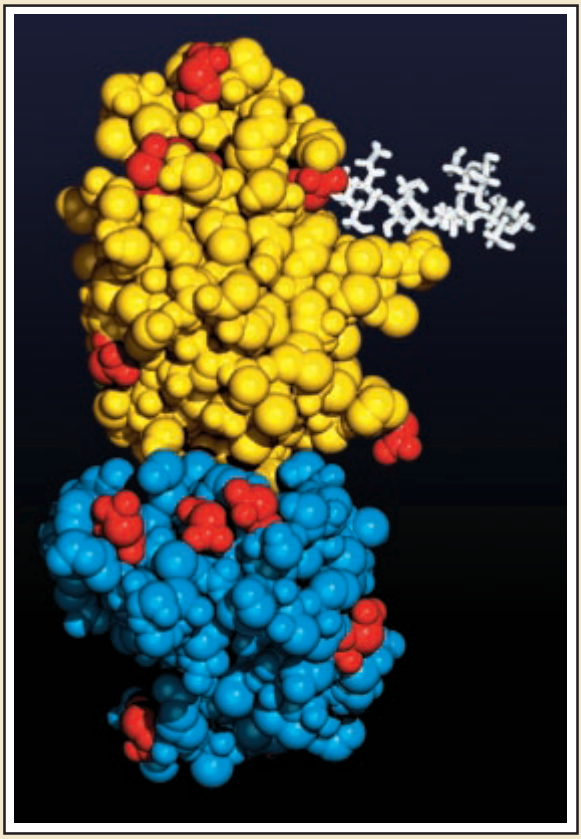
Separation of Proteins and Peptides by Capillary Electrophoresis: Application to Analytical Biotechnology



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Separation of Proteins and Peptides by Capillary Electrophoresis: Application to Analytical Biotechnology

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Part 2—Protein/Peptide Applications of CE to Analytical Biotechnology

About the Authors

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Thomas J. Pritchett is a Principal Scientist with Beckman Instruments, Inc., specializing in development and validation of CE and HPLC methods for the analysis of (glyco)proteins. He received a Ph.D. in Biological Chemistry from the University of California, Los Angeles, and did post-doctoral work at the Scripps Research Institute, La Jolla, CA. He was formerly manager of QC and Analytical Development at Cytel Corporation, an early-stage biopharmaceutical company in San Diego, CA. Previous to Cytel, Tom was associated with the Genetics Institute, Andover, MA, and Baxter Diagnostics, Inc., Miami, FL, where he was the founding member and manager of the Analytical Chemistry/Method Development group.

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Front Cover

The illustration on the cover depicts a computer-generated glycoprotein molecule. Color code: white, oligosaccharide; red, Asn residues; yellow and blue, other amino acids of ribonuclease B. Courtesy of Don Gregory, Molecular Simulations, Burlington, MA.

Other Beckman primers (Volumes I, II, III, and IV) on capillary electrophoresis:

Title	Beckman Part Number
Introduction to Capillary Electrophoresis	360643
Introduction to Capillary Electrophoresis of Proteins and Peptides	266923
Micellar Electrokinetic Chromatography	266924
Introduction to the Theory and Applications of Chiral Capillary Electrophoresis	726388

Acronyms and Symbols Used

The following acronyms and symbols may be found throughout this book.

ACE	affinity capillary electrophoresis
ACTH	adrenocorticotropic hormone
BGE	background electrolyte
CE	capillary electrophoresis
CEA	carcinoembryonic antigen
CGE	capillary gel electrophoresis
CGMP	current good manufacturing procedures
CHO	Chinese hamster ovary
CIEF	capillary isoelectric focusing
CMC	chemistry, manufacturing, and control
CTAB	cetyltrimethylammonium bromide
CTAC	cetyltrimethylammonium chloride
CZE	capillary zone electrophoresis
DAB	diaminobutane
DAP	diaminopentane
DMF	dimethylformamide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunoassay
EOF	electroosmotic flow
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte macrophage colony stimulating factor
HGH	human growth hormone
HPCE	high-performance capillary electrophoresis
HPIEC	high-performance ion exchange chromatography
HPLC	high-performance liquid chromatography
HPMC	hydroxypropylmethylcellulose
HSA	hexanesulfonic acid
IEF	isoelectric focusing
IFN-α	recombinant human interferon-α
IgG	immunoglobulin G
IND	investigative new drug application
ITP	isotachophoresis
LE	leading electrolyte
LIF	laser-induced fluorescence
μ	mobility

m/z	mass to charge
MAbs	monoclonal antibodies
MECC	micellar electrokinetic capillary chromatography
MHC	major histocompatibility complex
MS	mass spectrometry
MW	molecular weight
NDA	new drug application
PA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
pI	isoelectric point
PLA	product license agreement
rbst	recombinant bovine somatotropin
rhIL	recombinant human interleukin
rHuEPO	recombinant human erythropoietin
RIA	radiolabeled immunoassay
RMT	relative migration time
RP	reversed phase
rpst	recombinant porcine somatotropin
RSD	relative standard deviation
rtPA	recombinant human tissue plasminogen activator
SDS	sodium dodecyl sulfate
TE	terminating electrolyte
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TMR	tetramethylrhodamine
TNF	tumor necrosis factor
UV	ultraviolet
Vis	visible

Preface

The emergence of a new biotechnological industry utilizing hybridoma, peptide synthesis, and recombinant DNA techniques for the production of highly specialized biomolecules has increased the demand for sophisticated analytical instrumentation and methodologies. In pharmaceutical biotechnology, these analytical instruments and methods are used for the identification of chemicals and structures, control of purity, and assays of potency. Quantity, stability, heterogeneity and process consistency are important issues. In biological samples, proteins, nucleic acids, and polysaccharides are often present in very small quantities and sample sizes are often limited, requiring highly sensitive and selective separation techniques. The same is true for the analysis of complex samples of biological origin, *i.e.*, in clinical and diagnostic applications. Miniaturized, microcolumn separation techniques, such as micro-HPLC, capillary gas chromatography, or capillary electrophoresis (CE), are particularly suitable for the detection of analytes in very small, (sub)microliter-size volumes.

Because many samples of biological origin are quite complex, two or more inherently different yet complementary techniques are often used to perform qualitative and quantitative assays. The use of such complementary methods provides more confidence in the analytical results. HPLC and CE (or, in a broader sense, chromatography and electrophoresis) fulfill the above requirements. These techniques provide fully automated, computer-controlled, quantitative assays as well as high-resolution separations with fast analysis times. For example, whereas in (reversed-phase) HPLC, species are typically separated on the basis of hydrophobicity, in free-solution CE, the charge-to-mass ratio often plays a key role. This difference in separation mechanism is helpful in the characterization or elucidation of complex samples of biological origin. In addition, clean-up or purification of biomolecules is often done by preparative HPLC, thus requiring a second technique for purity control. CE, therefore, is a welcome addition to the toolbox of the bioanalytical chemist for solving problems related to proteins and peptides.

HPLC has undergone a tremendous development in the past two decades. This growth has been possible because of the advent of high-performance packing materials, suitable instrumentation, and a thorough theoretical understanding of separations. We are seeing a similar development in the field of CE. The development in CE of capillary pretreatment procedures and permanent coatings is similar to developments in chromatography decades ago. Research in CE—in its present form with fused-silica columns and on-column detection—is more than a decade old (Jorgenson and Lukacs, 1981, 1983), and

commercial CE instruments have been available since 1988. The field is moving rapidly, illustrated by the fact that already six annual symposia on CE have been held and a number of comprehensive textbooks (see, for example, the textbooks edited by Camillieri, 1993; Landers, 1994; and Guzman, 1993) recently have been published. The reviews by Karger (1993) and Landers *et al.* (1993) capture the state of the art of CE. A sampling of the type of protein-related applications for which CE has been utilized in analytical biochemistry and clinical/diagnostic assays is given below.

Applications of CE for Proteins and Peptides

Purity Assays

- QC of a manufacturing process
- Quantitation of contaminants or excipients
- Screening of samples prior to protein sequencing

Structural Studies

- Peptide mapping
- Two-dimensional methods, *i.e.*, HPLC-CE, IEC-CE
- Microheterogeneity of complex proteins *e.g.*, glycoproteins, monoclonal antibodies, histones, transferrins
- Oligomerization

Binding Studies

- Calcium, zinc-binding proteins
- Antigen-antibody immune complexes
  - Drug–protein complexes
  - Protein–DNA complexes

Process Analysis

- Quantitation of the final product
- Enzymatic digestion monitoring
- Derivatization (labeling) monitoring
- Deamidation, disulfide bridge reduction
- Conformational changes

Stability Studies

- pH, environment, temperature effects

Mobility Measurements

- (Semi-)empirical models for prediction of separation

Micropreparative Purification

See applications listed in Table 1-4

Clinical/Diagnostic

- Serum protein assays
- Hemoglobin variants; globin chains
- Neuropeptide isolation from brain tissue
- Apolipoproteins
- Cytokines, transferrins, metallothioneins
- Proteins in single cells
- Isoenzymes

As in HPLC, various separation modes are applicable to the analysis of proteins and peptides: capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MECC), capillary isoelectric focusing (CIEF), capillary gel electrophoresis (CGE), and displacement electrophoresis or isotachophoresis (ITP). Whereas in CE the separation of small peptides (in the CZE mode) often is relatively straightforward and well understood, it appears that no *single* strategy is applicable for large peptides and proteins. As might be expected, this is due largely to the wide diversity and complexity associated with these biomolecules. Thus, different strategies often work for different protein separation problems, hence requiring different CE separation modes. An introduction to the general principles of CE has appeared in another Beckman primer (part number 360643) and should serve as a quick reference guide and introduction to the present booklet on protein and peptide separations. Please contact your local Beckman office if you wish to order a copy.

This edition on the CE of proteins and peptides is a completely revised edition of the first printing of 1992. It is divided into two parts. Part I is an updated version of the first booklet, with added new material on coated capillaries, detectability enhancements, CIEF, and Affinity CE. It gives general background information on various strategies devised for protein separations. Part II discusses the specific application of the CE techniques to analytical biotechnology, while some clinical/diagnostic applications are also discussed. The objective is to show where and why CE can be used as a solution to a critical analytical need. Specifically, the role of capillary electrophoresis in product identity, quantity, purity, heterogeneity, stability, and process consistency is addressed.

In reading this primer, some familiarity with the basics of CE and protein structure and function is assumed. It is not meant as an all-inclusive, comprehensive review, but rather as a non-theoretical, practical guide which can be helpful as a reference or in designing separations. Relevant examples from our laboratories at Beckman Instruments in Fullerton, California, and from the literature are selected to serve as illustrations. **For easy reference, the articles and books cited in the text are listed alphabetically at the back of this primer.**

I: Introduction

Electrokinetic chromatography (EKC) is a family of electrophoresis techniques named after electrokinetic phenomena, which include electroosmosis, electrophoresis, and chromatography. Micellar electrokinetic chromatography (MEKC) is a mode of EKC in which surfactants (micelles) are added to the buffer solution. Surfactants are molecules which exhibit both hydrophobic and hydrophilic character. They have polar "head" groups that can be cationic, anionic, neutral, or zwitterionic and they have nonpolar, hydrocarbon tails. The formation of micelles or "micellization" is a direct consequence of the "hydrophobic effect." The surfactant molecules can self-aggregate if the surfactant concentration exceeds a certain *critical micelle concentration* (CMC). The hydrocarbon tails will then be oriented toward the center of the aggregated molecules, whereas the polar head groups point outward. Micellar solutions may solubilize hydrophobic compounds which otherwise would be insoluble in water. The front cover picture shows an aggregated SDS molecule. In the center of the aggregate, *p*-fluorotoluene is situated depicting the partitioning of a neutral, hydrophobic solute into the micelle. Every surfactant has a characteristic CMC and aggregation number, *i.e.*, the number of surfactant molecules making up a micelle (typically in the range of 50-100). (See also Table 1 and the discussion on page 10). The size of the micelles is in the range of 3 to 6 nm in diameter; therefore, micellar solutions exhibit properties of homogeneous solutions. Micellar solutions have been employed in a variety of separation and spectroscopic techniques. In 1980, Armstrong and Henry pioneered the use of micellar solutions as mobile phases for reversed-phased liquid chromatography (RPLC).

In the literature, MEKC is also often referred to as MECC (micellar electrokinetic capillary chromatography) since the separations are most often performed in a capillary tube. Other modes of EKC are cyclodextrin EKC (CDEKC), ion-exchange EKC (IXEKC), and microemulsion EKC (MEEKC). Cyclodextrin derivatives, polymer ions, and microemulsions are used in CDEKC, IXEKC, and MEEKC, respectively, instead of the micelles used in MEKC. The references listed on page 3 provide further detail on the differences between the various kinds of EKC techniques. In the following chapters, relevant references are listed in reverse chronological order after each chapter. All EKC techniques are based on the same

separation principle: the differential partitioning of an analyte between a two-phase system (*i.e.*, a mobile/aqueous phase and a stationary phase).

The same instrument that is used for capillary zone electrophoresis (CZE) is also used for MEKC. Both MEKC and CZE are modes of capillary electrophoresis (CE), as are capillary gel electrophoresis, capillary isoelectric focusing, and capillary isotachophoresis (for an introduction to CE, see the Beckman Primer *Introduction to Capillary Electrophoresis*, part number 360643). MEKC is different in that it uses an ionic micellar solution instead of the simple buffer salt solution used in CZE. The micellar solution generally has a higher conductivity and hence causes a higher current than the simple buffer does in CZE. MEKC can separate both ionic and neutral substances while CZE typically separates only ionic substances. Thus MEKC has a great advantage over CZE for the separation of mixtures containing both ionic and neutral compounds. However, in MEKC the size of the sample molecules is limited to molecular weights of less than 5000, whereas CZE has virtually no limitation in molecular size. The separation principle of MEKC is based on the differential partition of the solute between the micelle and water; CZE is based on the differential electrophoretic mobility.

Further Reading
(in reverse chronological order)

Janini, G. M., Isaaq, H. J. Micellar electrokinetic capillary chromatography: basic considerations and current trends. *J. Liq. Chromatogr. 15,* 927-960 (1992)

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Terabe, S., Otsuka, K., Ando, T. Electrokinetic chromatography with micellar solution and open-tubular capillary. *Anal. Chem. 57,* 834-841 (1985)

Terabe, S., Otsuka, K., Ichikawa, K., Tsuchiya, A., Ando, T. Electrokinetic separations with micellar solution and open-tubular capillaries. *Anal Chem. 56,* 111-113 (1984)

Armstrong, D. W., Henry, S. J. Use of an aqueous micellar mobile phase for separation of phenols and polynuclear aromatic hydrocarbons via HPLC. *J. Liq. Chromatogr. 3,* 657-662 (1980)

II: Separation Principle/Fundamentals

Figure 1 shows a schematic representation of the separation principle of MEKC. When an anionic surfactant such as sodium dodecyl sulfate (SDS) is employed, the micelle migrates toward the positive electrode by electrophoresis. The electroosmotic flow transports the bulk solution toward the negative electrode due to the negative charge on the surface of fused silica. The electroosmotic flow (EOF) is usually stronger than the electrophoretic migration of the micelle under neutral or alkaline conditions and, therefore, the anionic micelle also travels toward the negative electrode at a retarded velocity.

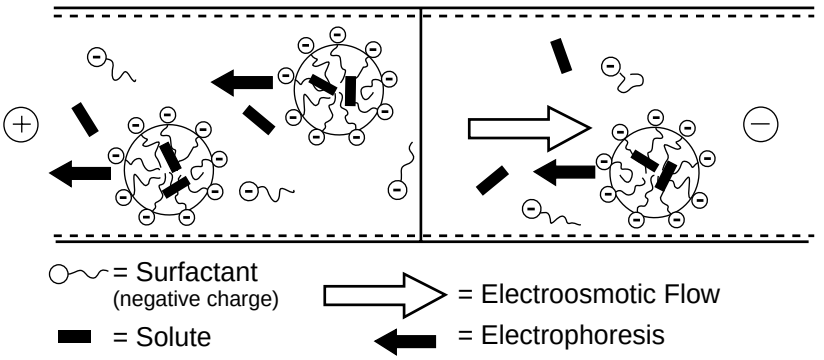

Figure 1. *Schematic of the separation principle of MEKC. The detector window is assumed to be positioned near the negative electrode.*

When a neutral analyte is injected into the micellar solution, a fraction of it is incorporated into the micelle and it migrates at the velocity of the micelle. The remaining fraction of the analyte remains free from the micelle and migrates at the electroosmotic velocity. The migration velocity of the analyte thus depends on the distribution coefficient between the micellar and the non-micellar (aqueous) phase. The greater the percentage of analyte that is distributed into the micelle, the slower it migrates. The analyte must migrate at a velocity between the electroosmotic velocity and the velocity of the micelle (see Figure 2A), provided the analyte is electrically neutral. In other words, the migration time of the analyte, t_R, is limited between the migration time of the bulk solution, t_0, and that of the

micelle, t_{mc} (see Figure 2B). This is often referred to in the literature as the *migration time window* in MEKC.

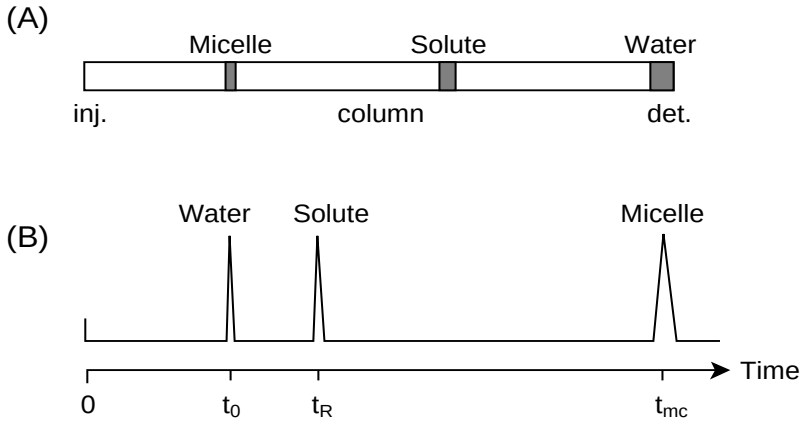

Figure 2. *Schematic of the zone separation in MEKC (A) and chromatogram (B). Reproduced with permission from Terabe,* et al., Anal. Chem. 57, *834 (1985).*

Capacity Factor

We can define the capacity factor, *k*', similarly to that of chromatography as

$$k' = \frac{n_{mc}}{n_{aq}} \tag{1}$$

where n_{mc} and n_{aq} are the amount of the analyte incorporated into the micelle and that in the aqueous phase, respectively. We can obtain the relationship between the capacity factor and the migration times as

$$t_R = \frac{1 + k'}{1 + (t_0 / t_{mc})k'} t_0 \tag{2}$$

The migration time of the analyte is equal to t_0 when $k' = 0$, or when the analyte does not interact with the micelle at all; the migration time becomes t_{mc} when *k*' is infinity or the analyte is totally incorporated into the micelle. Thus, the migration time window is limited between t_0 and t_{mc}.

When t_0 is infinity (electroosmosis is completely suppressed), equation (2) becomes

$$t_R = (1 + 1/k')t_{mc} \quad (3)$$

In this case, the bulk solution remains stationary in the capillary and the micelle migrates only by electrophoresis. If we define the capacity factor as the reciprocal of equation (1), equation (3) becomes identical with the relationship between t_R, t_0, and k' in conventional chromatography.

Figure 3 shows a typical example of MEKC separation. Eight electrically neutral compounds were successfully resolved in 17 min. The capacity factor scale is inserted in the figure to indicate the relationship between the migration time and the capacity factor. The capacity factor of infinity means that analyte has the same migration time as the micelle. Theoretical plate numbers calculated from the peak widths range from 200,000 to 250,000 which is typical for MEKC separations.

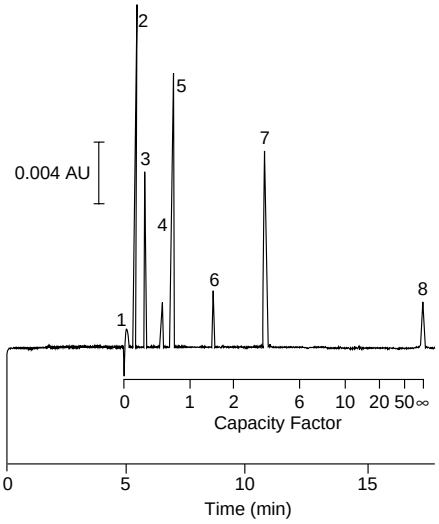

Figure 3*. Micellar electrokinetic chromatogram of a test mixture: 1 = methanol; 2 = resorcinol; 3 = phenol; 4 =* p*-nitroaniline; 5 = nitrobenzene; 6 = toluene; 7 = 2-naphthol; 8 = Sudan III. Conditions: capillary, 50 μm i.d. × 65 cm (effective length 50 cm); run buffer, 30 mM SDS in 50 mM phosphate/100 mM borate (pH 7.0); applied voltage, 15 kV; current, 33 μA; detection, UV absorbance at 210 nm; temperature, 35°C. Reproduced with permission from Terabe,* Trends Anal. Chem. 8, *129 (1989).*

Part 1

CE Techniques Applied to Proteins and Peptides

1.1 Prediction of Electrophoretic Mobility

The ability to predict the migration behavior of peptides and proteins under different experimental conditions (*e.g.*, changes in pH) would be extremely desirable as it would allow the optimization of separation conditions. For example, investigators could choose conditions such that a native protein could be distinguished from closely related variants or degradation products. Predictive models also might aid in the characterization of unknown species and in purity assays. This optimization approach could be applicable to the study of microheterogeneity of proteins such as glycoproteins, immunoglobulins, transferrins, and histone proteins.

