.)

Further studies by Chien *et al.* (1990) allowed the determination of cytosolic and vesicular dopamine in the same snail. Their results suggested that more than 98% of the dopamine was bound in vesicles and was not free within the cytoplasm. These results highlight the ability of separation-based techniques to provide spatially resolved chemical information at the single cell level. Olefirowicz and Ewing (1990a,b, 1991) have demonstrated direct detection of cytoplasmic levels of dopamine in single neurons using CE. Hydrofluoric acid was used to etch the outer diameter of the injection end of a 2–5 μm capillary to a point with an o.d. of 5–10 μm Fig. 14A). This point was then used to pierce the cell membranes and analyze the contents. Volumes of cytoplasm as small as 270 femtoliters could be withdrawn from invertebrate neurons.

Alternatively, CE capillaries of larger i.d. (25 μm) can be used to construct larger microinjectors to inject entire cells (Fig. 14B). The

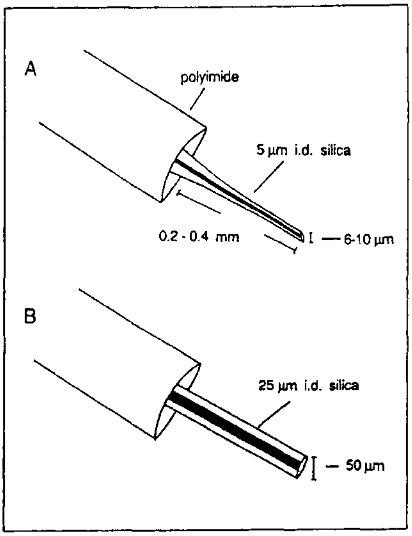

Fig. 14. Schematic depiction of two etched microinjectors. A: Injector for acquiring and injecting cytoplasmic samples, constructed at the high voltage end of a 5-μm i.d. electrophoresis capillary. B: Injector for acquiring and injecting whole cells, constructed at the high voltage end of a 25-μm i.d. electrophoresis capillary. (Reproduced with permission from Olefirowicz and Ewing, 1991.)

cells can then be lysed inside the capillary and their contents separated via electrophoresis. Using CEEC with these injectors, Olefirowicz and Ewing determined catecholamine and indoleamine neurotransmitters in single cells. Concentration detection limits for dopamine from a 50 picoliter injection are the order of 10^{-8} M. These injection systems are relatively simple and uncomplicated, thus enhancing their utility. However, a limitation of this method is that the injectors are too large to penetrate very small mammalian cells.

Human erythrocytes are among the smallest of mammalian cells and do not possess any extraordinarily high concentrations of electroactive or fluorescent species. Thus, a method having the capability to analyze the contents of an erythrocyte should make it possible to analyze nearly any mammalian cell, not simply very large invertebrate neurons or those containing high concentrations of electroactive species. The separation of hemoglobin species from single erythrocytes has been previously demonstrated by micro-electrophoresis on planar medium; however, the method was incapable of separating and detecting any of the low-molecular weight species present (Matioli and Niewisch, 1965). In addition, separation efficiency was quite low and no quantitation was attempted. In 1992, Hogan and Yeung reported direct separation and detection of cytoplasmic constituents of a single human erythrocyte by CE.

The experimental apparatus required for the analysis of single erythrocytes is shown in Fig. 15. Cells are manipulated and injected at the grounded end of the CE capillary. This end of the capillary is permanently mounted under the field of view of a microscope; a vial

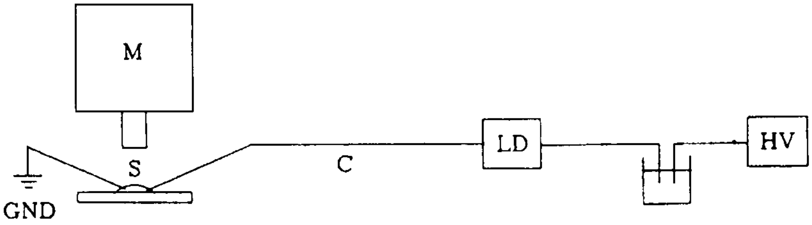

Fig. 15. Experimental layout of single-cell CE system. The CE system is operated with the grounded end (GND) of the CE capillary (C) immersed in the sample (S) placed under the field of view of the microscope (M). Detection is carried out on-column (LD) at a location close to the high voltage (HV) orifice of the column immersed in the running buffer (B). (Reproduced with permission from Hogan and Yeung, 1993.)