In general, *small* peptides[1] consisting of just a few amino acids are "well behaved" in CZE and their electrophoretic migration (mobility) can be predicted based on their mass (size) and charge characteristics. The charge of a small peptide can be estimated from the pK_a values of the individual amino acids. With this information, the migration of a small peptide in a particular CE buffer can be easily calculated (for example, by a computer program—see reviews by McCormick, 1994; Palmieri and Nolan, 1994). For *larger* peptides and proteins, calculation of the charge based on ionization constants is *not* trivial and cannot be calculated easily from the pK_as of the free amino acids. Aside from the mass-to-charge ratio, other factors which may affect mobility are hydrophobicity, primary sequence, conformational differences, and the chirality of amino acids.

To illustrate this point, let us examine the case of a number of nonapeptides with *identical* amino acid composition but *different* primary sequences. The sequences of the peptides are shown in Table 1-1. The question is whether or not these peptides can be separated by CE.

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[1] In this text, and in accordance with other textbooks, peptides containing ten or fewer amino acids are called oligopeptides (or small peptides). Peptides with a molecular mass of approximately 5000–7000 Daltons (approximately 50–70 amino acids) lie on the borderline between polypeptides and proteins.

Table 1-1. Six Nonapeptide Isomers and Their Sequences

Peptide	Amino Acid Sequence
5	NH_2-ALDYALAHR-COOH
6	NH_2-ALDYARLAH-COOH
7	NH_2-ALDYHALAR-COOH
8	NH_2-HALDYARLA-COOH
9	NH_2-ALDYHARLA-COOH
10	NH_2-DHAYLLAAR-COOH

In Figure 1-1, the zone velocity of the peptides is plotted versus the pH. The plot indicates that, at different pH values, these peptides should be separable. Apparently the proteins do *not* have the same mass-to-charge ratio and, with some fine-tuning (*i.e.*, pH manipulation), they *can* be resolved from each other. The net charge of a peptide can be modified by a small change in the pH of the buffer, particularly near the pK_as of the amino terminal, carboxy terminal, or side groups. Since all these peptides contain the same amino acids, the amino acid sequence can influence the pK_as of the ionizable side groups and, therefore, the migration behavior of the peptides. Under low-pH conditions (as are present in the CE runs of Figure 1-1), the Asp side group, as well as the carboxy terminal group, is titrated. The charge on peptide #10, which is unique in that it has an amino terminal group near the Asp and Arg near the carboxyl terminal, is evidently altered more strongly than the other peptides. This decreases the positive charge on peptide #10 and causes a reduced zone velocity.

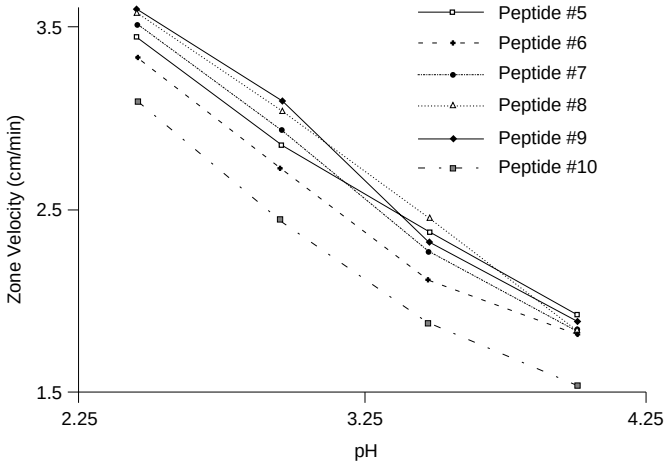


Figure 1-1. *Zone velocity of peptides plotted against pH. Linear sequences of the peptides are listed in Table 1-2. From Field* et al., Beckman Application Data Sheet DS-791 *(1991).*

Semi-empirical and theoretical models have been described to predict mobility in CE buffer systems. In 1966, Offord proposed the following equation relating the mobility (μ) of peptides with their valence (Z) and molecular mass (M):

$$\mu = k \cdot Z \cdot M^{-2/3}$$

where k is a constant. Thus a plot of mobility versus $Z \cdot M^{-2/3}$ should yield a straight line. This relationship was later confirmed by several researchers in the field of CE. The equation states that the frictional forces opposing the electrophoretic migration are proportional to the surface area of the species (assuming a spherical shape of the molecule with a radius proportional to the cube root of M). Researchers at Eli Lilly have extensively studied the mobility characteristics of peptides and proteins. An example from their data, derived from peptide fragments of an hGH digest, is shown in Figure 1-2A.

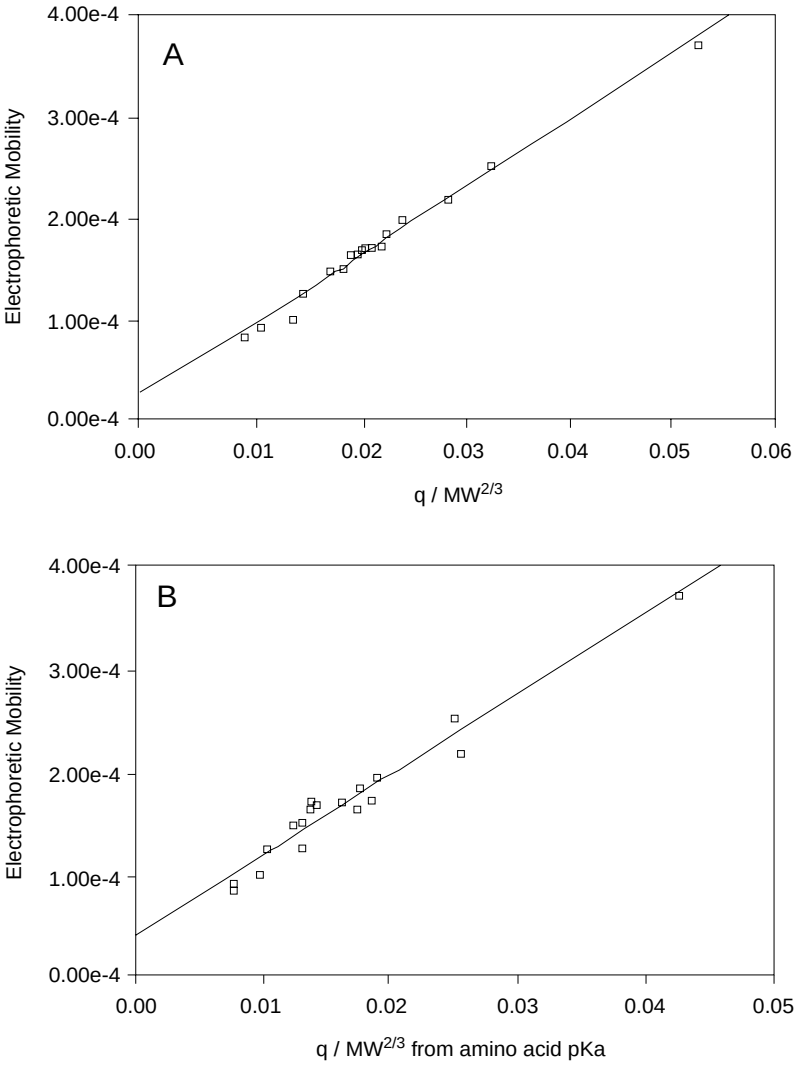


Figure 1-2. *Fit of electrophoretic mobility ($cm^2/V \cdot s$) versus the charge-to-size parameter for hGH digest separated in 0.1 M glycine, pH 2.35. (A) Data from pK_a values calculated at Eli Lilly. Correlation coefficient, 0.989. (B) Data based on pK_a values of the isolated amino acids. Correlation coefficient, 0.956. Data reproduced with permission from Rickard* et al., Anal. Biochem. 197, *197 (1991).*

Note that the charges in Figure 1-2A were calculated from ionization constants (pK_as consistent with those of typical peptides and proteins; for a detailed discussion and a list of ionization constants, see Rickard *et al.* (1991). A significant *lack of fit* was observed when the charges were calculated simply based on the pK_a values of the isolated amino acids. As a comparison, the plot based on these data is shown in Figure 1-2B.

Because mobility is defined as velocity per unit field strength, the above equation can also be re-written in the form of a relative migration time (relative to a standard reference peptide) as:

$$t/t_0 = K_0 \cdot Z^{-1} \cdot M^{2/3}$$

where t/t_0 is the relative migration time and K_0 is a constant pertaining to the reference peptide. Therefore, a plot of relative migration time versus $M^{2/3}/Z$ should also give a straight line as was found and published by several researchers (Deyl, 1989; Hjerten, 1989).

For closely related peptides, the above "Offord" equation can be used to estimate the optimum pH of the CE run buffer. This was shown by Bongers, *et al.* (1992) for synthetic peptides derived from human growth hormone releasing factor, a 44-residue peptide. The 11-residue peptides differed only by substitution of Asn, Asp, or β-Asp at a single residue. Titration curves (charge vs. pH) were constructed based on the pK data from Skoog and Wichman (1986) as illustrated in Figure 1-3. The curves reveal that it can be predicted that the optimum pH to separate the peptides should be between 3 and 5, as here the charge differences are maximized. Since the MWs of the peptides are approximately the same, their mass-to-charge ratios and, consequently, their mobilities differ the most in this pH range. The electropherogram shown at the bottom of Figure 1-3 shows that, indeed, baseline separation occurs at pH 4.3.

As shown above, the "Offord" charge/size parameter, $Z \cdot M^{-2/3}$ is related to the peptide mobility in CZE, and thus can be used to predict the relative migration order of peptides. This was verified by McCormick (1994) who used published data from Strickland and Strickland (1990) to calculate the charge-to-size parameter for 14 peptides with known sequence. The peptides varied widely in size and composition. A commercially available computer program (IntelliGenetics, Mountain View, CA) was used for the calculation of charge and $Z \cdot M^{-2/3}$. Exactly as predicted, the CE migration time order of the 14 peptides correlated well (inversely) with the order of calculated $Z \cdot M^{-2/3}$ values. Thus, the use of such procedures would be a valuable tool in the determination of peak identity for peptide separations in CZE.

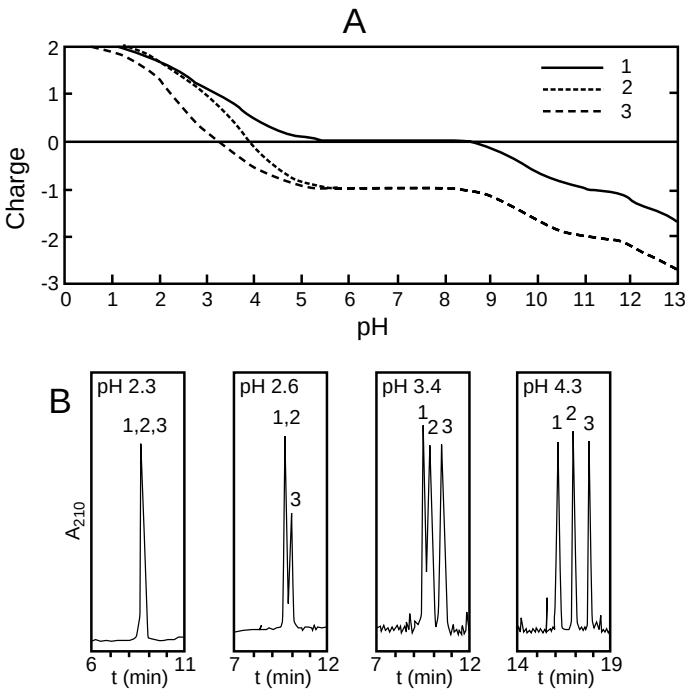


Figure 1-3*. (A) Calculated charge versus pH profiles and (B) electropherograms at varying pH for the synthetic model peptides (Leu[27], Asn[28]) GRF(22-32)-OH, 2, and (Leu[27], β–Asp[28])-GRF(22-32)-OH, 3. The pH of the 25-mM Na_2HPO_4/H_3PO_4 running buffer is shown in the upper left corner of each panel. Reprinted with permission from Bongers* et al., J. Liq. Chromatogr. 15, *1115 (1992).*

Deyl (1989) demonstrated that, for a large number of collagen proteins which varied considerably in molecular weight, the relative migration time is linearly related to their pIs. This relationship was studied in untreated fused-silica capillaries within the pH range of 6.9 to 10.5 and included data replotted from other researchers. Other investigators (Compton, 1991) have modeled the mobility of proteins in CE and applied it to protein microheterogeneity analysis. In this study, mobility was found to be a continuous function of $M^{-1/3}$ to $M^{-2/3}$, depending on the magnitude of M and the ionic strength of the buffer. Research on the modeling of mobility of peptides and proteins in CE is ongoing and various approaches are being investigated at this time.

1.2 Buffer Selection and pH Control in CZE

As pointed out in Section 1.1, the choice of a run buffer is very important in CE because it determines the charge on the analyte molecule and, therefore, its migration velocity. Briefly, the characteristics of a useful CE buffer are:

1. Good pH control/buffer capacity
2. Low conductivity
3. Good UV transparency (especially in the low UV region)

To obtain high-performance separations and special selectivities, the addition of certain compounds to the buffer is often required. These additives will be discussed in detail in Sections 1.3 and 1.4 dealing with peptides and proteins, respectively. The type of buffer, its ionic strength, and its pH can be varied and optimized for a particular separation problem. With untreated fused-silica capillaries, the use of high-pH buffers generally produces fast separations because the electroosmotic flow (EOF) is high. At a low pH, peptides migrate primarily on the basis of their charge-to-mass characteristics and the EOF is diminished. Buffers based on sodium phosphate, citrate, acetate, or combinations thereof are frequently used in CE. Typical buffer concentrations are 20 to 200 mM. Borate (pH range 7.5 to 10), in particular, is a popular buffer in CE for a wide variety of applications. This buffer has an inherently low conductivity (its use in CE has been reported with concentrations of 500 mM). Furthermore, borate is known to complex with diol groups which facilitate analysis of sugars and glycoproteins (Landers, 1993). Often, the conductivities associated with phosphate, citrate, and acetate buffers are relatively high, necessitating the selection of smaller i.d. capillaries or the adjustment of electric field strength conditions. Such limitations can be greatly minimized by employing efficient capillary cooling designs such as circulating liquids used in Beckman's P/ACE™ system. In certain cases, low conductivities can be achieved by using zwitterionic buffers, *i.e.*, the so-called "Good's" buffers (see Sigma catalog, 1993, page 1556). Several examples of the utility of these buffers in CE are discussed below.

1.2.1 Ohm's Law Plot

To estimate the maximum voltage that can be applied during a CE run, an "Ohm's Law" plot can be constructed (Nelson *et al.*, 1989). It is also a useful procedure to compare run buffers (or even CE cooling systems) in terms of current and Joule heat generation. By recording the current at each applied voltage (*e.g.*, at 1-minute intervals), an ideal Ohm's Law plot should yield a

straight line. This is the case when the heat generated inside the capillary is adequately dissipated (aided by the cooling system of the CE instrument). Deviations from linearity are indicative of inadequate Joule heat dissipation. At the voltage where linearity is lost, the heat dissipation capacity of the system has been exceeded. As a rough rule of thumb, the heat generated with a CE run buffer should not exceed ≈ 5 W/m. Figure 1-4 shows Ohm's Law plots for three buffers, *i.e.*, 100 mM phosphate, pH 2.5; 100 mM borate, pH 8.3; and 100 mM CAPS, pH 11.0. It can be seen that, while the borate buffer yields a straight line over 0 to 30 kV, deviation from linearity occurs much earlier, *i.e.*, at a lower voltage, with CAPS and phosphate. At an applied voltage of 20 kV, the power generated with CAPS and phosphate is 5.88 and 10.07 W/m, respectively. Therefore, it is advisable to use lower voltages for these buffers during CE runs. On the other hand, with borate, only 0.58 W/m is generated. At 20 kV, the recorded current was only 10 mA for borate, while 100 and 150 mA for CAPS and phosphate, respectively.

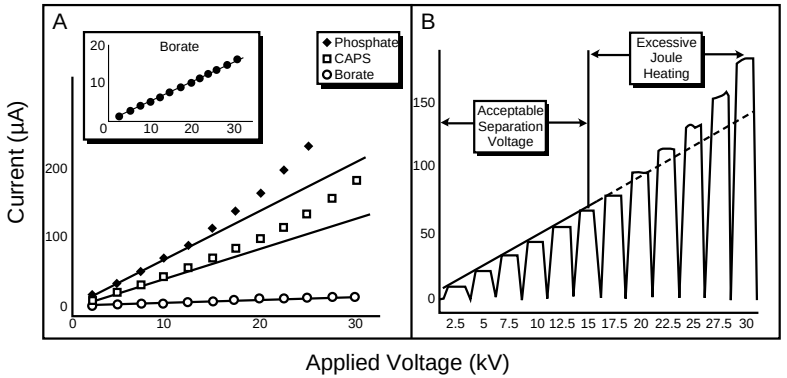


Figure 1-4. *Ohm's Law Plot. (A) Plot of observed current vs. applied voltage for each of three buffers. The voltage was incremented at 2.5 kV/min. Buffers were 100 mM phosphate, pH 2.5, made by dilution of phosphoric acid and titration with NaOH; 100 mM borate, pH 8.3, made by titrating 25 mM sodium tetraborate with 100 boric acid; and 100 mM CAPS, pH 11.0, made by titration of the appropriate concentration dissolved in water with NaOH. The inset shows the borate data plotted on an expanded scale. (B) Direct plot of current vs. applied voltage for 100 mM CAPS, pH 11.0. Voltage is incremented by 2.5 kV/min. A straight line drawn through the front edge of the plateau illustrates the ability of the cooling system to dissipate the heat generated by the passage of current. The departure from linearity indicates the excessive increase in current at the applied voltage, and is a reflection of the increase in capillary temperature. Reprinted with permission from Oda and Landers,* Handbook of Capillary Electrophoresis, *Landers (Ed.), Boca Raton: CRC Press, 1994.*

While it is relatively easy to make up CE run buffers, it is now possible to buy ready-to-go CE buffers from various sources (*e.g.*, Fluka, ISCO, Scientific Resources; Beckman sells a number of useful CE buffers designed for use with coated and untreated capillaries and in conjunction with application-specific kits).

1.2.2 Buffer Composition, pH Control

Camilleri and co-workers (1991) have suggested the use of deuterium oxide (D_2O) instead of water when making a run buffer. The increased viscosity of a D_2O buffer is favorable with respect to resolution of closely spaced peaks. To illustrate this point, Figure 1-5 shows the separation of a tryptic digest in both water and D_2O buffers. While the cost of D_2O (≈ $70.00 per 100 g) is relatively high compared to water, CE only requires 1 to 2 mL of buffer per experiment (≈ 5 runs). Thus, expensive buffers can be used in CE rather economically.

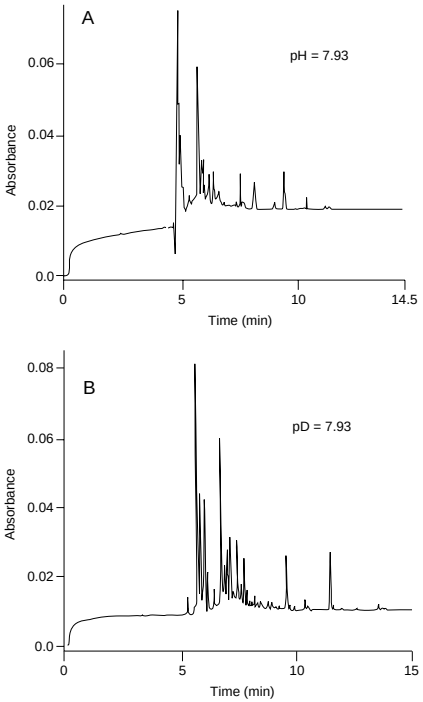


Figure 1-5. *Comparison of CE separations of tryptic digests of salmon calcitonin in (A) water (pH 7.93) and (B) D_2O (pD 7.93). Reproduced with permission from Camilleri* et al., Anal. Biochem. 198, *36 (1991).*

In practice, manipulation of the pH of a buffer is a powerful tool used to control the charge on a peptide (or protein) and, hence, the resolution between neighboring peaks in an electropherogram. This point is illustrated in Figure 1-6.