of running buffer completes the electrophoretic circuit. An expanded view of the injection region of the system is shown in Fig. 16. The capillary and the ground electrode are permanently mounted in three-dimensional micromanipulators so the analyst can control their motions while viewing the capillary under the microscope. A cell is injected for analysis by applying a pulse of vacuum to the opposing end of the capillary when the injection orifice of the capillary is near the cell of interest. Once within the capillary, the cell typically adheres to the fused silica walls of the capillary and can then be lysed. The contents of the cell are then separated by electrophoresis and detected via laser-induced fluorescence.

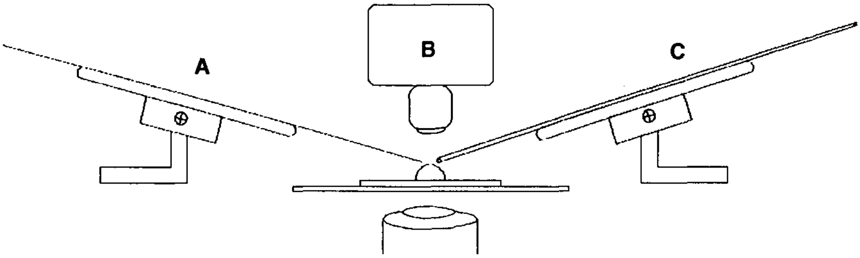

Fig. 16. Expanded view of the cell injection region of the CE system. A, ground electrode; B, 100X microscope; C, CE capillary. A cell is selected for injection by manually positioning the orifice of the capillary close to the cell of interest and applying a pulse of vacuum to the capillary. (Reproduced with permission from Hogan and Yeung, 1992.)

Laser-induced fluorescence detection is a technique with the ability to detect both quite low concentrations of analyte (10^{-12} M) and very small absolute quantities of analyte (10^{-21} moles) (Chen *et al.*, 1991). Unfortunately, few compounds possess appreciable innate fluorescence at easily accessible excitation wavelengths. One method of circumventing this limitation is to derivatize the analyte species of interest. However, the 90 femtoliter cell volume of the erythrocyte makes ordinary derivatization procedures problematic.

One novel approach to micro-derivatization is to use the semipermeable membrane of the cell to regulate the flux of species through the "reaction vessel," *i.e.*, the cell itself. Small non-polar derivatization reagents applied to the macroscopic exterior phase surrounding the cell readily partition through the lipophilic cell membrane where they are available for reaction with the appropriate analyte functionalities. The exterior phase with its excess reagents and contaminants is removed via centrifugation. The derivatized product, if larger or more polar than the original reagent, will not migrate back through the cell membrane. As an example of the utility of this concept, monobromobimane (mBBr) was used to derivatize thiol-containing species within functioning erythrocytes at physiological conditions. Figure 17 shows an electropherogram of the free intracellular thiols within a single human erythrocyte after mBBr derivatization. The lower limit of detection for the method was 5.9 attomoles (4.8×10^{-8} M) of injected GSH standards.