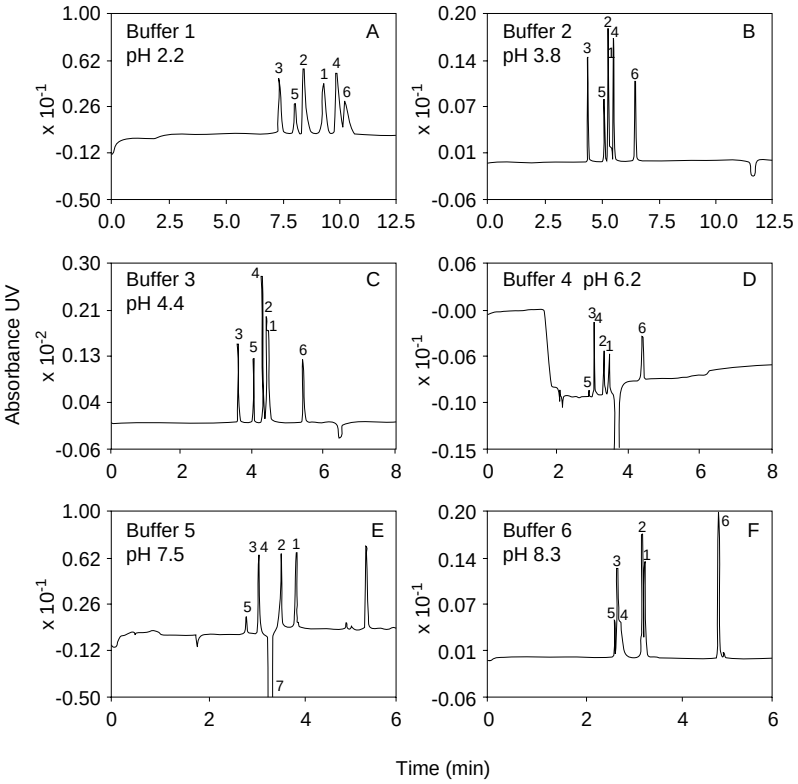


Figure 1-6*. CE of peptides separated with the buffers listed in Table 1-2. UV detection at 214 nm. Peak identification of the ACTH fragments: (A) 4-9; (B) 5-9; (C) 6-9; (D) 7-9; (E) 8-9; (F) 4-6. Reproduced with permission from van de Goor* et al., J. Chromatogr. 545, *379 (1991).*

In this example from Van der Goor *et al.* (1991), a mixture of six peptides was separated using different buffers ranging in pH from 2.2 to 8.3. The buffers and some of their properties are listed in Table 1-2. In this experiment, the peptide samples were dissolved in water prior to injection into the CE instrument.

Table 1-2. Operational Buffer Systems

Buffer No.	pH	Concentration*	Conductivity (mS/cm)	EOF·10^{-5} ($cm^2/V \cdot s$)	UV Absorbance (AU at 214 nm)
1	2.2	25 mM phosphate + KOH	4.03	< 3.2	0.000
2	3.8	20 mM formate + alanine	0.95	16.4	0.0002
3	4.4	20 mM α–aminocaproate + acetic acid	0.84	29.7	0.007
4	6.2	20 mM histidine + MES	0.39	52.8	0.059
5	7.5	40 mM imidazole + MOPS	0.73	57.6	0.057
6	8.3	100 mM borate + KOH	1.90	67.9	0.000

* MES = 2-(N-morpholino)ethanesulphonic acid;
MOPS = 3-(N-morpholino)propanesulphonic acid.

The peptides were synthetic fragments derived from the hormone ACTH and ranged in size from two to six amino acids. Several points can be noted:

1. Whereas all six panels show fast separations, the choice of a buffer with relatively high conductivity (25 mM phosphate—see panel A) results in somewhat broader peaks. This may be due to inadequate zone focusing using the 25 mM phosphate concentration (100 mM would result in sharper peaks—see the later section on "Detectability Enhancements"). Also, Joule heating in the capillary (relative to the other buffers) may play a role. In contrast, panels B and C show sharp, highly efficient peaks (theoretical plate counts of 200,000 to 300,000 are obtained).
2. Going from low to high pH, the electroosmotic flow (EOF) increases. Note: the EOF can be observed as the negative peak in the electropherograms; this negative peak is the result of water which is not associated with buffer ions and, therefore, has a decreased absorbance, migrating as a neutral species.
3. Selectivity is greatly affected by pH (*e.g.*, note the position of peak 4 relative to the other peaks).
4. The use of high-UV-absorbing buffers decreases the linear dynamic range of the detector and therefore leads to smaller peak heights. Note the smaller peak heights resulting from the choice of MES/histidine and MOPS/imidazole buffers.

In another paper by Langenhuizen and Janssen (1993), the effectiveness of different buffers was studied for the separation of pharmaceutical peptides. The selected buffers were:

- 25 mM phosphoric acid, adjusted to pH 2.20 with 1 M NaOH
- 20 mM formic acid, adjusted to pH 3.80 with β-alanine
- 20 mM L-histidine, adjusted to pH 6.20 with MES
- 50 mM Tris, adjusted to pH 7.50 with acetic acid
- 100 mM boric acid, adjusted to pH 8.30 with 1 M NaOH

Adrenocorticotropic hormone (ACTH), endorphins, cholecystokinin and fragments thereof were chosen as model compounds. It was found that the optimum buffer pH depends strongly on the pI values of the peptides. For peptides with an acidic character, the neutral pH region was preferred for optimum separation. Basic and neutral peptides were best separated in the low pH region.

1.3 Separation of Peptides in Free Solution

In physiology and medicine, peptides play important roles as hormones or neurotransmitters, and epitopes for such receptors as major histocompatibility complex (MHC) molecules. Many microorganisms produce peptides, often with antibiotic activity. Some peptides are very toxic (*e.g.*, phalloidin, a cyclic peptide originating from a mushroom). Examples of peptides with growth-promoting activity are the streptogenins. Other peptides act as enzyme inhibitors (*e.g.*, pancreatic trypsin inhibitor). A wide variety of biologically active peptides has been isolated from plants. The analysis of synthetically made peptide analogs is important to the biopharmaceutical industry in the development of new therapeutic agents. The analysis of peptides may also be part of extensive protein characterization schemes. For example, this is the case in peptide mapping applications where proteins are enzymatically or chemically cleaved into smaller subunits and subsequently analyzed by several methods such as slab gel electrophoresis, HPLC, thin-layer chromatography, or CE. Peptide mapping is important in a quality control environment.

During recent years, CE in free solution, as opposed to CE in a gel matrix (see Section 1.6), has been successfully applied to peptide separations. As we will see, many of the separation strategies used for peptides also work for

proteins. However, being more complex, proteins often require special pretreated capillaries and conditions. This is due in part to the adsorption problems encountered with proteins. MECC conditions—also considered a free-solution technique—generally work better for peptides than for proteins, although protein applications have been reported. The CGE and IEF techniques, on the other hand, are almost exclusively used with proteins.

1.3.1 Types of Capillaries

In the vast majority of peptide separations by CE, uncoated, fused-silica capillaries may be employed. For peptides, coated capillaries are used less than in conjunction with proteins but may be useful in some cases. Coated capillaries suitable for protein and peptide separations (*e.g.*, the eCAP™ Neutral and Amine capillaries) will be discussed in more detail in Section 1.4. Generally, it is a good idea to pretreat the uncoated capillary with sodium hydroxide (≈ 0.1 M) followed by rinsing the capillary with buffer and/or water before the actual run and repeat this rinsing procedure after each or several runs. The rinsing of the capillary with a strong base removes adsorbed contaminants. These contaminants may influence the magnitude of the EOF—and, therefore, run-to-run reproducibility— during subsequent runs. On the capillary wall surface, siloxane bonds are hydrolyzed to free silanol groups, the number of which determines the charge on the capillary wall and the magnitude of EOF at the existing buffer pH. Good run-to-run reproducibility is generally obtained by rinsing the capillary with a consistent rinse protocol (either after each run or after a number of runs); this ensures that—if changed—the EOF always returns to a certain, constant value at the start of a new run.

1.3.2 Use of Buffer Additives to Optimize Peptide Separations

In addition to pH control and buffer selection, one can use a number of buffer additives to optimize selectivity and fine-tune a separation. The reviews by Schwartz *et al.* (1993), Palmieri and Nolan (1994), and McCormick (1994) provide additional information on this subject.

1.3.2.1 Ionic Surfactants *(e.g.*, SDS or CTAB)

An effective way to achieve better selectivity—especially for neutral solutes—is the addition of micelle-forming reagents to the buffer. These can be either anionic (*e.g.*, SDS) or cationic (*e.g.*, CTAB). The resulting separations, known as micellar electrokinetic capillary chromatography (MECC—see Terabe, 1989), resemble reversed-phase HPLC in that the analytes partition between a

mobile phase (*i.e.*, the background electrolyte) and a pseudo-stationary phase (the "micellar phase," *e.g.*, SDS, which migrates against the EOF). In MECC, the detergent is added to the buffer above its critical micelle concentration (*e.g.*, with SDS, generally a concentration of 50 to 100 mM is used.) Note: with cationic surfactants such as CTAB, the polarity of the power supply must be reversed because the EOF changes direction. This occurs because these surfactants bind to the negatively charged silanol sites on the fused-silica surface and effectively changes the charge on the wall from negative to positive. Another Beckman CE primer authored by Terabe (P/N 266924) describes the MECC technique in more detail.

1.3.2.2 Non-Ionic Surfactants

This strategy is often useful when the above-described MECC approach fails (*e.g.*, when peptides with subtle differences in hydrophobicity are being separated). The non-ionic surfactant may provide a better balance between the electrostatic and hydrophobic forces controlling the separation. For example, in work done in our lab on the separation of analogs of growth hormone releasing peptide (all consisting of six amino acids), two analogs which had the same mass-to-charge ratio where separated by adding 20 mM polyoxyethylene-10 (Sigma Co.) to the buffer.

1.3.2.3 Ion-Pairing Reagents

Short-chain, ion-pairing reagents *(e.g.*, hexanesulfonic acid (HSA)) have been used in HPLC for protein and peptide separations. Research in our lab indicates that this reagent is also particularly effective in CE for hydrophobic peptides that are difficult to separate. For example, a 30 mM sodium phosphate buffer, pH 2.5, with 100 mM HSA buffer was used for the separation of two proprietary synthetic peptides (Figure 1-7). The mechanism by which resolution enhancement occurs is by a hydrophobic pairing between the short alkyl chain of the sulfonate and hydrophobic surfaces on the peptide at HSA concentrations below the critical micelle concentration. This results in an increase in negative charge on the peptide surface and a corresponding increase in migration time. Improvement in resolution is seen as the peptide-associated HSA induces a repulsion between peptides that would otherwise be attracted by hydrophobic forces. Yet another mechanism may be at work, in that the HSA can also ion-pair as in the traditional HPLC application, decreasing the surface positive charge on the peptides and any wall interaction with the negatively charged capillary surface. Both hydrophobic forces and ion pairing may be at work, since either association would demonstrate the experimentally observed result of increased resolution and migration times.

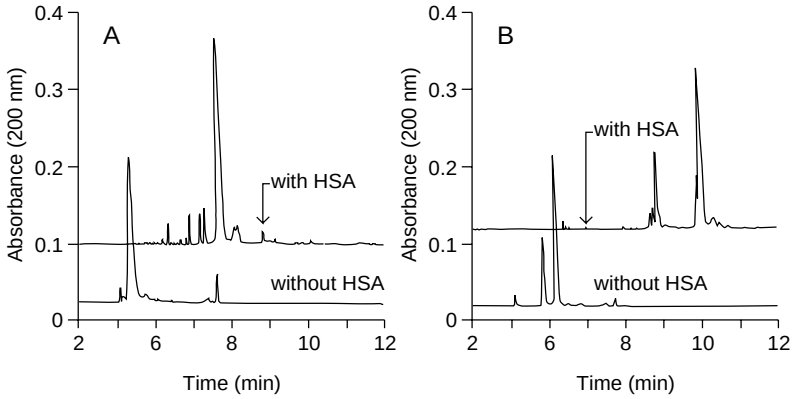


Figure 1-7. *Comparison of separations of two proprietary synthetic peptides (A) with and (B) without 100 mM hexanesulfonic acid added to the buffer. A 30 mM sodium phosphate, pH 2.5 buffer was used. Reproduced with permission from McLaughlin* et al., J. Liq. Chromatogr. 15, *961 (1992).*

1.3.2.4 Cyclodextrins

It has been mentioned (Novotny *et al*., 1990) that addition of cyclodextrins significantly enhances sensitivity of the fluorescence detection of derivatized amino acids and peptides. These additives improve resolution for peptide separations—presumably due to host–guest interactions with the cyclodextrin cavities). The use of these additives was first explored in HPLC (and later in CE by Terabe, 1989) for the separation of chiral substances such as pharmaceuticals.

1.3.2.5 Organic Solvents

Small amounts of organic solvents (approximately 0 to 30% methanol or acetonitrile, 1 to 2% THF) can be added to the buffer. This is a well-known practice in MECC when dealing with small molecules and is often used to increase analyte solubility in the buffer. Adding organic solvents to the buffer causes a decrease in the EOF due to a decreased zeta potential, resulting in a lower current and less Joule heat generation. In Figure 1-8, the MECC separation of a mixture of enkephalins is shown with and without 5% acetonitrile. A much better separation is obtained using acetonitrile as a buffer additive.

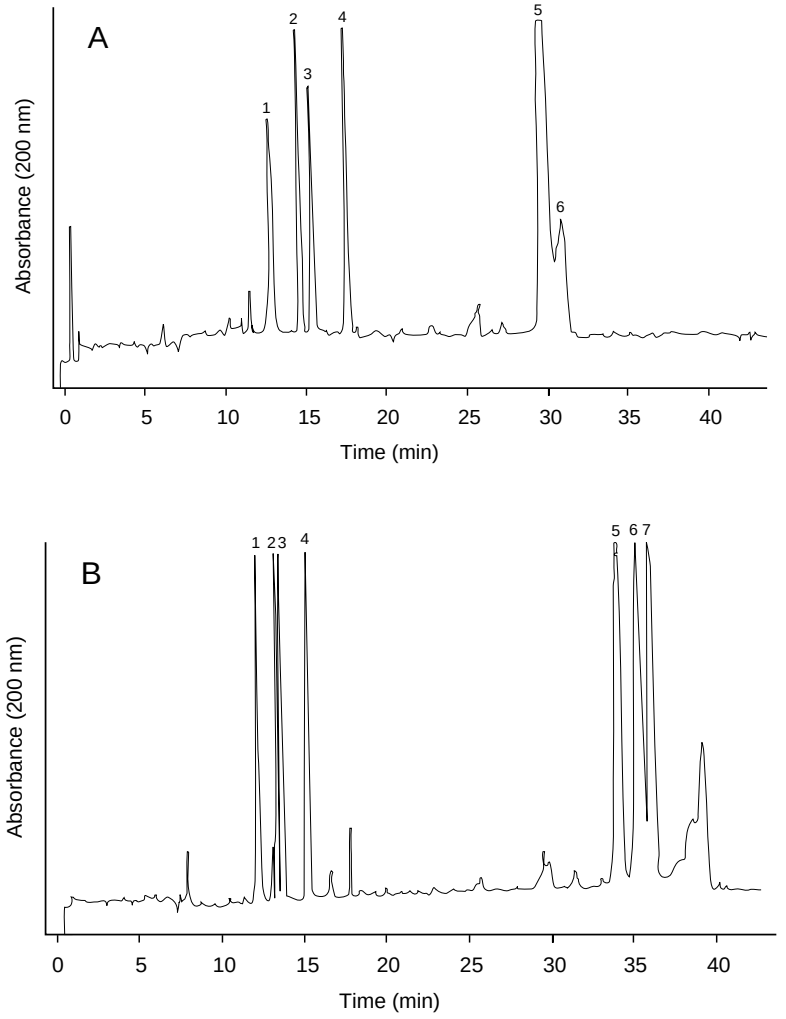


Figure 1-8*. MECC of enkephalin analogs (A) without and (B) with the addition of 5% acetonitrile to the buffer. Capillary, 65 cm × 75 μm, 100 mM sodium borate, 100 mM SDS, pH 8.5, 15 kV, 25°C, 200 nm. Peak identification: (1) metsulfoxide enkephalin; (2) methionine enkephalin; (3) [ala^2] methionine enkephalin; (4) leucine enkephalin; (5) leucine enkephalin amide; (6) leucine enkephalin-arg; (7) proenkephalin.*

1.3.2.6 Divalent Amines

While adsorption on the capillary wall is usually not a problem with small peptides, larger peptides and proteins can be irreversibly adsorbed on the capillary wall. This is one of the major problems encountered with CE of proteins (see also the following Section 1.4 on proteins). Efforts to reduce adsorption have been aimed at modifying the interfacial double layer at the wall (*i.e.*, to modify its zeta potential). This can be done by adding small amounts (0 to 5 mM) of cationic, divalent amines to the buffer. 1,4-diaminobutane (DAB) (see Lauer and McManigill, 1986; Stover *et al*., 1989), 1,5-diaminopentane (DAP), and morpholine (Nielsen *et al*., 1989) have been used for this purpose. Much larger amounts of amine (30 to 60 mM) are useful for protein separations (Bullock and Yuan, 1991). Figure 1-9 shows the effect of adding 5 mM DAP to the buffer, resulting in better selectivity and sharper peptide peaks.

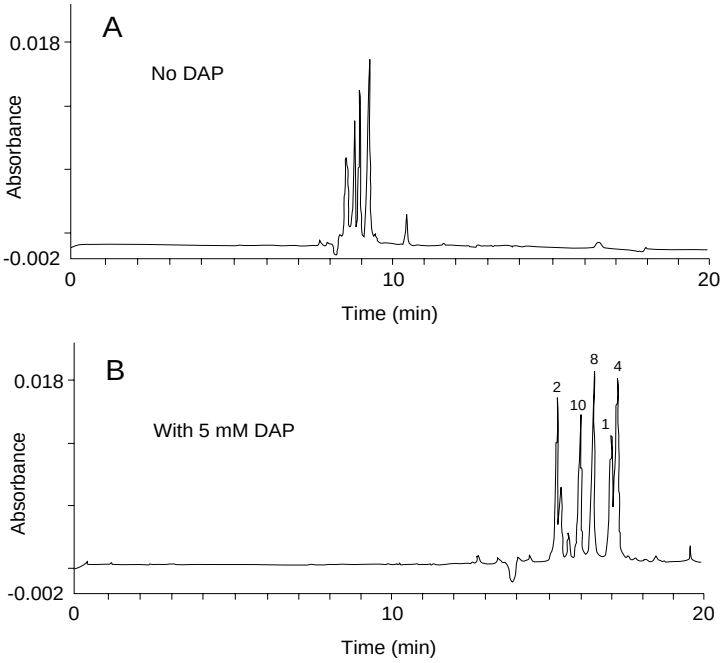


Figure 1-9. *Separation of a mixture of five synthetic nonapeptides. (A) Without DAP added to the buffer, four peaks can be discerned. (B) With 5 mM DAP added to the buffer, all five peaks are resolved. Peak identification, see Field* et al., Beckman Application Data Sheet DS-791.

1.3.2.7 Complexing Reagents

Cohen *et al.* (1987) found that metal ions enhance the resolution of nucleic acids and amino acids in MECC buffers. In a similar fashion, 0 to 30 mM zinc perchlorate has been used as a complexing reagent for histidine-containing peptides (Stover *et al*., 1989). Metal ions originating from zinc or copper salts can interact with certain N, O, or S atoms on proteins or peptides. This causes the mobility of these species to decrease relative to species in which no complexation takes place. On the other hand, trace amounts of EDTA are sometimes used to complex *undesirable* trace metals during electrophoresis (Ludi *et al*., 1988).

1.4 Separation of Proteins in Free Solution

1.4.1 Adsorption

Proteins have the unfortunate property of sticking to many different surfaces, including metals, plastics, and glass. In *chromatography*, concerns regarding the adsorption of proteins on packing materials or ancillary equipment have led to the development of "biocompatible" instrumentation. One of the main problems associated with protein separations using *CE* on untreated fused-silica capillaries is adsorption by the charged sites of proteins on fixed, negatively charged sites (silanol groups) on the capillary wall. This process leads to band broadening and results in far lower actual plate numbers (a measure of the efficiency of the separation) than would be expected on the basis of theory. The electrostatic interaction of proteins with the wall is schematically depicted in Figure 1-10.

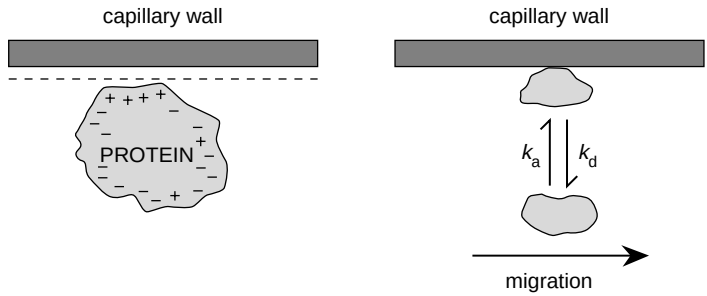


Figure 1-10. *Schematic of electrostatic interactions of proteins with the negatively charged capillary wall. The adsorption-desorption process affects peak width as migration occurs. Reproduced with permission from Novotny* et al., Electrophoresis 11, *735 (1990).*

The adsorption and desorption processes have specific rate constants (k_a and k_d, respectively) which ultimately affect the band width of the analyte zone as migration progresses. Protein adsorption may also occur through *hydrophobic* forces if the stability of the protein in solution is affected by changes in its environment (*e.g.*, by changes in pH or temperature). In solution, proteins are held together by H-bonding and hydrophobic forces. When unfolding occurs, hydrophobic areas may be exposed, resulting in aggregation or hydrophobic adsorption. Reagents such as urea or guanidine HCl are often used by protein chemists to force this process of denaturation. β-mercaptoethanol is used to break the disulfide bonds which hold the polypeptide chain together, as illustrated for ribonuclease in Figure 1-11. The following sections deal with strategies to prevent undesirable protein adsorption on the capillary wall surface when working with CE in the free-solution mode.

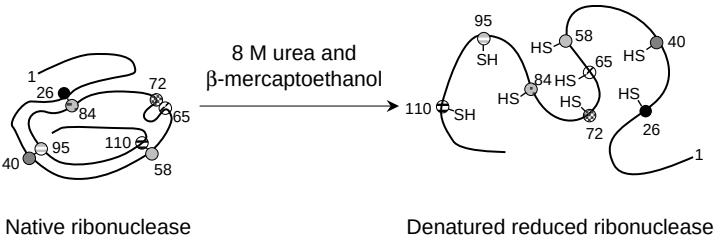


Figure 1-11*. Denaturing of ribonuclease by treatment with β-mercaptoethanol in 8 M urea.*

1.4.2 Strategies to Prevent Protein Adsorption

1.4.2.1 High- and Low-pH CE Buffer Conditions

Several investigators (Lauer and McManigill, 1986; McCormick, 1988) have tried to overcome the electrostatic adsorption of proteins on the capillary wall simply by working at extremes of pH. Under these conditions, the silanol groups on the wall surface are either negatively charged or neutral. In solution, proteins exhibit acid–base behavior and their net charge is dependent on the pH of the buffer. The net charge is zero at the characteristic isoelectric point (where the number of positive charges is balanced precisely by the number of negative charges). This is illustrated in Figure 1-12 for a protein with a pI of 7.0.

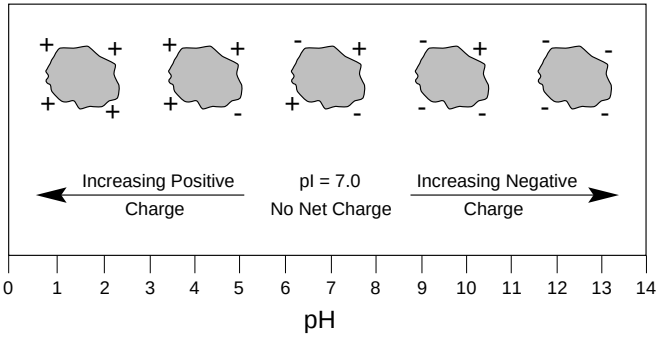


Figure 1-12*. Schematic of the effect of buffer pH on the net protein charge. At pH 7.0 (in this case, the pI of the model protein), there is no net charge on the protein. Increasing net positive and net negative charge is obtained by decreasing and increasing the pH of the buffer, respectively.*

At *high pH* (*e.g.*, pH approximately 10), the pI of the protein typically is less than the pH of the buffer (an exception being a very basic protein). Therefore, *both* the protein and the capillary wall are negatively charged, and the adsorption process is minimized as a result of a charge repulsion effect. Note that, at high pH, a relatively high EOF is generated. Theoretically, resolution is improved when the EOF is suppressed and balanced against the electrophoretic migration (*e.g.*, by means of buffer additives—see Section 1.4.2.3).

This is also the case when working at the other extreme with *low pH* conditions (*e.g.*, pH approximately 2). In this situation, the capillary wall is

protonated while the proteins are positively charged, again minimizing electrostatic interactions. However, while these approaches are useful in some cases, working at the pH extremes is not always feasible for the following reasons:

- It precludes the study of many native molecular conformation interactions. For example, at the pH extremes, unfolding and/or aggregation of proteins may occur which could lead to the undesirable appearance of multiple and/or broad peaks. In addition, at high pH, deamidation or peptide bond scission may occur.
- The biological activity of the protein may be very different under extreme pH conditions, precluding enzymatic assays.
- At very high pH (greater than 11), dissolution of the silica (wall material) becomes an issue.
- The electrophoretic mobility of proteins tends to be very sensitive to certain pH regions. The largest number of titratable groups occurs in the region of pH < 6 or pH > 9.

As a result of the enormous diversity in proteins, a wide range of run conditions at varying pH values needs to be available.

Two basic strategies have been applied in CE to address the above issues: (1) a *"static"* approach through permanent modification of the capillary wall by suitable coatings, and (2) a *"dynamic"* approach using certain buffer additives. In the latter, the capillary wall is dynamically modified each run by masking the charged sites.

1.4.2.2 Permanent Capillary Coatings

As CE is maturing, coated capillaries specifically designed for protein separations are becoming more available. A recent survey by Majors (1994) describes the latest introductions of companies at the 1994 Pittsburgh Conference. Wehr (1993), Swerdberg (1994), and Guzman's book (1993, several chapters) have reviewed column technology for CE. Some of the major suppliers of CE instrumentation, including Beckman, also sell coated capillaries. However, some of these capillaries are designed to fit only in certain instruments, so it advisable to check first if these capillaries will fit directly in a P/ACE system. Specialty companies such as J & W Scientific, Supelco, Scientific Glass Engineering, Scientific Resources, and MetaChem Technologies also provide coated capillaries. Beckman has introduced several coated capillaries which are available either by themselves or as part of a method development kit. In general, the 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. Hydrophobic coatings, such as those frequently used

in GC, are less suitable for protein CE separations. A sampling of coating procedures is summarized in Table 1-3. In most cases, the capillary is first deactivated by means of a silanization reagent and is then coated with a functional group.

Table 1-3. Some Capillary Coatings for Protein Separations

Coating Type	**Investigator**	**Reference**
Methylcellulose	Hjerten	*J. Chromatogr. 347,* 191 (1985)
Polyacrylamide (through Si-O-Si-C bonds)	Hjerten	*J. Chromatogr. 347,* 191 (1985)
Polyacrylamide (through Si-C bonds)	Novotny	*Anal. Chem. 62,* 2478 (1990)
3-glycidoxypropyltrimethoxysilane	Jorgenson	*Science 222,* 266 (1983)
Epoxy-diol, maltose	Poppe	*J. Chromatogr. 480,* 339 (1989)
Polyethyleneglycol	Poppe	*J. Chromatogr. 471,* 429 (1988)
Polyvinylpyrrolidone	McCormick	*Anal. Chem. 60,* 2322 (1988)
Aryl-pentafluoro(aminopropyl-trimethoxy)silane	Swerdberg	*Anal. Biochem. 185,* 51 (1990)
α-lactalbumin	Swerdberg	*J. High Res. Chromatogr. 14,* 65 (1991)
Polyether	El Rassi	*J. Chromatogr. 559,* 367 (1991)
Hydrophilic, C1, C8, C18	Dougherty	*Supelco Reporter*, Vol. X, No. 3 (1991)
Ion exchangers, polyacrylamide	Engelhardt	*J. Microcol. Sep. 3,* 491 (1991)
Neutral, hydrophilic	Karger	*J. Chromatogr. 652,* 149 (1993)
Hydrophilic & hydrophilic polymers	Lee	*Anal. Chem. 65*, 2747 (1993)

It is interesting to mention that, as early as 1967, Hjerten demonstrated the utility of polyacrylamide or methylcellulose polymer coatings for glass tubes in free-solution electrophoresis. In 1985, Hjerten and Zhu described a capillary surface modification which has been frequently cited in the CE literature. Before the advent of commercial CE coatings, the "Hjerten" coating was the most widely used among practitioners of CE. A bifunctional silane was used to first derivatize the surface, after which a hydrophilic polymer (polyacrylamide) was covalently attached. This type of coating minimizes solute adsorption and strongly suppresses the EOF (the negative charge on the wall is "neutralized"). However, an often-heard complaint was that these home-made capillaries were not stable in the long run and, therefore, would not yield reproducible migration times.

Beckman recently introduced a similar type of neutral coating (eCAP Neutral Capillary, P/N 477441) designed for protein separations in the pH 3 to 8 range (use of solutions with pHs outside this range may damage the capillary). This capillary also should prove useful for certain peptide separations. A Neutral Capillary Method Development Kit (P/N 477445) includes three buffers designed for use in the normal- or reversed-polarity mode. In applications requiring normal polarity, the citrate, pH 3.0 buffer, and the citrate/MES, pH 6.0 buffer are most useful for proteins with pIs > 4.0 and 6.7, respectively. The tricine, pH 8.0 buffer is designed for applications in the reversed-polarity mode. Note that, with this type of coating, migration is mainly by electrophoretic flow as the EOF is minimized (≈ 5% of the EOF determined for untreated fused silica). The excellent stability of these neutral capillaries is demonstrated in Figures 1-13 and 1-14 for acidic and basic proteins, respectively. The Neutral Capillary is also suitable for CIEF applications (see Sections 1.7, 2.2, and 2.3).

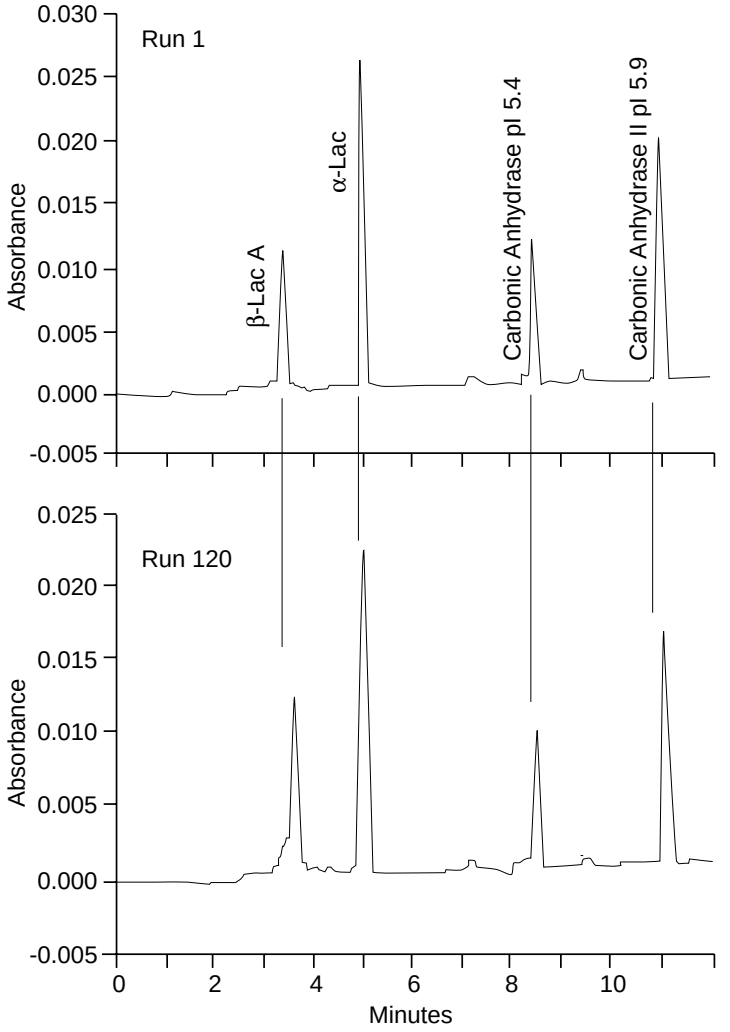


Figure 1-13*. e-CAP Neutral Capillary stability. 1st and 120th run of acidic proteins (% RSD for absolute migration time). β-lactoglobulin (2.99), α-lactoglobulin (0.45), carbonic anhydrase II, pI 5.4 (1.00) and pI 5.9 (1.30).*

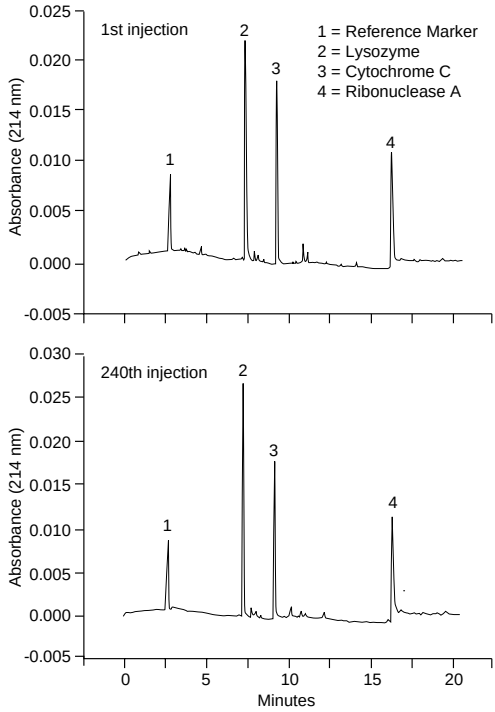


***Figure 1-14**. e-CAP Neutral Capillary stability. 1st and 120th run of basic proteins (% RSD for absolute migration time). Lysozyme (0.47), cytochrome C (0.51), myoglobin (1.05), ribonuclease A (0.96).*

A second type of coating (eCAP Amine Capillary, P/N 477431) available from Beckman utilizes a polyamine-modified surface. A strong cationic charge is created on the capillary wall and, consequently, the EOF is reversed. Basic analytes should be repelled from the wall surface; hence, adsorption is minimized for cationic peptides, proteins, and other positively charged analytes. This capillary must be operated in the reversed-polarity mode as, otherwise, the EOF is in the direction away from the detector. Figure 1-15 shows the reversed order of elution when the amine capillary is used instead of the untreated capillary. In addition, the analysis time on the coated capillary is much shorter than with the untreated capillary. In the Beckman Amine Capillary Kit (P/N 477430), three buffers are supplied which will yield relatively fast run times: acetate, pH 4.5; MES, pH 6.0; and Tris pH 8.0. Also included are two phosphate buffers (pH 2.5 and 7.0) which will reduce the positive surface charge on the capillary wall through ion pairing, and consequently, the EOF.

The effect of the phosphate concentration (varied from 0 to 300 mM) on the mobility of a neutral marker (benzyl alcohol) is demonstrated in Figure 1-16. Typically with the phosphate buffers, peak efficiency and resolution are superior to those obtainable with non-phosphate buffers. The reduced surface charge on the wall is especially advantageous for acidic analytes, as electrostatic interaction will be minimized.

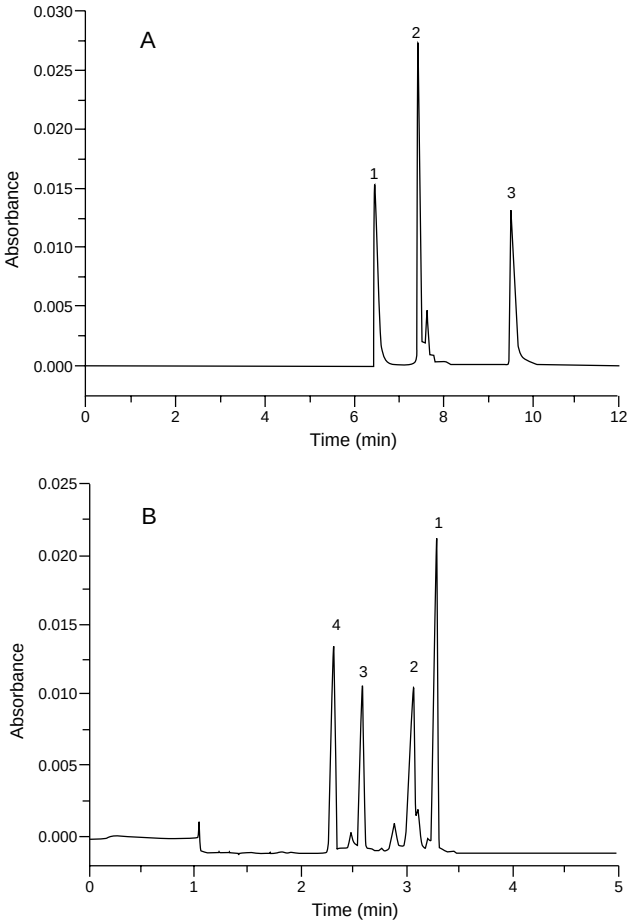


Figure 1-15. *(A) Separation of (1) lysozyme, (2) cytochrome C, and (3) ribonuclease A on fused-silica capillary. (B) the eCAP Amine Capillary improves the resolution, speed of separation, and reverses the migration order. Benzyl alcohol (4) is a marker.*

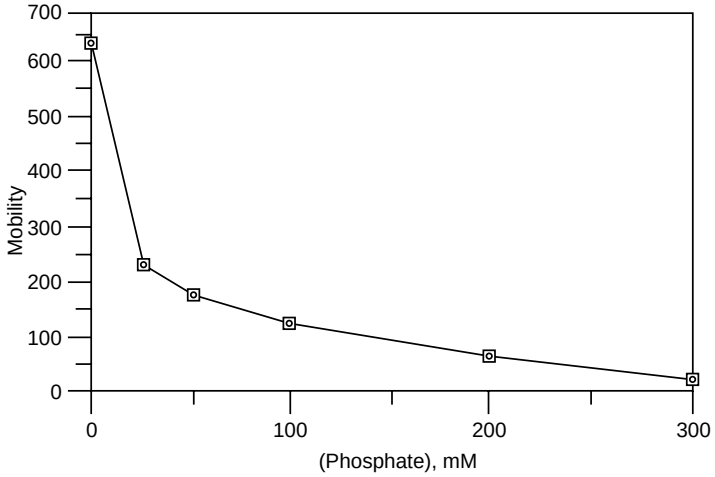


***Figure 1-16**. Mobility vs. phosphate buffer concentration*

Whereas coated capillaries may dramatically reduce protein adsorption, it still may be necessary to clean the capillary in between runs. This is necessary when severe peak broadening occurs and/or poor precision is observed. The extent of capillary cleanup is dependent on the type and number of samples exposed to the capillary. With the Neutral capillary, a recommended regeneration procedure involves rinsing with 0.1 N HCl (30 to 60 seconds) followed by a 1.5-minute rinse with run buffer. For the Amine capillary, rinsing with 1 N NaOH is recommended in between runs. However, with "dirty" samples, a rinse procedure consisting of 1 N HCl, 1 N NaOH, and Amine Regenerator solution (5 minutes each) may be required.

1.4.2.3 Dynamic Capillary Coating: Buffer Additives

The dynamic approach for capillary coating involves the use of buffer additives. A number of ways have been suggested to reduce protein adsorption on the capillary wall, conceivably by interfering with the protein–wall ion-exchange mechanism.

1.4.2.3.1 High-Salt or High-Ionic-Strength Buffers

Lauer and McManigill found that a relatively large amount of salt (*i.e.*, 0.25 M K_2SO_4) led to improved separation efficiency. Under these conditions, salt competes with protein for adsorption sites. An issue of concern with this ap-

proach is the absorptivity (purity) of high salt buffers in the low UV region. A variation of this method was applied by Chen (Beckman, Fullerton, CA) who used 0.5 M sodium phosphate buffers, pH 5 to 10, for the separation of protein standards in the pI 5.2 to 10.5 range. Short, small-i.d. (25-μm) capillaries were used, resulting in fast, efficient separations. With standard-size capillaries (75 to 100 μm i.d.), the use of high-ionic-strength buffers may result in intolerable Joule heat generation. (In Figure 1-9, for example, the 75 μm i.d. capillary could *not* be used with buffer concentrations exceeding 0.125 M.) However, 25 μm i.d. capillaries dissipate heat much more efficiently and, therefore, are preferable with high-ionic-strength buffers. The 0.5 M sodium phosphate buffers are UV transparent and can be used with low-UV detection. An example of this separation system applied to milk analysis is shown in Figure 1-17. Urea was added to the buffer to prevent aggregation of the caseins.