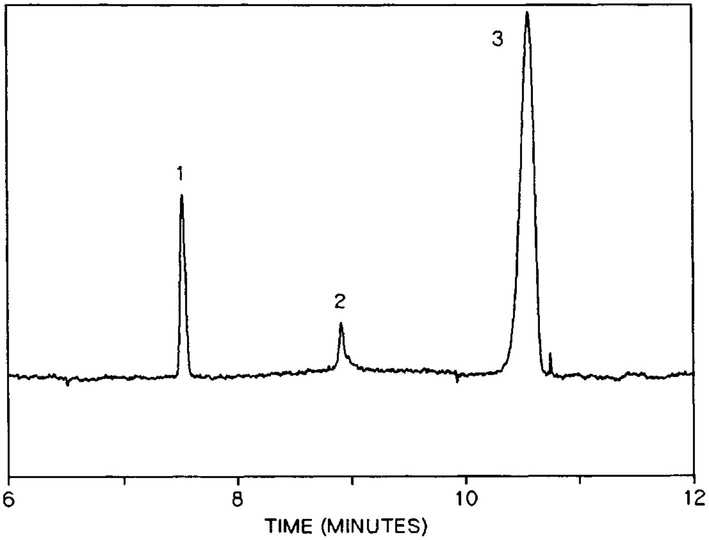

Fig. 17. Electropherogram of mBBr-derivatized contents of a single human erythrocyte using direct fluorescence detection. The peaks are: 1 = peak associated with unreacted mBBr; 2 = unidentified intracellular thiol; 3 = glutathione (GSH). (Reproduced with permission from Hogan and Yeung, 1992.)

It must be recognized that derivatization methods, regardless of their degree of refinement, will not be useful in all instances. Indirect detection methods permit the sensitive detection of species that have no inherently useful detection properties and offer the potential for general detection of charged species. Of particular interest are those intracellular species which possess no useful detection properties, such as inorganic ions. Indirect fluorescence detection records the passage of a non-fluorescent analyte through the detection zone by the charge displacement effects of the analyte upon the fluorescent co-migrating buffer ions (Kuhr and Yeung, 1988a,b). Figure 18 shows the results of a determination of sodium and potassium from a single erythrocyte (Hogan and Yeung, 1992). The determination of Na^+ and K^+ within single erythrocytes via indirect fluorescence detection can be achieved with minor instrumental modifications to the apparatus described here.

As the erythrocyte is among the smallest of mammalian cells, this method should be widely applicable to nearly any mammalian cell. The microderivatization concepts introduced may allow simple derivatization of species such as amino acids, proteins, and thiols within mammalian cells. Indirect fluorescence detection shows promise for the general detection of species lacking useful detection properties without undertaking the process of derivatization (Fig. 18).

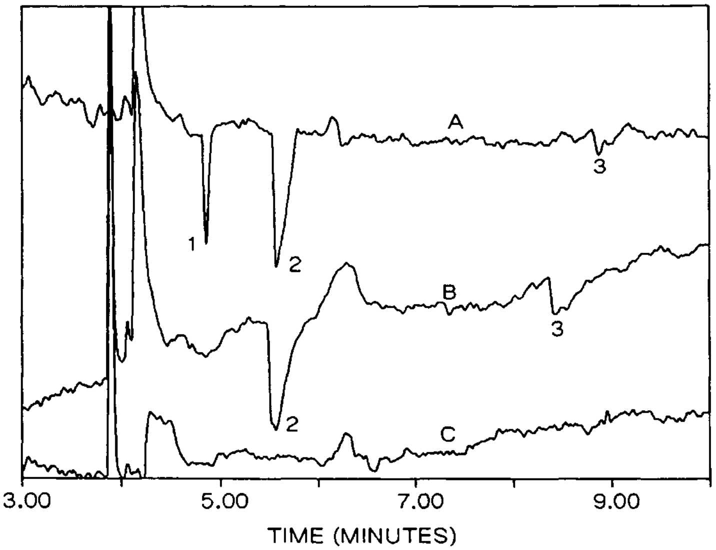

Fig. 18. Electropherograms using indirect fluorescence detection. A: 2 s electroinjection of 45 μM standards where 1 = Li (11.7 femtomoles injected), 2 = Na (10.7 femtomoles injected), 3 = K (6.5 femtomoles injected). B: 1 human erythrocyte injected. C: blank of extracellular matrix. (Reproduced with permission from Hogan and Yeung, 1992.)