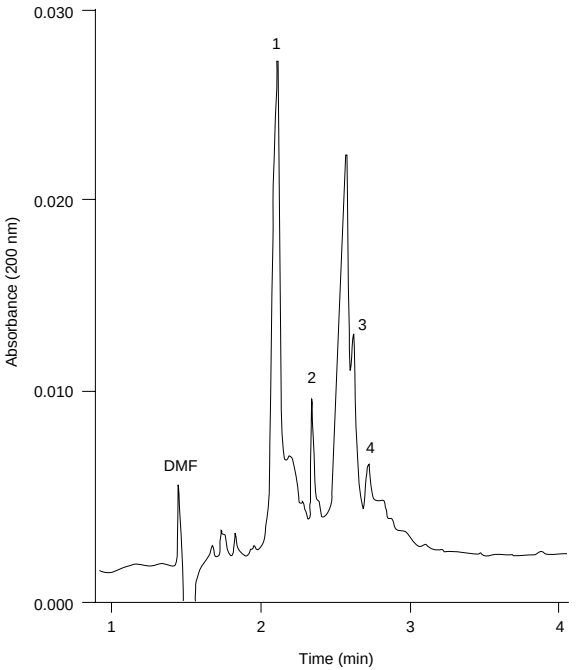


Figure 1-17*. CE of nonfat milk on a 23 cm × 21 μm capillary. Buffer, 0.5 M sodium phosphate, 4 M urea, pH 7.0. Peak identification: (1) β-casein; (2) α-lactalbumin; (3) α-casein and β-lactoglobulin B; (4) β-lactoglobulin A. Dimethylformamide (DMF) was added as an EOF marker. From Chen,* Beckman Application Data Sheet DS-818 *(1991).*

Phosphate buffers dynamically modify the capillary wall by converting residual silanols on the capillary surface to phosphate complexes that are more easily protonated (McCormick, 1988). The resulting dynamically coated surface exhibits a different selectivity than the untreated fused-silica capillary.

1.4.2.3.2 Zwitterionic Salts

Instead of using ionic salts, Bushey and Jorgensen (1989) proposed the use of zwitterionic salts as a buffer additive. Zwitterions such as betaine, sarcosine, and triglycine do not contribute to the conductivity and, consequently, can be used at relatively high concentrations (up to 2 M). A similar approach was taken by workers at Waters Associates, who used n-propyl(trimethyl)ammonium sulfate in concentrations up to 1 M. The quaternary ammonium functionality of the zwitterions interacts with the negatively charged silanol groups on the surface; they contain sulfate and carboxyl groups as the anionic components. Zwitterionic salts are effective over a wide pH range and are limited by the pK_as of the titratable groups. In many cases, however, the initial concentration must be in excess of 1 M to be effective. Alternatively, primary aminophosphoryl reagents exert their effect at lower concentrations and provide evidence of increased ion-pair stability when compared to phosphate buffers alone (see Chen, F. T. *et al.*, 1992). This may be due to the increased hydrogen bonding potential for these additives compared to the quaternary ammonium compounds.

In Figure 1-18, 250-mM O-phosphorylethanolamine was used as the zwitterionic additive to separate five protein standards. This reagent is widely available, low in cost, and UV transparent. It can be seen that, *with* the additive, considerably better peak shape is obtained, particularly for β-lactoglobulin A and myoglobin. It is apparent from the two pK_as (*i.e.*, 5.8 and 9.4) that the effective working range is limited to pHs between 6 and 9.

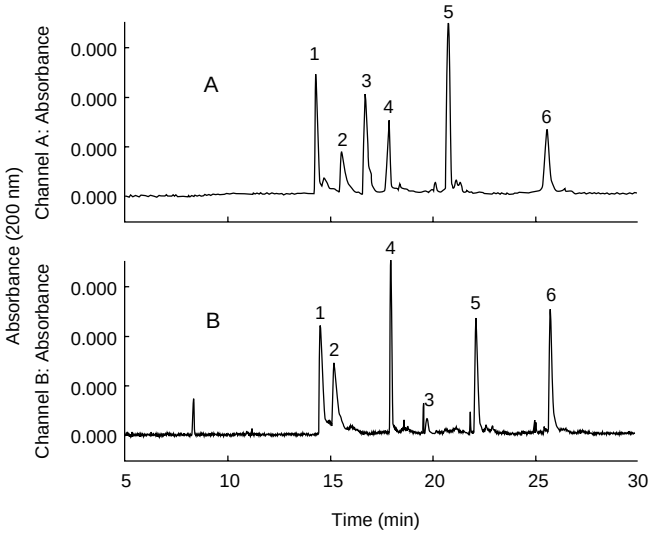


Figure 1-18*. Effect of zwitterionic additive (O-phosphorylethanolamine) on separation efficiency. (A) run buffer, 250 mM sodium phosphate, pH 6.0; (B) 100 mM sodium phosphate, plus phosphorylethanolamine, pH 5.8. Peak identification: (1) lysozyme; (2) cytochrome C; (3) EOF masker; (4) myoglobin; (5) carbonic anhydrase; (6) β-lactoglobulin A. Reproduction with permission from Chen* et al., J. Liq. Chromatogr. 15, *1143 (1992*).

1.4.2.3.3 Divalent, Cationic Amines

As mentioned earlier regarding peptide separations (Figure 1-9), divalent amines suppress solute–capillary wall interactions. For protein separations, 1,4-diaminobutane, 1,5-diaminopentane, and 1,3-diaminopropane have been used for this purpose. The latter additive (in relatively large quantities of 30 to 60 mM) appeared particularly suitable in conjunction with moderate amounts of salt. Figure 1-19 shows the effect of varying amounts of 1,3-diaminopropane on the resolution of basic proteins in a run buffer of pH 7.0. Increasing the amine concentration in the buffer results in longer separations as the EOF becomes increasingly suppressed.

This method is well suited for basic proteins. Acidic proteins (pI 5.4 to 7.4) showed considerable band broadening, however.

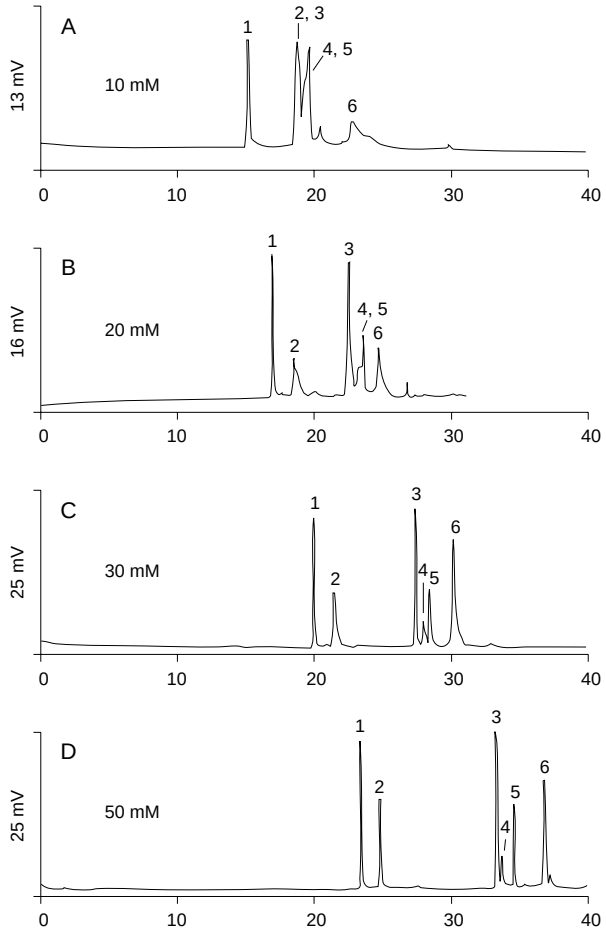


Figure 1-19*. Effect of adding 1,3-diaminopropane on peak shape and resolution in a pH 7.0 phosphate buffer. Peak identification: (1) lysozyme; (2) cytochrome C; (3) ribonuclease A; (4) trypsinogen; (5) α-chymotrypsinogen A; (6) rhuIL-4. Reproduced with permission from Bullock and Yuan,* J. Microcol. Sep. 3*, 241 (1991).*

1.4.2.3.4 Non-Ionic Surfactants

Hydrophobic proteins are often difficult to analyze by CE due to their interaction with the (coated) capillary wall. Towns and Regnier (1991) showed that non-ionic surfactants such as Brij35 and Tween20 can be used to dynamically coat deactivated (bonded-phase) capillaries, thus allowing high-efficiency separations for hydrophobic proteins. At Supelco, Dougherty *et al.* used a similar approach with their hydrophobically bonded "CElect" capillaries and the non-ionic surfactant Brij35. The detergent coating makes the capillary wall surface less hydrophobic and, therefore, more suitable for hydrophobic protein separations.

Typically, the procedure of preparing the capillary involves rinsing the bonded-phase capillary (by pressure) for approximately 2 hours with aqueous buffer containing 5% of the desired detergent (*e.g.*, Brij35); 0.001% detergent is added to the run buffer during the CE runs. The latter helps to maintain a constant detergent concentration on the capillary wall surface. Figure 1-20 shows the separation of three hydrophobic proteins—lysozyme, cytochrome C, and ribonuclease A—analyzed on a CElect C18 capillary dynamically coated with Brij35.

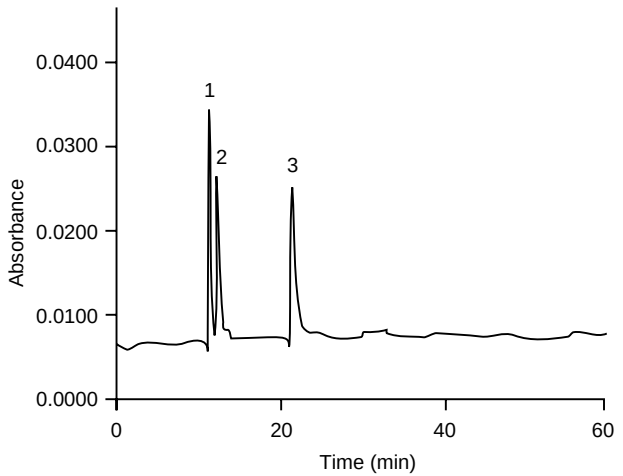


Figure 1-20. *Separation of basic proteins using a CElect-H2 (C18) capillary coated with Brij35. Run buffer: 10 mM sodium phosphate, pH 7.0, 0.001% Brij35. Field strength: 300 V/cm. Peak identification: (1) lysozyme; (2) cytochrome C; (3) ribonuclease A. Figure reproduced with permission from Ann Dougherty, Supelco, Bellefonte, PA.*

1.4.2.3.5 Charge Reversal Reagents

Another way to reduce adsorption of cationic proteins is to add to the buffer certain surfactants or polymers which reverse the charge on the wall from negative to positive (note: this is also the case with the Amine capillary from Beckman except here a permanent coating is created). Consequently, the EOF is also reversed which necessitates reversal of the power supply. Figure 1-21 from Emmer *et al*. (1991) depicts this situation for a fluorocarbon surfactant, FC-134 (3M Company), and is thought to involve a bilayer formation of hydrophobic chains at the wall.

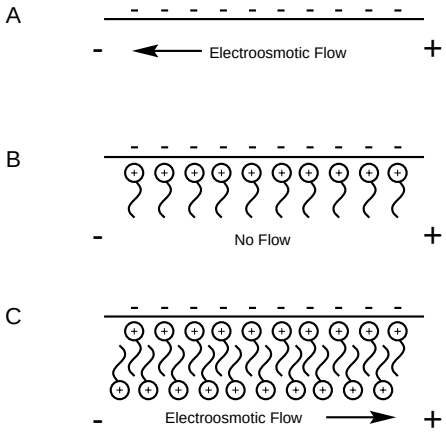


Figure 1-21. *Schematic of the charge reversal process at the capillary wall. (A) No surfactant added. (B) Electrostatic interaction of the positively charged surfactant headgroup to the negatively charged silanol sites of the capillary wall. (C) Admicellar bilayer formation by hydrophobic interaction between the nonpolar chains, resulting in a reversal of the electroosmotic flow. Reproduced with permission from Emmer* et al., J. Chromatogr. 547*, 544 (1991).*

Due to the hydrophobic behavior of the fluorocarbon chain, interaction with proteins is minimized. High-efficiency separations were obtained in a relatively low-ionic-strength buffer (10 mM phosphate, pH 7) as illustrated in Figure 1-22. With this approach, high-efficiency separations at a neutral pH are feasible without creating high current conditions which may adversely affect separation performance.

A similar approach, involving charge reversal through a cationic, polymeric surfactant was investigated by Wiktorowicz and Colburn (1990) and is commercially available as MicroCoat from Applied Biosystems. For dynamic coating of the capillary wall, the additive solution is rinsed through the capillary prior to analysis. Subsequently, excess surfactant is removed by rinsing with run buffer (periodic replenishment is required).

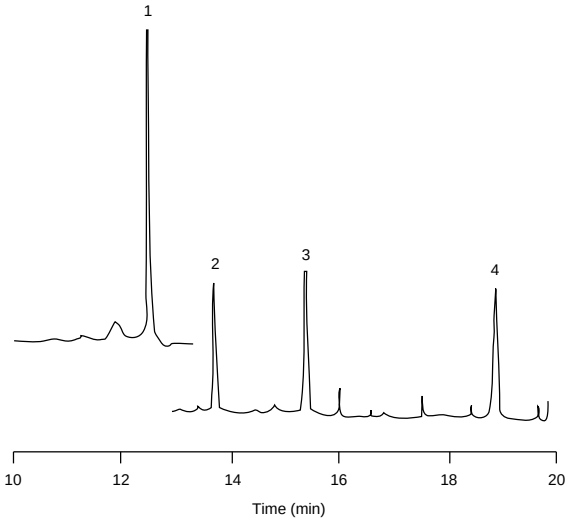


***Figure 1-22**. Electropherogram of four model proteins using the charge reversal system depicted in Figure 1-21. Buffer: 0.01 M phosphate, pH 7.0 with 50 µg/mL FC-134 added. Peak identification: (1) myoglobin (run separately); (2) ribonuclease A; (3) cytochrome C; (4) lysozyme. Reproduced with permission from Emmer* et al., J. Chromatogr. 547, *544 (1991).*

1.4.2.3.6 EOF and/or Adsorption Suppressors

Small amounts of ethylene glycol or cellulose derivatives, *e.g.*, hydroxypropylmethylcellulose (HPMC), have been used by several research groups to significantly increase the resolution of protein separations. From previous studies in isotachophoresis (Everaerts *et al*., 1976), it is known that low concentrations (0.01 to 0.03%) of these additives decrease the zeta potential at the fused-silica wall and may assist (by shielding) in preventing undesirable adsorption. In one CE report (Gordon *et al*., 1991), ethylene glycol was effective by adding it to the sample (as opposed to adding it to the buffer). Higher con-

centrations of these additives (*e.g.*, 0.5% methylcellulose or 10 to 30% ethylene glycol) substantially increase the viscosity of the buffer and cause a molecular sieving effect through the formation of an entangled polymer network (see Section 1.6). Figure 1-23 from Lindner *et al.* (1992) shows the utility of applying 0.03% HPMC to the separation of rat liver core histone proteins. Histones are a class of complex, basic proteins (pI 11 to 12) which show considerable microheterogeneity.

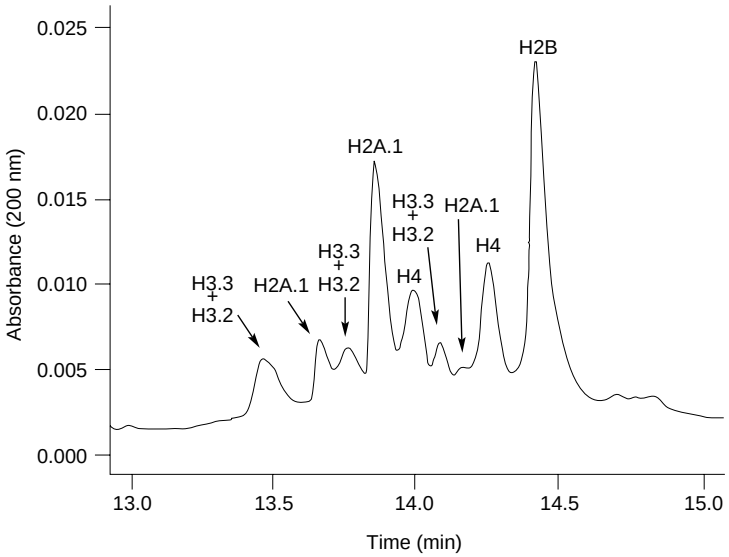


***Figure 1-23**. Separation of rat liver core histones. Buffer: 110 mM phosphate, pH 2.0, with 0.03% HPMC added. Histone peaks are labeled as indicated in the figure. Reproduced with permission from Lindner* et al., J. Biochem. 283, *467-471 (1992).*

1.4.3 Protein Modification: Use of Ionic Surfactants and Urea

1.4.3.1 Ionic Surfactants—MECC Conditions

The anionic surfactant SDS can bind to proteins in a variety of modes. SDS binds to proteins in a constant ratio of 1.4 g detergent per gram of protein, yielding a rod-shaped, SDS–protein micelle complex. All molecules, in theory, possess the same charge-to-mass ratios and can be separated according

to relative molecular weight by SDS-polyacrylamide gel electrophoresis (see Section 1.6). However, differences in SDS saturation levels may occur as a consequence of disulfide bond formation, amino acid composition, and glycosylation. Nolan and Palmieri (1994) exploited this phenomenon in CE for the separation of a glycosylated protein (avidin) and a nonglycosylated protein (streptavidin). In this example (Figure 1-24), SDS was added to both the sample (1%) and the run buffer (0.1%). By contrast, this protein pair could not be resolved in a simple borate buffer. Note that differences in mobility result from different levels of SDS saturation.

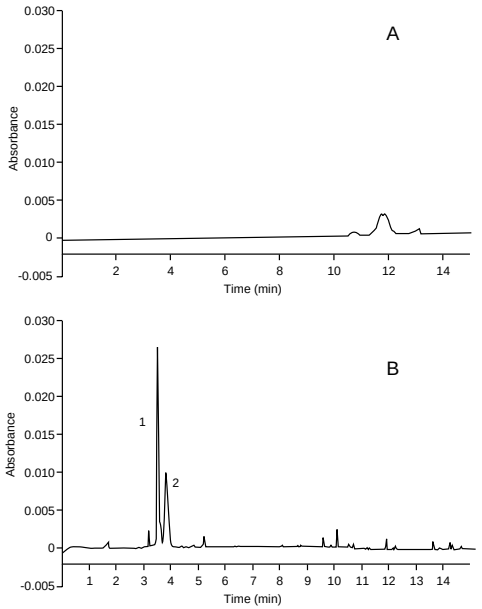


Figure 1-24*. SDS-containing run buffer for the separation of glycosylated and nonglycosylated proteins. Capillary, 50-µm i.d. × 27-cm length; wavelength, 200 nm; proteins, streptavidin (peak 1) and avidin (peak 2) stocks made 1 mg/mL each in 25% phosphate-buffered saline (PBS). (A) Run buffer, 500 mM sodium borate, pH 8.5; sample preparation, diluted 1:3 in water for analysis; voltage, 0- to 10-kV linear ramp over 20 min, normal polarity (toward cathode). (B) Run buffer, 200 mM sodium phosphate, pH 2.0, 0.1% SDS; sample preparation, stocks diluted 1:3 in 1% SDS, 5% 2-mercaptoethanol, 2.5 mM Tris-glycine (pH 8.9), 10% glycerol, and boiled for 5 min; voltage, 10 kV, constant, reverse polarity (toward anode). Reprinted with permission from Palmieri and Nolan,* Handbook of Capillary Electrophoresis, *Landers (Ed.), Boca Raton: CRC Press, 1994.*

SDS can also be used to break up protein–carbohydrate–lipid complexes. This is shown in Figure 1-25 where 50% methanol extracts of wheat grains were separated with 1% SDS (approximately 35 mM) in the borate buffer. In this application, the electrophoretic profile obtained from an extract or complex sample is more important than knowing precisely the identity of all species in that sample. The electropherogram shows that three distinctly different patterns are obtained for the three varieties of wheat. The run buffer employed, rich in acetonitrile (20%), was most effective in the resolution of the many protein peaks resulting from the methanolic extraction of the wheat kernels.

It is interesting to mention that, strictly speaking, the separation mechanism with protein–SDS free-solution electrophoresis is not MECC, even though the SDS concentration in the buffer may exceed the critical micelle concentration (8 mM for SDS). The large SDS–protein complexes do not partition in the micelles as do smaller molecules. However, in the literature, protein separations with SDS buffers are often classified as MECC separations (Vinther *et al*., 1992; Arentoft *et al*., 1993).