The limitations of this single cell analysis method are the requirement for low ionic strength CE running buffer solutions for indirect detection and the physiological restrictions on reaction conditions available for micro-derivatization. On-column derivatization methods involving non-physiological conditions could provide additional flexibility in the types of species amenable to this approach.

3.5 Strengths and Limitations of the Use of CE for Cellular Analysis

The use of CE has allowed the extension of cellular analysis to the scale of the single human erythrocyte. CE analysis of the contents of isolated cells is helping researchers to quantitate a variety of organic species simultaneously from within single isolated cells. This multiparameter information should provide clues to biochemical processes that other single parameter techniques are unable to supply.

Limitations of the use of a separation technique to probe the contents of cells include a generally limited degree of spatial resolution within the cell itself. Also, these separation techniques provide no temporal dynamics since they represent only snapshots of the physiological status of the cell at a given point in time.

Future work could provide insight into many cellular processes of interest by applying similar techniques to the study of important mammalian cells such as tumor cells, cells of the immune system, and mammalian neurons. Such applications would provide clear benefits in the understanding of such heterogeneous tissues.

Likewise, applications of various detection schemes to allow the quantitation of a wider variety of intracellular species would be of considerable utility. Electrochemical detection has proven useful for the detection of the catecholamine neurotransmitters, yet the range of analytes accessible to this method should be extended. Fluorescence derivatization of intracellular species has been demonstrated only on cellular thiols. Determination of peptides, proteins, amino acids, and nucleic acids from within the cellular matrix is also of considerable interest. It should be noted that UV-excited native fluorescence of proteins (Lee and Yeung, 1992a) and nucleic acids (Milofsky and Yeung, 1993) has been demonstrated at levels compatible with single cell detection. In fact, Lee and Yeung (1992b) have now demonstrated the analysis of proteins within erythrocytes. Native protein fluorescence was used to detect the analytes, removing the need for derivatization of these tyrosine- and tryptophan-containing proteins. Methods of detecting a wide variety of species without derivatization should be useful if properly incorporated into an analytical instrument. Likewise, the use of indirect detection methods may find utility in these applications due to the excellent mass detection limits of the technique. Substantial improvements in system stability and resistance to matrix effects will be required before rugged assays can be developed for such complicated *in vivo* environments.

4. Conclusions

Methods described for monitoring the chemistry of single cells and small localities within an *in vivo* environment are essential for a full understanding of the mechanisms involved in biological activity. An integrated use of these tools could provide clearer views of cellular roles in physiology and disease, as well as providing clues to synergistic effects between cellular populations.

5. References

Adams, R. N. (1976). *Anal. Chem.*, *48*, 1128A–1138A.

Adams, F., R. K. W. Schwarting, F. Boix and J. P. Huston (1991). *Brain Res.* *553*, 318–322.

Albritton, E. C. (1952). *Standard Values in Blood*, p. 56. Saunders, Philadelphia.

Ammann, D. (1986). *Ion-Selective Microelectrodes*. Springer-Verlag, Berlin.

Ben-Sasson, S., D. Patinkin, N. B. Grover and F. Doljanski (1974). *J. Cell Physiol.*, *84*, 205–214.

Benveniste, H. (1989). *J. Neurochem.*, *52*, 1667–1679.

Benveniste, H. and P.C. Hüttemeier (1990). *Prog. Neurobiol.*, *35*, 195–215.

Bito, L., H. Davson, E. Levin, M. Murray, and N. Snider (1966). *J. Neurochem.*, *13*, 1057–1067.

Boutelle, M. G., L. K. Fellows, and C. Cook (1992). *Anal. Chem.*, *64*, 1790–1794.

Caprioli, R. M. and S.-N. Lin (1990) *Proc. Natl. Acad. Sci. USA.*, *87*, 240–243.

Chen, D. Y., H. P. Swerdlow, H.R. Harke, J. Z. Zhang and N. J. Dovichi (1991). *J. Chromatogr.*, *559*, 237–246.

Chien, J. B., R. A. Wallingford and A. G. Ewing (1990). *J. Neurochem.*, *54*, 633–638.