Alternatively, SDS may interact with proteins in limited amounts. SDS may bind and ion-pair, bind and modify the net charge, or bind and modify the tertiary structure (or activity). In most cases, these interactions tend to be specific and, depending on the experimental conditions, may or may not alter the expected mobility. Since SDS binds in a reversible manner, it is important to adjust the concentration of this additive in order to maintain equilibrium during separation. Typically, concentrations of 0.5 to 10 mM SDS are used and the optimum level is determined by titration. For example, Harrington *et al.* (1991) used 0.5 mM SDS in the borate run buffer to characterize enzyme–antibody conjugates. Plasma apolipoproteins (HDL, LDL) were characterized by Tadey and Purdy (1993) using a pH 8.3 borate buffer to which 0.1% SDS was added. Strege and Lagu (1993) separated model proteins with coated capillaries in the presence of anionic (SDS, 0.3%) or cationic (CTAC, 0.1%) surfactants. Thus, as shown in these examples, differential SDS binding may be exploited in CE to separate proteins by increasing differences in the charge-to-mass ratio.

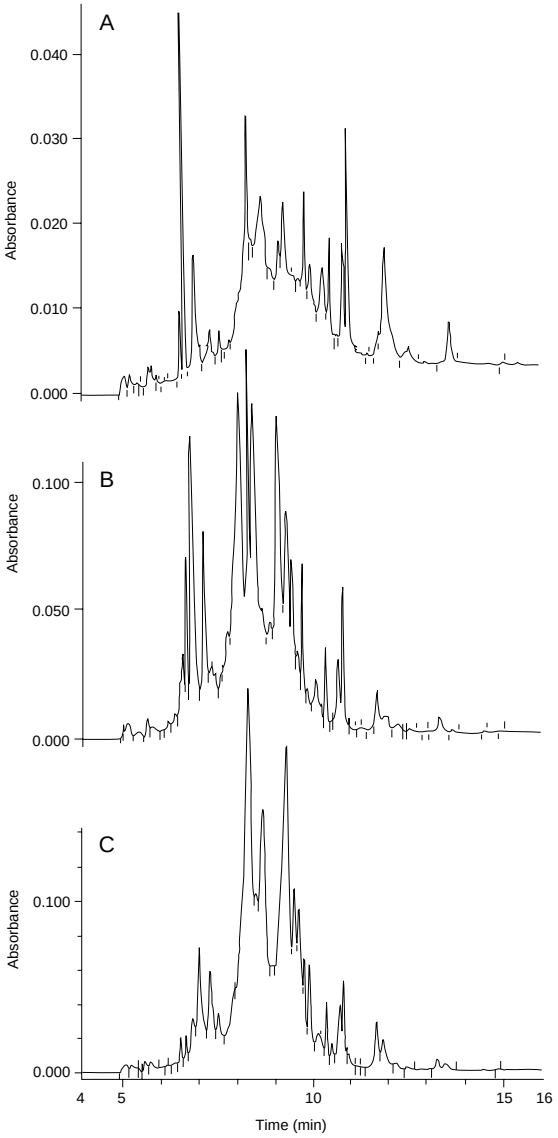


Figure 1-25. *Profiling of wheat varieties. Capillary: 57 (50) cm × 50 μm; 30°C; 200 nm; buffer: 0.06 M sodium borate, pH 9.0, 1% SDS, 20% acetonitrile added. Three wheat varieties are shown: (A) Galahead; (B) Avalone; (C) Mercia.*

1.4.3.2 Denaturants (Urea)

In many cases, urea has been found to be an effective denaturant for electrophoresis. Urea is included in the sample and/or run buffers to reduce conformer formation and prevent aggregation. In relatively high concentration (4 to 8 M), urea prevents aggregation while protein structure is randomized so that a single, symmetrical peak is observed on the electropherogram. Because urea has a significant absorbance in the low UV range, it is recommended to keep the concentration of urea in the run buffer as low as possible to preserve the linearity of the dynamic range of the UV detector. However, it is still possible to add higher concentrations of urea to the sample buffer when the EOF is low because urea is uncharged at run conditions and will not migrate with the component of interest. An example of the use of urea is the work of Josic *et al.* (1990) on hydrophobic membrane proteins. This class of proteins tends to precipitate in aqueous solution. Urea (7 M) was effective in preventing precipitation which led to increased precision in migration times and peak heights. In other work, Strege and Lagu (1993) used a urea-containing buffer to study protein folding.

1.5 Detectability Enhancements: Matrix Effects, Sample Stacking, and ITP Preconcentration

Almost all commercially available CE instruments are equipped with a UV-visible absorbance detector. For most protein and peptide assays, the UV-Vis detector provides more than adequate sensitivity. However, because of limited sample loadability and, furthermore, a short detector pathlength (typically 20-100 μm), concentration sensitivity with CE is limited in trace analysis ($\approx 10^{-6}$ M). Research has been carried out to improve the detection limits in CE. To achieve this goal, basically two strategies have been applied. The first involves a detection scheme other than UV absorbance, *i.e.*, laser-induced fluorescence (LIF). The second strategy makes use of sample preconcentration techniques through suitable chemistries and will be discussed next.

1.5.1 Laser-Induced Fluorescence Detection (LIF)

Unless the natural fluorescence of a compound can be exploited, it is necessary to derivatize the analyte with a suitable fluorescent label. The majority of CE-LIF applications involve pre-capillary labeling with reagents similar to those used with fluorescence detection in HPLC. However, whereas it is relatively easy to derivatize and analyze at high amino acid concentrations, it becomes increasingly difficult at low levels ($< 10^{-7}$ M), as either the labeling efficiency decreases or the background signal increases. Proteins may present additional complications as multiple sites can be tagged; the resulting labeled species are usually electrophoretically separable. An interesting new development is the employment of fluorescently labeled immunochemicals. These probes act as highly selective tagging reagents and may circumvent the problems encountered with labeling of protein analytes at low levels (see the discussion of Figures 2-7 and 2-14 in Part 2.) Full discussion of LIF detection is beyond the scope of this book, but readers are referred to a recent review by Schwartz *et al.* (1994) and chapters in the textbooks by Guzman (1993) and Landers (1994).

1.5.2 Effect of Sample Matrix; Stacking of Sample Components

Samples from biological origin frequently contain significant amounts of salts or buffer ions. When injected into the CE instrument, these "matrix" ions can dramatically influence resolution and detectability. It is not unusual to find that separations performed on standards in relatively clean sample matrices are superior to the separations in more complex environments, *i.e.*, "real" samples. Thus, it appears that the composition of the sample is important with respect to the peak efficiency of the analyte under investigation.

The above phenomenon is related to the relative conductivities of the sample zone and the run buffer. Figure 1-26 shows the effect of the ionic strength of the run buffer on the migration and peak height of a standard mixture of bioactive peptides. The sample components were dissolved in 0.03% TFA; the run buffer was sodium phosphate. In changing from 0.025 M to 0.125 M, an increase in peak efficiency and peak height can be seen.

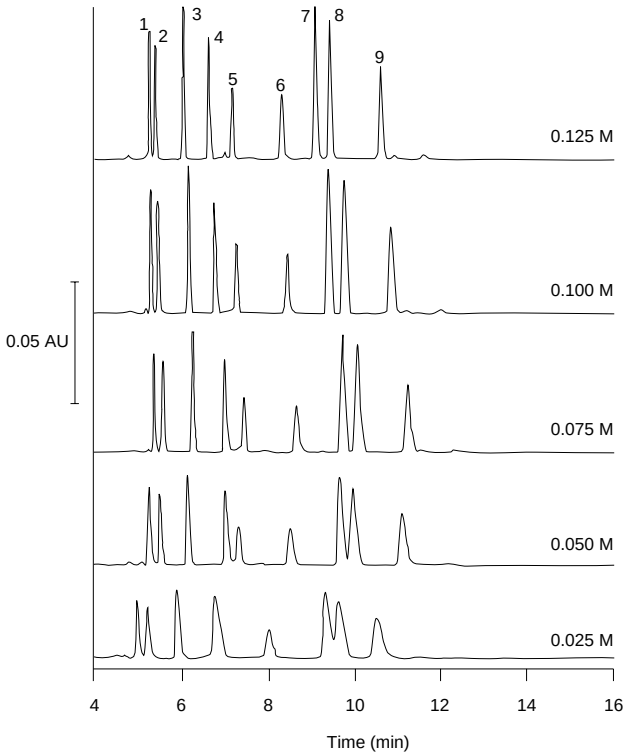


Figure 1-26*. Effect of the buffer ionic strength on peak shape and migration time of peptides. Buffer: sodium phosphate, pH 2.44; 30 kV; 20°C; 200 nm; 57 (50) cm × 75 µm capillary. Peak identification: (1) dynorphin; (2) bradykinin; (3) angiotensin II; (4) TRH; (5) LHRH; (6) bombesin; (7) leu-enkephalin; (8) met-enkephalin; (9) oxytocin. From McLaughlin* et al., Beckman Technical Information Bulletin TIBC-106 *(1991).*

The greater the difference in conductivity between the sample zone and the run buffer, the greater the focusing (also referred to as sample stacking). Under typical conditions, a 5- to 10-fold increase in detection sensitivity can be obtained by using sample stacking. In this case, the electric field in the sample zone is relatively high, causing the analytes to migrate rapidly until they reach the interface between the sample buffer and the run buffer (see Figure 1-27). This causes the sample to be "stacked" at that interface. Thus, the sample should be applied in a medium of relatively low conductivity. If the opposite is the case, uneven migration and zone spreading will result. Thus, for samples

which often contain salts, it is desirable to select a buffer with a relatively high ionic strength. Note: the use of small-i.d. capillaries, *i.e.*, 25 to 50 μm, is favorable in this respect as they permit better heat dissipation; an efficient capillary cooling system is also beneficial. High-ionic-strength buffers are also used in protein separations (see Section 1.4.2.3.1). In addition to zone-focusing effects based on differences in *conductivity*, differences in *pH* between the zones may further contribute to the sharpening of the peaks. Figure 1-28 provides a schematic example of a sample solution which has a pH that is greater than the pI of the peptides injected into a low-pH buffer. The diagram shows that deprotonated, negatively charged peptides stack up against the sample–buffer interface.

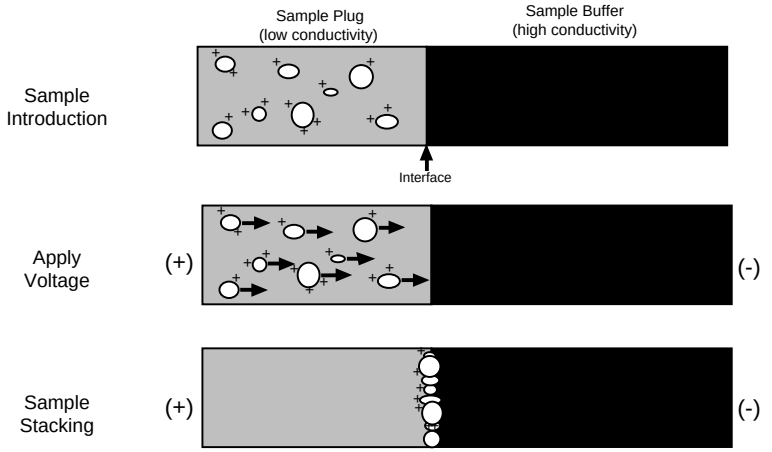


Figure 1-27*. The sample "stacking" mechanism. Sample ions have an enhanced electrophoretic mobility in a lower conductivity environment (*i.e., *elevated local field strength). When a voltage is applied, sample ions in the sample plug instantaneously accelerate toward the adjacent separation buffer zone where, on crossing the boundary, a higher-conductivity environment (lower field strength) causes a decrease in electrophoretic velocity and "stacking" of the sample ions into a buffer zone smaller than the original sample plug. Reprinted with permission from Oda and Landers,* Handbook of Capillary Electrophoresis, *Landers (Ed.), Boca Raton: CRC Press, 1994.*

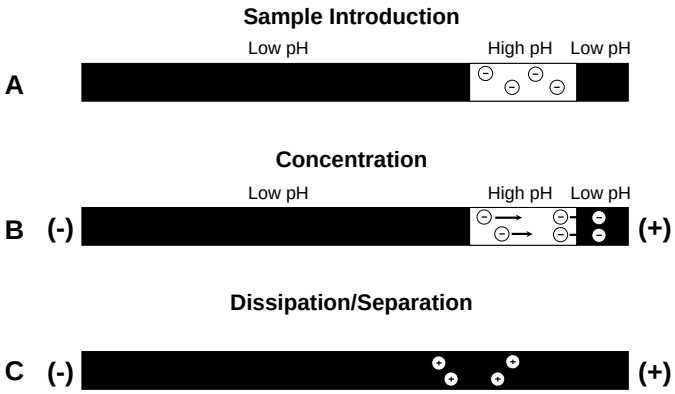


Figure 1-28*. Electrophoretic stacking of peptides. (A) Sample is applied to the capillary in a solution with a pH higher than the pI of the peptides, resulting in negatively charged peptides. (B) Electric field is applied, resulting in stacking of peptides at the buffer/sample zone interface. (C) After dissipation of the pH step gradient, peptides move toward the cathode. Reproduced with permission from Abersold* et al., J. Chromatogr. 516, *79 (1990).*

As discussed earlier, sharper peaks also result in better detectability. For example, significantly lower detection limits were obtained by exploiting zone focusing effects. By dissolving peptides in water or 0.03% TFA and using a pH 2.5 phosphate buffer, a 5- to 10-fold increase in signal can be obtained compared to a sample matrix consisting of run buffer. For the highest sensitivity, the sample injection volume should be maximized (as a rule of thumb, not to exceed 2% of the total capillary volume). The reviews by Chien and Burgi (1992) and Albin *et al.* (1993) give further information on sample concentration techniques.

The effect of salts dissolved in the sample matrix on peak shape is demonstrated in Figure 1-29. This situation is commonly encountered when samples are prepared by ion-exchange or reversed-phase HPLC. In the example in Figure 1-28, three peptides at a concentration of 100 μg/mL were dissolved in the presence of 30 mM and 100 mM NaCl. A 100 mM borate run buffer, pH 9.2, was used. As can be seen, inefficient, broad peaks were obtained with 100 mM salt in the sample. Doubling the injection time from 10 seconds to 20 seconds only marginally increased the peak height. Similar, salt-related, peak-broadening effects also occur under acidic conditions (data not shown here).

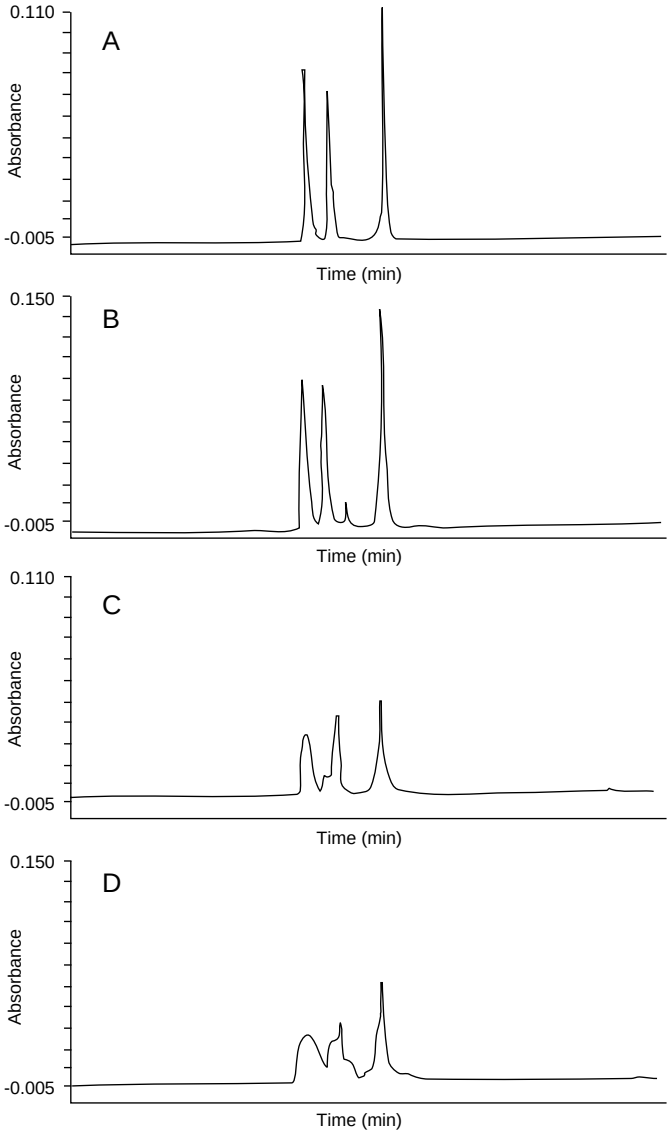


***Figure 1-29**. Effect of salts in the sample zone on peak height and peak shape of bioactive peptides. (A) 30 mM sodium chloride, 10 s injection. (B) 30 mM sodium chloride, 20 s injection. (C) 100 mM sodium chloride, 10 s injection. (D) 100 mM sodium chloride, 20 s injection. Reproduced with permission from Satow* et al., HRC, J. High Resolut. Chromatogr. 14, *276 (1991).*

Because of the effects described above, desalting of the sample may be required. When CE protocols require electrokinetic injection (with gel-filled columns, for instance), desalting is often required as only a relatively small amount of sample is introduced into the capillary otherwise. This occurs because salt ions compete with the analyte ions during the electrophoretic sample introduction process. Protein and nucleic acid samples are frequently desalted using procedures based on ultrafiltration, dialysis, or centrifugation. A micro-concentrating/desalting device, consisting of a small disc with reversed-phase packing material suitable for small peptides, is commercially available. Another device, based on ultrafiltration and centrifugation, is ideally suited for the small sample volumes typically encountered in CE (Amicon, Beverly, MA). Filters with different molecular mass cut-offs are available from the manufacturer.

Finally, it should be mentioned that, under carefully selected sample/run buffer conditions, the addition of salt to the sample matrix may actually benefit zone sharpening. This case (*e.g.*, Beckers and Everaerts, 1990) involves the creation of a temporary isotachophoretic (ITP) zone during the injection process prior to the electrophoretic migration of the species. The use of ITP as a preconcentration technique will be discussed next.

1.5.3 ITP Preconcentration

Isotachophoresis (ITP) or displacement electrophoresis is an electrophoretic technique which can be used as a concentration method for dilute samples. The concentration of the sample is adapted to that of the leading zone according to the Kohlrausch regulating function (dating back to 1897!). In contrast to CZE, where dilution of the sample takes place due to dispersion, ITP is inherently a focusing technique, as is isoelectric focusing. In the literature, different approaches have been described to couple ITP to CZE (*e.g.*, Wanders and Everaerts, 1994; Foret *et al*., 1992 and 1993; Schwer and Lottspeich, 1992). The preconcentration step can be performed either in a dual- or single-column mode. The dual-column mode involves ITP in a pre-capillary with subsequent transfer of the concentrated zone to an analytical capillary where the separation of the individual zones takes place in a CZE mode. While it has been shown that several milliliters of sample can be introduced with this approach, the dual-column system cannot be easily implemented in commercial CE systems. In the single-column configuration, a discontinuous buffer system is employed. ITP takes place only at the beginning of the experiment, and, after a change in conditions, separation in the CZE mode takes over. With this approach, the injection volume, which can be focused and analyzed with on-column ITP preconcentration, is typically 10 to 100 times higher than in normal-mode

CZE: 100 to 1000 nL can be injected into a 75-µm-i.d. capillary. The increase in sample loadability translates directly to an increase in sample detectability. The use of preconcentration appears to be particularly useful when the sample contains not just proteins but also other ions; the latter often is the case with real samples. The following section discusses two methods—one suited for basic proteins, the other for acidic proteins—in which ITP preconcentration is used in P/ACE instrument (Foret *et al*., 1993). The schemes of the two basic electrolyte arrangements for the on-column ITP sample preconcentration used in this study are depicted in Figures 1-30 and 1-32 as Methods A and B.

1.5.3.1 Method A: For Basic Proteins

In this case, the sample is injected into the column filled with a leading electrolyte (LE) as shown in step I of Figure 1-30. Next, the injection end of the capillary is placed in an electrode reservoir containing the terminating electrolyte (TE). Then the voltage is applied, and the sample components having mobilities intermediate to those of the LE and TE stack into sharp ITP zones as shown in step II. In practice, the final concentrations in focused ITP zones are close to the concentration of the leading electrolyte, irrespective of the original sample concentration.

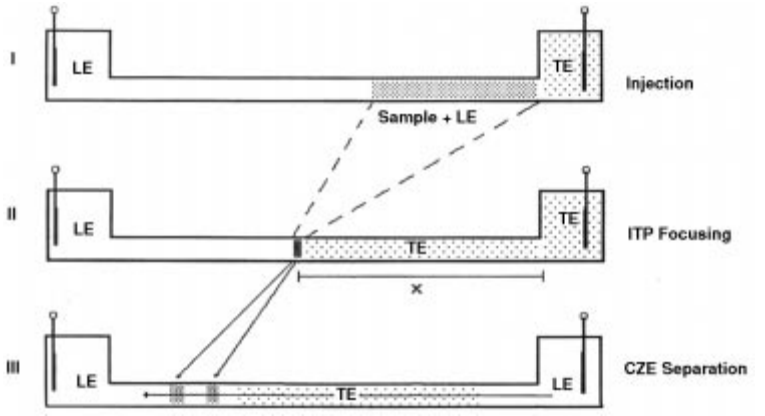


Figure 1-30*. Schematic illustration of transient ITP sample preconcentration in CE. Method A: replacement of the terminating electrolyte. From: Foret* et al.*, Beckman Technical Information Bulletin A-1740 (1993).*

After achieving the ITP steady state, the buffer reservoir containing the terminating electrolyte is replaced by a reservoir containing the leading electrolyte, resulting in destacking of ITP zones, shown in step III. At this point, individual species begin to move in a zone-electrophoretic mode. The separation of basic proteins according to method A is shown in Figure 1-31. The sample volume was approximately 450 nL. After 4 minutes of ITP migration, the anode electrode reservoir (on the injection side of the capillary) was replaced by a buffer reservoir containing leading electrolyte. As can be observed in Figure 1-31, excellent peak shape and resolution of proteins is obtained within a short run time.

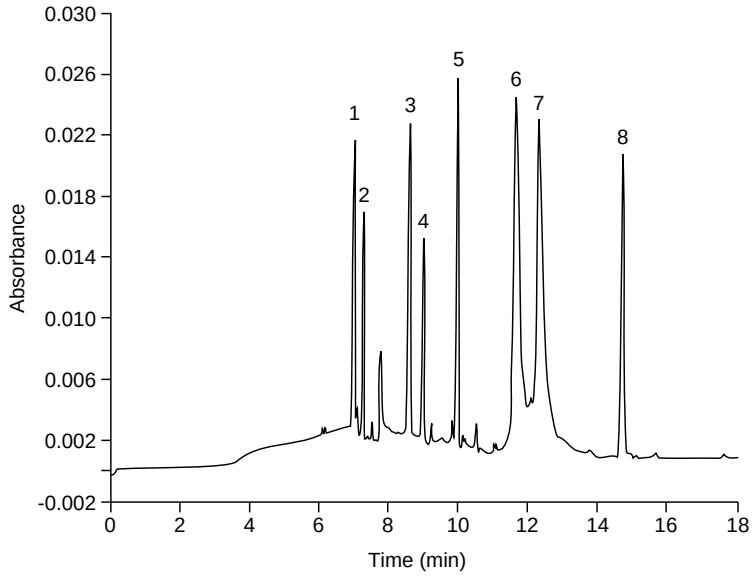


Figure 1-31. *Cationic CZE separation of basic proteins with ITP sample preconcentration. BGE = leading electrolyte: 0.02 M triethylamine + acetic acid, pH 4.3; Terminating electrolyte: 0.01 M HAc, applied for 4 min. Capillary: 50 cm × 75 mm, coated. Constant current mode: 15 mA, 17-28 kV. Sample: 1) lysozyme, 6.3 × 10^{-7} M; 2) cytochrome C, 3.1 × 10^{-7} M; 3) ribonuclease A, 4.7 × 10^{-8} M, 4) myoglobin 5.3 × 10^{-7} M; 5) α-chymotrypsinogen, 1.6 × 10^{-7} M; 6) β-lactoglobulin A, 3.1 × 10^{-7} M; 7) β-lactoglobulin B, 3.1 × 10^{-7} M; 8) carbonic anhydrase, 1.6 × 10^{-7} M. Analytes dissolved in BGE. Injected volume: 450 nL. From Foret* et al., Beckman Technical Information Bulletin A-1740 *(1993).*

1.5.3.2 Method B: For Acidic Proteins

Method B consists of a single electrolyte (the "background" electrolyte—BGE) used for both ITP preconcentration and CZE separation (Figure 1-32). The BGE acts as a terminating electrolyte when the sample itself contains ions with high electrophoretic mobility which can serve as a leading zone during transient ITP migration. Biological samples usually contain such ions (*e.g.*, sodium, potassium, or ammonium ions in the form of chlorides, sulfates, or phosphates); however, partial desalting may still be desirable in cases when the salt concentration is high. The best results will be obtained with the salt concentration in the range 5×10^{-3} to 5×10^{-2} M. At higher salt concentrations, longer capillaries must be used for completion of the separation. After sample injection and application of the electric current, the leading ions from the sample (having higher mobility) form a zone with an asymmetric leading and sharp rear boundary, resulting in a non-uniform electric field; consequently, ITP stacking of the sample ions is achieved. During the migration, the leading zone broadens due to electromigration dispersion and its concentration decreases. At a certain concentration of the leading zone, the sample bands destack and move with independent velocities in the zone-electrophoretic mode.

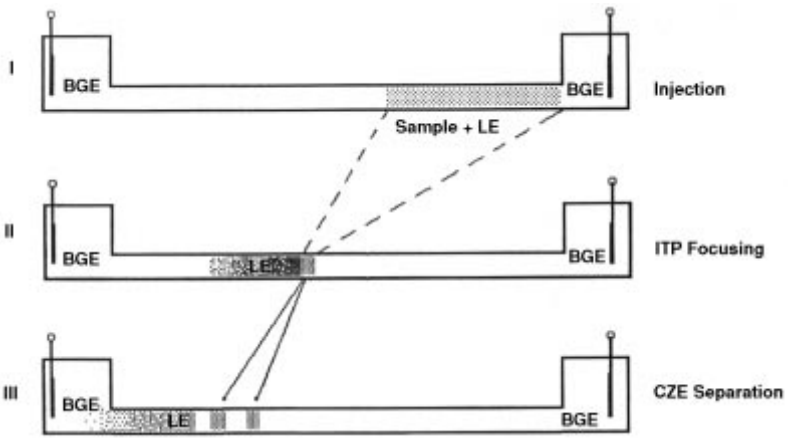


***Figure 1-32**. Schematic illustration of transient ITP sample preconcentration in CE. Method B: sample contains leading ions. LE = leading electrolyte, TE = terminating electrolyte. From Foret* et al., Beckman Technical Information Bulletin A-1740 *(1993).*

Since zones with different conductivities may develop during the ITP step, it is convenient to work at constant current. This will keep electric field strength in the BGE constant and, thus, also maintain constant velocity of protein zones during the detection, assuring good reproducibility and quantitation. The use of transient ITP for preconcentration of acidic proteins according to method B is shown in Figure 1-33. In this case, 1 µL of the sample was injected. The protein zones preconcentrated behind the zone of chloride, thus forming a transient leading electrolyte during the early stages of the migration.

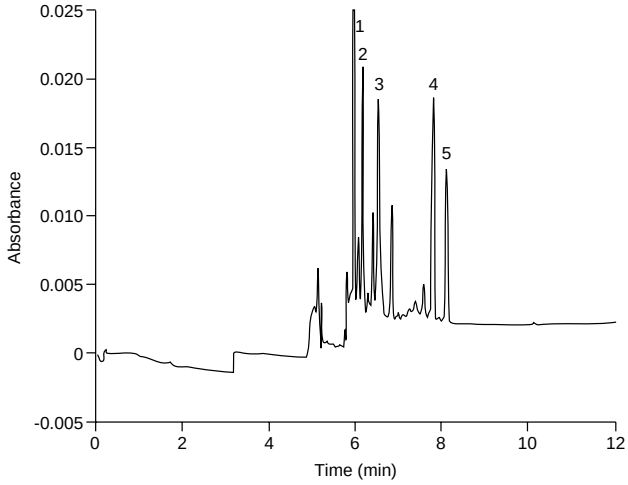


Figure 1-33*. Anionic CZE separation of 1 mL of the sample of acidic proteins dissolved in 5 mM Tris-HCl. Sample: 1) glucose-6-phosphate dehydrogenase, 1.25×10^{-8} M; 2) trypsin inhibitor, 1.6×10^{-7} M; 3) β-lactoglobulin B, 1.2×10^{-7} M, 4) L-asparaginase, 1.9×10^{-8} M; 5) α-lactalbumin, 4.4×10^{-8} M. BGE: 0.02 M TAPS-TRIS, pH 8.3. Capillary: 40 cm × 75 µmm, coated. Constant current mode: 7 mA, 22 kV. From Foret* et al., Beckman Application Information Bulletin A-1740 *(1993).*

The above examples have shown that transient ITP preconcentration can easily be adopted with the P/ACE instrument. The methods are reproducible with typical RSDs of migration times ≈ 0.5 to 1%. Sample volumes up to 1 µL can be effectively preconcentrated in a 75-µm-i.d. coated capillary. Consequently, detection limits of proteins can be substantially improved (detection limits below 10^{-8} M or 0.1 µg/mL are typical).

1.6 SDS Capillary Gel Electrophoresis (SDS-CGE)

Gels such as those made of agarose and cross-linked polyacrylamide (PA) were originally used as an anticonvective medium in slab gel electrophoresis. The gel structure creates a molecular sieving effect, allowing separations to be performed based on the size of the molecules. The technique of SDS-PAGE is the most popular among all electrophoretic techniques. SDS binds to polypeptide chains such that, in theory, similar charge densities and constant mass-to-charge ratios of different proteins are obtained. As a result of the presence of the gel, separation then takes place based solely on the basis of the size of the proteins. SDS-PAGE is frequently used to analyze the purity of peptides and proteins and also to determine apparent molecular weight (MW) by means of a comparison with calibration standards. Earlier approaches of SDS-PAGE in capillaries were described by Cohen and Karger (1987), Widhalm *et al*. (1991), and Tsuji (1991).

Beckman recently developed a new system for the SDS-CGE of proteins. A proprietary, linear polymer formulation (UV transparent at 214 nm) is used in conjunction with 100-µm-i.d. coated capillaries. In CE, UV-transparent polymers such as dextran or polyethylene glycol are preferred over polyacrylamide as a sieving medium (Ganzler *et al*., 1992; Guttman *et al*., 1992, 1993; Lausch *et al*., 1993). Samples can be introduced by means of pressure injection while, after completion of the run, the gel matrix can be replaced by using the pressure-rinsing feature of the P/ACE instrument. With an "in-between-runs" rinsing procedure, excellent reproducibility can be achieved for proteins of up to ≈ 200,000 Daltons (Tsuji, 1993).

Figure 1-34 shows the separation of seven protein standards (size range from 14,200 to 205,000 Daltons) in approximately 15 minutes on a 27-cm-length capillary. A semilog plot of the MW of the standards versus the inverse of the relative migration time (RMT) exhibits excellent linearity (Figure 1-35). With the use of Gold™ Molecular Weight software, estimation of the MW of unknown proteins is easily obtained.

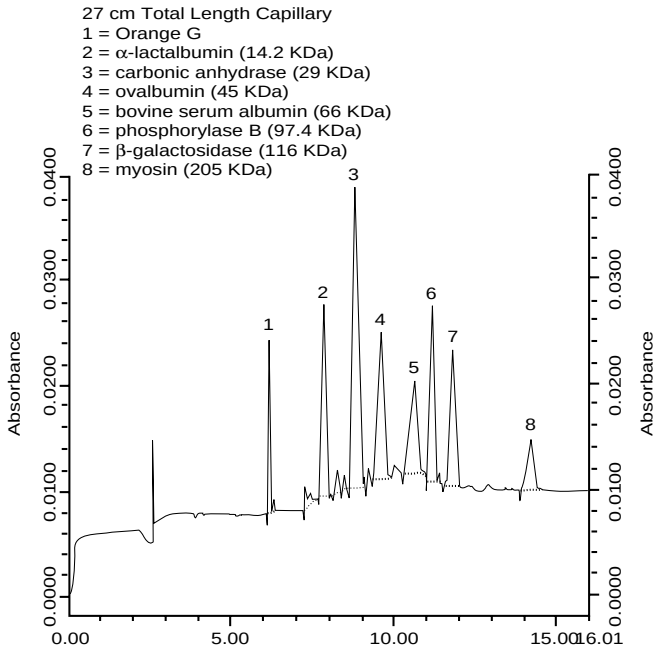


Figure 1-34. *Standard test mix separation on an eCAP-SDS 14-200 capillary*

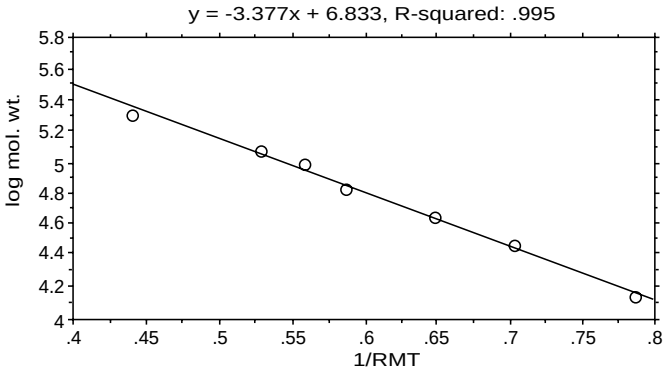


Figure 1-35*. Molecular weight standard curve*

For proteins which do *not* bind to SDS in the 1 to 1.4 ratio (*e.g.*, glycoproteins), generally inaccurate estimates are obtained. In this case, a Ferguson plot can be constructed to account for this nonideal behavior. First, the RMTs of standards at different gel concentrations are measured. A log (1/RMT) vs. the gel buffer concentration plot yields a retardation coefficient, K_R, for each of the standard proteins (Figure 1-36). Next, a plot of log MW vs. the square root of K_R can be used for estimation of MWs (Figure 1-37). The Ferguson method is a rather time-consuming and labor-intensive method when used with SDS-PAGE. However, using CE, this method is easy to perform in an automated fashion (for further details see the Beckman eCAP SDS 14-200 Kit, P/N 477420, and a recent publication by Werner *et al.* (1993).

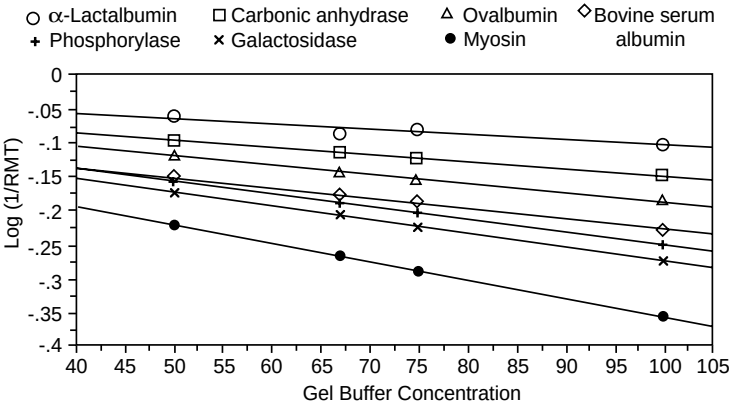


***Figure 1-36**. Ferguson plot of seven standard proteins*

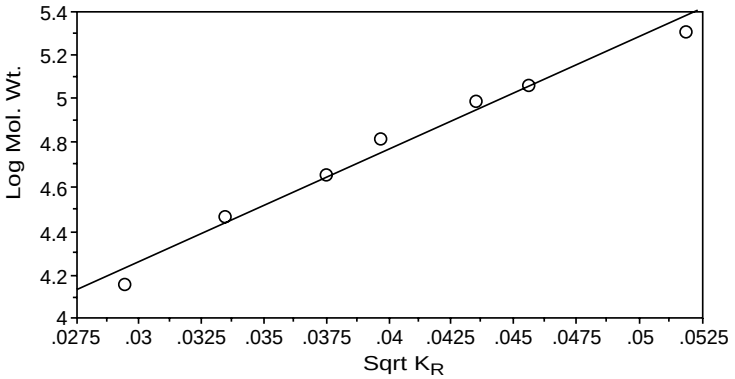


***Figure 1-37.** K_R plot used to determine molecular weights of proteins*

While SDS-CGE is in many respects very similar to SDS-PAGE on slab gels (*e.g.*, sample preparation), a number of distinct advantages can be noted:

- CE analyses are fast, reproducible and quantitative.
- In SDS-CGE, the gel can be replaced at least 100 times.
- Direct, on-line UV detection is feasible.
- There is no need to stain or de-stain the proteins. It is known that, with slab gels, the staining reagent Coumassie Blue does not bind to proteins in a stoichiometric ratio; therefore quantitation with slab gels is not always reliable.

1.7 Capillary Isoelectric Focusing (CIEF)

1.7.1 Principle of IEF

In isoelectric focusing (IEF), amphoteric molecules such as proteins are separated by electrophoresis in a pH gradient generated between the cathode and anode. The technique takes advantage of the fact that each protein has a different pH at which it is electrically neutral; its isoelectric point (pI). Briefly, the principle of IEF separation is as follows. Under the influence of an electric field, charged species will start to migrate through the electrophoresis medium (gel or solution). If the sample component has a net *negative* charge, migration is toward the anode. During the migration, the sample encounters progressively lower pH, thus picking up more positive charge. Eventually, a zone is reached where the net charge is zero. At this point (at the pI), migration stops and the sample component is focused in a tight zone. Likewise, if a component has a *positive* charge, migration will be toward the cathode. Thus, each sample component migrates to its own isoelectric point. In classical IEF techniques, the separated zones are usually visualized by means of staining. IEF is a true electrophoretic *focusing* technique, as is isotachophoresis, *i.e.*, the separated zones are s*elf-sharpened* during the electrophoretic separation. Protein molecules diffusing out of a focused zone will acquire a charge and are pulled back into the center of the zone where the net charge is zero. IEF is most often applied to proteins (including enzyme isoforms, polyclonal, and monoclonal antibodies, hemoglobin variants, and r-DNA-made proteins), although peptides, whole cells and subcellular particles, viruses, and bacteria also have been studied by IEF. The technique is particularly useful to estimate the pI of an unknown protein through calibration with known protein standards. Many applications of IEF in the biomedical/clinical fields have been reported (Righetti, 1983).

1.7.2 Classical IEF

In IEF, the pH gradient, which is key to the success of the technique, is provided by molecules called "carrier ampholytes." In the mid 1960's, the Swedish researchers Svensson and Vesterberg were able to synthesize molecules with the exact properties needed for IEF (*e.g.*, good conductivity at their isoelectric points and good buffering capacity). The ampholytes consist of polyamino-polycarboxylic acids with slightly different pI values; their average molecular mass is approximately 750 Daltons. Ampholyte mixtures in wide and narrow pH ranges can be purchased from various commercial sources. Typically, polyacrylamide or agarose gels are used as anticonvective media, similar to types of slab gel electrophoresis. It is also possible to *immobilize* pH gradients on a suitable matrix such as polyacrylamide. In this case, the buffering groups of the pH gradient are acrylamide derivatives which are copolymerized into the gel matrix. This IEF technique with immobilized pH gradients was introduced in 1982 by Bjellqvist, Righetti, and co-workers. IEF yields typically higher resolution than do other modes of electrophoresis. With immobilized pH gradients, proteins differing by 0.001 pI unit have been separated. Routinely, IEF provides resolution of 0.1 to 0.01 pI units. For even higher resolution, IEF can also be combined with other modes of electrophoresis, *e.g.*, with SDS-PAGE or immunoelectrophoresis ("two-dimensional electrophoresis").

1.7.3 Capillary Isoelectric Focusing (CIEF)

CIEF offers the potential to combine the high resolving power of conventional gel IEF with the advantages of modern CE instrumentation. Small-diameter capillaries with efficient dissipation of Joule heat permit the use of relatively high field strengths for rapid separations. Thus far, all CIEF separations have been carried out in *free solution* (*i.e.*, without a gel, as in classical IEF). In CIEF, as in classical IEF, proteins are separated in a pH gradient created by ampholytes under the influence of an electric field. As will be discussed below, the key to high performance is to effectively displace the protein zones out of the capillary without introducing band broadening. Direct, on-line UV detection is feasible, without the requirement for staining of the focused protein zones (most often, detection is at 280 nm as the ampholytes absorb in the low UV region). With the P/ACE system, CIEF can be performed automatically, allowing unattended analysis of multiple samples.

1.7.3.1 Two-Step CIEF Methods: Focusing and Mobilization

Hjerten and Zhu (1985) were the first to develop IEF in capillaries. A two-step process was performed: first, proteins were focused in the capillary. After completion of this process (monitored by a drop-off in current), displacement ("mobilization") of the zones out of the capillary took place by means of changing the chemical composition of anolyte or catholyte solution (*i.e.*, by adding acid, base, or salt). In this method, termed "chemical mobilization," the change in anolyte or catholyte causes a shift in the pH gradient, resulting in migration of the zones past the detection point in the capillary. CIEF with chemical mobilization was further optimized and applied to a variety of substances including monoclonal antibodies (Wehr *et al*., 1990), hemoglobin variants (Zhu *et al*., 1992), human serum transferrin (Kilar and Hjerten, 1989), and glycoforms of recombinant proteins (Yim, 1991). The method involves the use of coated capillaries to eliminate the electroosmotic flow (EOF) and to prevent undesirable adsorption of proteins to active sites on the capillary wall.