Church, W. H. and J. B. Justice, Jr. (1987). *Anal. Chem.*, *59*, 712–716.

Cooper, B. R., J. A. Jankowski, D. J. Leszczyszyn, R. M. Wightman and J. W. Jorgenson (1992). *Anal. Chem.*, *64*, 691–694.

Delgado, J. M. R. (1962). In: *Pharmacological Analysis of Central Nervous Action* (Paton W. D. M., ed.), pp. 265–292. Pergamon Press, Oxford.

Delgado, J. M. R., F. V. DeFeudis, R. H. Roth, D. K. Ryugo and B. K. Mitruka (1972). *Arch. Int. Pharmacodyn., 198*, 9–21.

Dodd, P. R. and H. F. Bradford (1974). *J. Neurochem., 23*, 289–292.

Ewing, A. G., T. G. Strein and Y. Y. Lau (1992). *Acc. Chem. Res., 25*, 440–447.

Fox, R. H. and S. M. Hilton (1958). *J. Physiol.(Lond.), 142*, 219–232.

Gaddum, J. H. (1961). *J. Physiol. (Lond.)*, 155, 1P.

Gibaldi, M. and D. Perrier (1982). In: *Pharmacokinetics* (Gibaldi, M. and D. Perrier, eds.), pp. iii–iv. Marcel Dekker, New York.

Hamberger, A., I. Jacobson, B. Nyström and M. Sandberg (1991). *J. Intern. Med., 230*, 375–380.

Hernandez, L., N. A. Guzman and B. G. Hoebel (1991). *Psychopharmacology, 105*, 264–268.

Hogan, B. L. and E. S. Yeung (1992). *Anal. Chem., 64*, 2841–2845.

Hogan, B. L. and E. S. Yeung (1993). *Trends Anal. Chem., 12*, 4–9.

Hogan, B. L., S. M. Lunte, J. F. Stobaugh and C. E. Lunte (1994). *Anal. Chem., 66*, 596–602.

Horning, M. G. (1983). In: *Drug Metabolism and Distribution: Current Reviews in Biomedicine* (Lamble, J. W., ed.), pp. v–viii. Elsevier Biomedical Press, Amsterdam.

Jankowski, J. A., S. Tract and J. V. Sweedler (1995) *Trends Anal. Chem., 14*, 170–176.

Justice, J. B., Jr. (1987). *Voltammetry in the Neurosciences*, Humana Press, Clifton, New Jersey.

Kennedy, R. T. and J. W. Jorgenson (1989). *Anal. Chem., 61*, 436–441.

Kennedy, R. T., R. L. St. Claire, J. G. White and J. W. Jorgenson (1987). *Microkhim. Acta*, 37–46.

Kennedy, R. T., M. D. Oates, B. R. Cooper, B. Nickerson and J. W. Jorgenson (1989). *Science, 246*, 57–63.

Kubitschek, H. E. (1958). *Nature, 182*, 234–235.

Kuhr, W. G. and E. S. Yeung (1988a). *Anal. Chem., 60*, 1832–1834.

Kuhr, W. G. and E. S. Yeung (1988b). *Anal. Chem., 60*, 2642–2646.

Lada, M. W., G. Schaller, M. H. Carriger, T. W. Vickroy and R. T. Kennedy (1995). *Anal. Chim. Acta, 307*, 217–225.

Lee, T. T. and E. S. Yeung (1992a). *J. Chromatogr., 595*, 319–325.

Lee, T. T. and E. S. Yeung (1992b). *Anal. Chem., 64*, 3045–3051.

Lent, C. M., R. L. Mueller and D. A. Haycock (1983). *J. Neurochem., 41*, 481–490.

Lönnroth, P. (1991). *J. Intern. Med., 230*, 363–364.

Lönnroth, P. and U. Smith (1990). *J. Intern. Med., 227*, 295–300.

Lunte, C. E., D. O. Scott, and P. T. Kissinger (1991). *Anal. Chem., 63*, 773A–780A.

Lushbaugh, C.C., N. J. Basmann and B. Glascock (1962). *Blood, 20*, 241–248.

Maidment, N. T., D. R. Brumbaugh, V. D. Rudolph, E. Erdelyi, and C. J. Evans (1989). *Neuroscience, 33*, 549–557.

Malone, M. A., H. Zuo, S. M. Lunte and M. R. Smyth (1995). *J. Chromatogr. A, 700*, 73–80.

Matioli, G. T. and H. B. Niewisch (1965). *Science, 150*, 1824–1826.

McAdoo, D. J. (1978). In: *Biochemistry of Characterized Neurons* (Osborne, N. N, ed.), pp. 19–45. Pergamon, New York.

McCaman, R. E., D. Weinrich and H. Borys (1973). *J. Neurochem., 21*, 473–476.

Milofsky, R. E. and E. S. Yeung (1993). *Anal. Chem., 65*, 153–157.

Olefirowicz, T. M. and A. G. Ewing (1990a). *J. Neurosci. Meth., 34*, 11–15.

Olefirowicz, T. M. and A. G. Ewing (1990b). *Anal. Chem., 62*, 1872–1876.

Olefirowicz, T. M. and A. G. Ewing (1991). *Chimia, 45*, 106–108.

Osborne, N. N., A. C. Szczepaniak and V. Neuhoff (1973). *Int. J. Neurosci., 5*, 125–131.

O'Shea, T. J., M. W. Telting-Diaz, S. M. Lunte, C. E. Lunte and M. R. Smyth (1992a). *Electroanalysis, 4*, 463–468.

O'Shea, T. J., P. L. Weber, B. P. Bammel, C. E. Lunte, S. M. Lunte and M.R. Smyth (1992b). *J. Chromatogr., 608*, 189–195.

Roda, A., S. Girotti, B. Grigolo, S. Ghini, G. Carrea, R. Bovara, I. Zini and R. Grimaldi (1991). *Biosensors Bioelectronics, 6*, 21–29.

Tellez, S., N. Forges, A. Roussin and L. Hernandez (1992). *J. Chromatogr. Biomed. Appl., 581*, 257–266.

Ungerstedt, U. (1991). *J. Intern. Med., 230*, 365–373.

Ungerstedt, U. and A. Hallström (1987). *Life Sci., 41*, 861–864.

Ungerstedt, U., M. Herrera-Marchintz, U. Jungnelius, L. Ståhle, U. Tossman, and T. Zetterström (1982). In: *Advances in Dopamine Research* (Kotisaka M., ed.), pp. 219–231. Pergamon, New York.

Van der Kuil, J. H. F. and J. Korf (1991). *J. Neurochem., 57*, 648–654.

Wages, S. A., W. H. Church and J. B. Justice, Jr. (1986). *Anal. Chem., 58*, 1649–1656.

Wallingford, R. A. and A. G. Ewing (1987a). *Anal. Chem., 59*, 678–681.

Wallingford, R. A. and A. G. Ewing (1987b). *Anal. Chem., 59*, 1762–1766.

Wallingford, R. A. and A. G. Ewing (1988). *Anal. Chem., 60*, 1972–1975.

Weinstein, R. S. (1974). In: *The Red Blood Cell* (Surgenor, D. M., ed.), p. 232. Academic Press, New York.

Westerink, B. H. C. (1992). *TRACS., 11*, 176–182.

Wightman, R. M., L. J. May and A. C. Michael (1988). *Anal. Chem.*, *60*, 769A–779A.

Yeung, E. S. (1994) *Acc. Chem. Res., 27,* 409–414,

Zetterström, T., L. Vernet, U. Ungerstedt, U. Tossman, B. Jonzon, and B. B. Fredholm (1982). *Neurosci. Lett., 29*, 111–115.

Zhou, S. Y., H. Zuo, J. F. Stobaugh, C. E. Lunte and S. M. Lunte (1995). *Anal. Chem.*, *67*, 594–599.

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