Instead of the above-described chemical mobilization, focused zones can also be moved past the detection point by hydrodynamic means. In their early experiments, Hjerten and Zhu (1985) used a low-flow pump to displace the capillary contents while leaving the voltage on to maintain resolution of the focused protein zones. Recently, the principle of this method was implemented in a modern CE instrument by Chen and Wiktorowicz (1992) and Nolan (1993), using vacuum and pressure mobilization, respectively. The former workers demonstrated excellent linearity of a pI vs. mobility plot in the pH range of 2.75 to 9.5. Figure 1-38 shows the principle of the method employed by Nolan. A plug of sample is introduced into a 27 cm × 50 mm capillary. The catholyte containing 20 mM sodium hydroxide is backflushed just past the detection point. The base acts as a "blocking agent," *i.e.*, focusing of basic proteins will not extend beyond the detection window which would prevent their detection. After the focusing step (completed in approximately 3 minutes), a low-pressure rinse is applied, moving the focused zones past the detection point (Figure 1-38B). During this step, the electric field is turned on to maintain the sharpness of the bands. This CIEF system yielded sharply focused protein peaks and was found to be reproducible (RSDs for migration times < 0.6%). Calculated pI values of proteins correlated well with reported pIs.

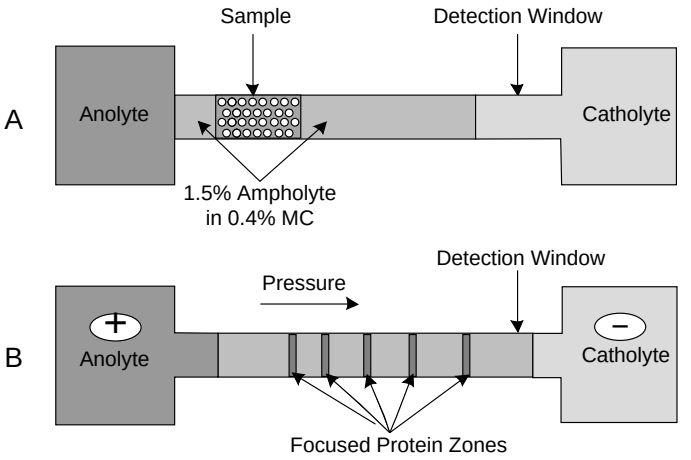


Figure 1-38. *Principle of CIEF by simultaneous pressure/voltage mobilization. A) Catholyte is backflushed past the detection point and a sample plug is introduced into the coated capillary (no high voltage). B) Focusing of sample is complete and the sample components are driven toward the detector by a low-pressure rinse. High voltage is applied during this step.*

1.7.3.2 One-Step CIEF Methods: Simultaneous Focusing and Mobilization

The need for a simple, reproducible method has led to a one-step CIEF method which involves utilizing the EOF for mobilization of the focused zones (Mazzeo *et al*., 1992; Thormann *et al*., 1992; Yao and Regnier, 1993; Pritchett, 1994). In one variant, Thormann *et al.* (1992) introduced a large plug of sample (approximately 6% of the capillary volume) dissolved in an ampholyte solution into a 90-cm-length, untreated capillary which was filled with catholyte (20 mM sodium hydroxide, 0.3% hydroxypropylmethylcellulose). After placing the capillary between the catholyte and the anolyte (10 mM phosphoric acid), the electric field was turned on, causing the formation of a pH gradient and focusing of the protein zones; simultaneously, the sample was swept towards the detection point by the EOF.

The method of Mazzeo and Krull (1992) is different from the above-mentioned method of Thormann in that, initially, the *entire* capillary is filled with sample and ampholytes. Good results were achieved with commercially

available (Supelco) C8 coated capillaries. Yowell, *et al*. (1993) used this one-step CIEF method for the QC analysis of recombinant protein formulations. Pritchett (1994) applied the methods to the qualitative and quantitative analysis of monoclonal antibodies. The principle of their method is schematically shown in Figure 1-39. After filling the capillary with the sample mixture (which contained the basic "blocking agent," TEMED), the capillary was placed between the inlet (catholyte, 20 mM sodium hydroxide) and outlet (anolyte, 10 mM phosphoric acid). Subsequently, the voltage was turned on, focusing the protein zones in the *short* (7 cm) end of the capillary (note: the TEMED blocks the long (40 cm) end of the capillary). Simultaneously, the EOF drives the zones past the detection point in the *reverse* direction.

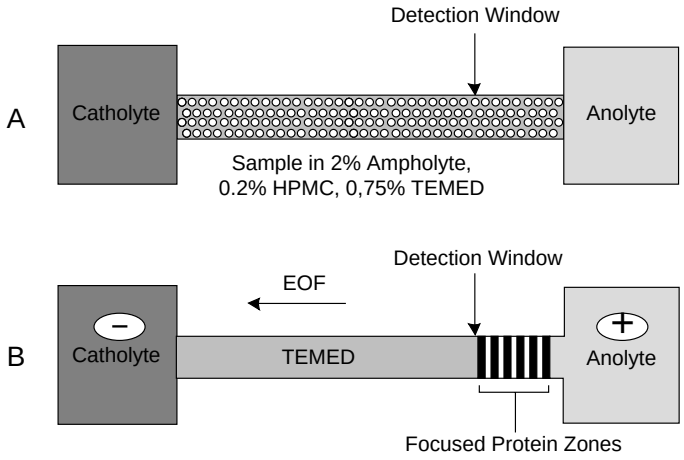


Figure 1-39. *Principle of the one-step CIEF method with EOF mobilization. (A) Coated capillary is filled with sample. (B) Under high voltage, proteins are focused in the short end of the capillary and mobilized past the detection point by the EOF.*

CIEF has the potential to replace many of the established, classical IEF procedures. CIEF has advantages over classical IEF with slab gels with regard to time savings, sample preparation, quantitation, and reproducibility. In addition, no staining or destaining of the bands needs to be performed as the detection takes place directly on the capillary, in *real time*.

1.8 Micropreparative CE

Because CE involves separations in small-i.d. capillaries, the technique has found limited use as a micropreparative tool. In comparison with HPLC, CE is *a priori* more limited with respect to the maximum sample load (*i.e.*, the amount of sample which can be loaded onto the capillary without causing excessive zone broadening). Consequently, the amounts of material collected from capillaries are relatively small, which constrains further manipulation and analysis. However, a growing number of publications have recently indicated that, under proper conditions, CE can be a useful tool for protein chemists. In 1988, Cohen *et al.* have shown the utility of micropreparative CE for the purification of oligonucleotides (800 ng of a primer was collected and subsequently used as a probe) and peptides. While gel-filled capillaries were used in this work, CE in the free-solution mode can also be utilized for micropreparative work. Table 1-4 lists a number of applications in which CE has been used in the micropreparative mode.

Table 1-4. Micropreparative CE Applications

- Peptide purification from digests prior to microsequencing
- Amino acid compositional analysis after hydrolysis
- Slab gel electrophoresis (radioactivity labeling)
- Identification of fractions by mass spectrometry
- Enzymatic activity analysis of fractions collected by HPLC or CZE

Fraction collection with CE is different from HPLC in that the capillary must stay in contact with a solution containing water or buffer and the electrode *(i.e.*, the electric field, which drives the separation, must be maintained). This is probably the most commonly used way to collect fractions from CE runs. Alternatively, the fraction to be collected can be mobilized by utilizing the low-pressure (0.5 psi) rinsing capability of the P/ACE system (Gagnon, 1991). Yet another possibility is to connect the capillary to a syringe pump for a "dynamic" elution after switching off the field (as was done by Camilleri *et al., 1991*). The latter approach was applied successfully for sequencing peptides at the low-picomole level.

Basically, three strategies are conceivable for micropreparative CE: collection from a single run with standard capillaries, collection by performing multiple runs, and collection from large-diameter capillaries.

1.8.1 Collection from a Single Run with Standard Capillaries

When enough analyte is present in the sample or when the sample can be preconcentrated, a *one-time* collection can be performed on a standard capillary of 50 to 75 μm i.d. Typically, the initial concentration of peptide required is from 1 to 10 mg/mL to allow further analysis by spectroscopic or chemical means. Banke *et al.* (1991) have shown that, when enzymatic amplification is applied, even minor components of a fermentation broth can be analyzed by means of collection from 75-μm-i.d. capillaries. In this application, quantities as small as 3 ng could be identified as an alkaline protease of the subtilisin family.

1.8.2 Collection by Performing Multiple Runs

To increase the amount of collectable material, *multiple* runs can be performed with the *same* collection vial. The concentration of the targeted analyte should increase in proportion to the number of runs. A prerequisite is that adequate run-to-run reproducibility has been obtained. More detailed information on programming the CE instrument for micropreparative work can be found in Beckman *Technical Information Bulletin TIBC-105* (Biehler and Schwartz, 1991).

1.8.3 Collection from Large-Diameter Capillaries

The loadability on a capillary is roughly proportional to the square of its diameter. Thus, for example, seven times the sample load can be collected using a 200-μm-i.d. capillary than when using a 75-μm-i.d. capillary. However, the penalty paid for increasing the diameter is increased Joule heat generation. Ultimately, this is the limiting factor in the separation performance. A good capillary cooling system for heat dissipation decreases this problem.

The feasibility of this approach (Smith and Ohms, 1992) is illustrated in Figure 1-40 with the collection of peptides from a tryptic digest (β-lactoglobulin). The sample load on the micropreparative capillary was approximately 100 pmol of digest and a 50 mM sodium phosphate run buffer, pH 2.7, was used. It can be seen that the integrity of the individual peaks is largely maintained when scaling up from the analytical (75 μm i.d.) to the micropreparative capillary (200 μm i.d.). However, the analysis time is much longer because a lower voltage was applied to prevent excessive Joule heating. After collection, various fractions were subjected to microsequence analysis. The results of the sequence analysis are shown in Table 1-5. Recoveries of the peptides ranged from 10 to 75%.

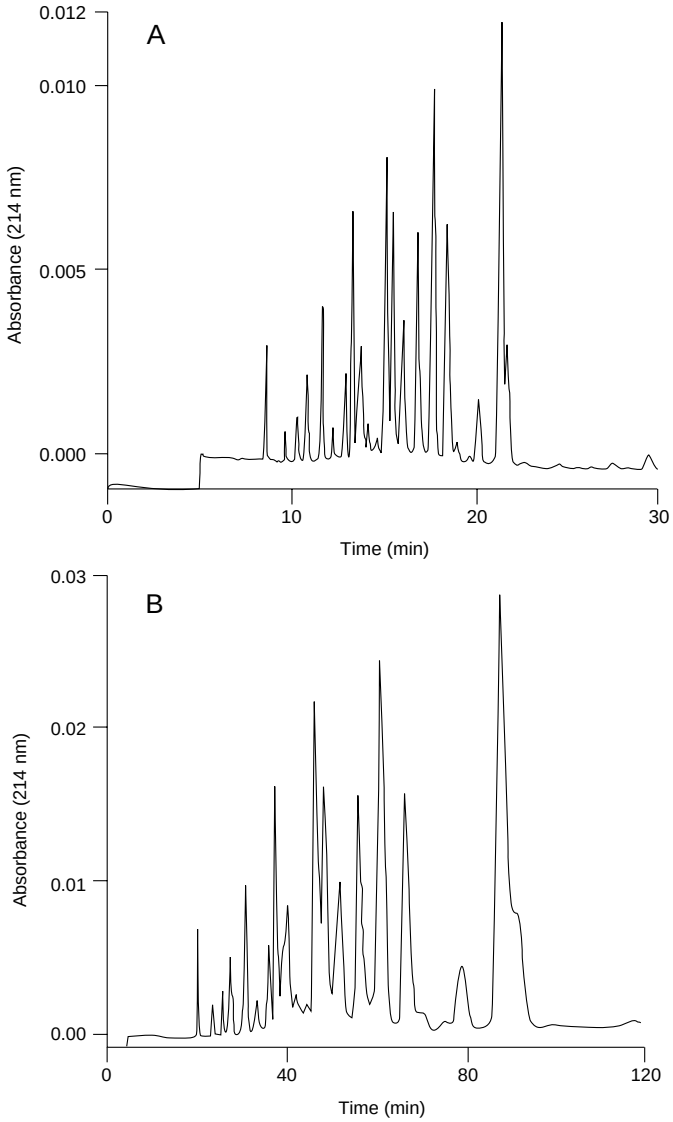


Figure 1-40*. Comparison of analytical and micropreparative CE of a β-lactoglobulin tryptic digest. (A) analytical CE with a 75-µm-i.d capillary. (B) micropreparative CE with a 200-µm-i.d. capillary. See text for details. From Smith and Ohms,* Techniques in Protein Chemistry III *(1992).*

*Table 1-5. Recovery of Peptide from β-Lactoglobulin Tryptic Digest**

Frac No.	Amino Acid Sequence	Yield (pmol)	Recovery %	Purity %
8	TK	70	70	> 90
11	ALK	35	35	50
	TMK	35	35	50
13	LQK	50	50	80
15	FPK	40	40	> 90
18	VYEELKPTPEGDDLEILLQK	25	25	> 90
21	TPEVDDEALEK	40	40	75
28	SLAMAASDISLLD(AQSAPLR) VAGT(W)Y	35	35	65

* Data from Smith and Ohms, *Techniques in Protein Chemistry III*, 1992.

In a later paper, Kenny *et al.* (1993) used 150-μm-i.d. capillaries for fraction collection prior to microsequencing at the low picomole level. A comparison with HPLC was made. The results indicate that, for the peptides selected, the recovery with CE was superior to that with HPLC at load levels below 50 picomoles. With CE, recoveries in the 60 to 70% range at the 5- to 10-picomole level were routinely obtained, whereas with HPLC yields were less than 10% at the 50-picomole level.

1.9 Affinity Capillary Electrophoresis (ACE)

Affinity electrophoresis is a technique which has been used for the characterization of biomolecules and for the analysis of specific interactions with of biomolecules with affinity ligands, including antibody–antigen interactions (for a review, see Takeo, 1987). When CE is used to study receptor–ligand interactions, the technique is referred to as affinity capillary electrophoresis (ACE). While still in its infancy, it is clear that ACE is rapidly becoming an important tool for the bioanalytical/clinical researcher. At the HPCE 1994 meeting in San Diego, a complete session was devoted to applications in this field.

1.9.1 Receptor-Ligand Studies

The binding of a ligand (protein, peptide, small molecule) to a receptor (typically a protein or peptide) is schematically shown in Figure 1-41.

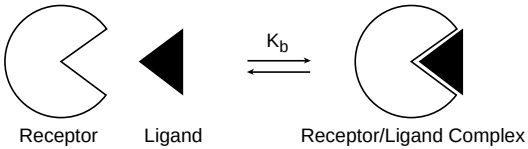


Figure 1-41. *Binding of a ligand to a receptor*

The protein–ligand complex has different charge-to-mass characteristics than the unbound protein which permits its separation by CE. By measuring the migration time as a function of the concentration of the ligand present in the CE run buffer, the equilibrium constant K_b can be measured (Chu *et al.*, 1992). Traditionally, such affinity measurements have been performed with techniques such as equilibrium dialysis or spectroscopy. However, these methods are often labor intensive and require labeling or depend on secondary reagents for quantitation. ACE has several potential advantages:

- Only a small amount of material is required.
- The receptor need not be highly purified and its concentration does not need to be known.
- ACE allows, in principle, simultaneous determination of several ligands.
- ACE has short run times and is precise and quantitative.

Interactions between receptors and ligands have been studied by a number of workers and various CE-based reaction schemes have been devised. For example, Kajiwara (1991) used ACE for the analysis of conformation and interaction of metal-binding proteins such as calmodulin and parvalbumin (calcium-binding) and carbonic anhydrase (zinc-binding). Using EGTA as a Ca^{2+}-chelating agent in the run buffer, the binding shift of soluble calcium binding proteins was investigated by Huch and D'Haese (1993). In the case of calmodulin, binding of Ca^{2+} resulted in a change in protein conformation and subsequent interaction with various target enzymes. The electrophoretic change upon adding the chelating agent for parvalbumin is shown in Figure 1-42. Whereas in the presence of Ca^{2+} a single protein peak is obtained (panel A), adding EGTA to the buffer results in a double peak and shift toward longer migration times (panel B). Potential applications of this assay include the diagnosis of pathological alterations of skeletal muscle.

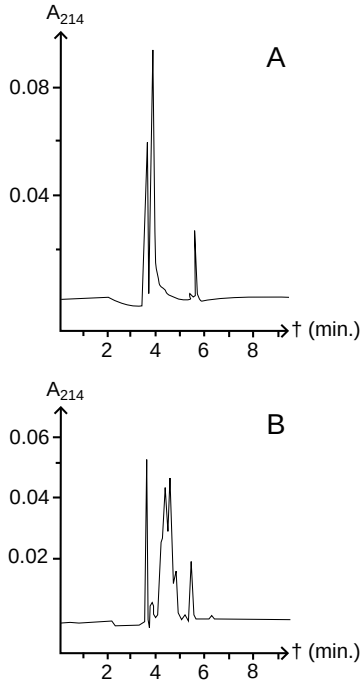


Figure 1-42. *CE profiles of parvalbumin from white skeletal muscle of the chub (Leuciscus cephalus) in the presence of 0.1 mmol L^{-1} Ca^{++} (A) or 10 mmol L^{-1} EGTA (B) Voltage: 20 kV (Current 1: 10 µA). Temperature: 30°C. Injected amount of protein: 4 ng in 2 nL (≈ 0.3 pmol). From Huch and D'Haese,* Discovery, *Spring 1993, Beckman Instruments, Inc.*

In other recent applications of ACE, Honda *et al.* (1992) determined the association constant of monovalent-mode protein–sugar interaction. Heegaard and Robey (1992) used CE to evaluate the binding of anionic sugars to synthetic peptides. Chu *et al.* (1992), as well as Biehler and Jacobs (1993), applied ACE to study molecular recognition with low-molecular-weight receptors. Kraak *et al.* (1992) tested three different reaction schemes for determination of binding constants resulting from protein–drug interactions. Frontal analysis gave the best results for a bovine serum albumin–warfarin model system. Finally, Kuhn *et al.* (1994) used ACE for determination of lectin–sugar interactions.

1.9.2 Antibody-Antigen Interactions

Immune complexes can also be studied by ACE. Nielsen *et al*. (1991) investigated MAb-antigen complexation with hGH as the antigen. Immunoassays based on ACE and laser-induced fluorescence (LIF) detection have also been described recently. An example of this approach—digoxin assay in serum—will be discussed later in Part 2. LIF detection, in contrast to UV detection, permits the (ultra-) low detection of labeled analytes required for immunoassays. P/ACE can be coupled to various laser light sources, permitting optimum selectivity and sensitivity. [A recent review (Schwartz *et al*., 1994) describes various applications of the P/ACE-LIF detector, including those of proteins, peptides and amino acids.] Immunoassays are based on the specific chemical reaction between an antibody and its corresponding antigen. Quantitation involves the separation and detection of antibody–bound antigen from the free antigen or antibody, depending upon the analytical scheme employed. The antigen–antibody reaction-equilibrium has a slow off-rate, resulting in the formation of an observable—by CE—complex (this is different in some of the examples mentioned in Section 1.9.1). In a competitive binding immunoassay, a labeled antigen and the antigen of interest compete for an appropriate amount of antibody. Differentiation between the (labeled) complex and the free (labeled) antigen can be accomplished by CE-LIF. In a noncompetitive assay, a known amount of fluorescently labeled antigen is added to the sample which forms a complex specifically with the antibody. The feasibility of both types of immunoassay with CE-LIF was demonstrated by Schultz and Kennedy (1993). A He-Cd laser emitting at 442 nm was used to determine Fab fragments of a monoclonal antibody and human insulin. Shimura and Karger (1994) used a fluorescently labeled antibody fragment to tag an antigen (methionine–recombinant human growth hormone). In this competitive immunoassay, a 488-nm Ar-ion laser was used with detection limits down to 5×10^{-12} M.

Part 2

Protein/Peptide Applications of CE to Analytical Biotechnology

2.1 Introduction

2.1.1 The Importance of Analytical Chemistry to Biotechnology

Biotechnology products, especially protein and peptide biopharmaceuticals, must be analyzed and characterized as rigorously and completely as current technology allows. Such extensive analysis is driven by the complexity of the molecules, and is needed to meet the demands of good science, ethical business practice, and regulatory requirements. As we will discuss in this part, the separation power and versatility of CE make it an ideal solution for many of the analytical challenges provided by proteins and peptides.

An important goal of analytical biotechnology is to have adequate characterization, specification, and control assays for the Chemistry, Manufacturing, and Control (CMC) section of a regulatory filing such as an IND (investigative new drug application), PLA (product license agreement), or NDA (new drug application). The importance of analytical methods can be seen in the fact that, according to biotechnology consultant and ex-Food and Drug Administration (FDA) official Peter Hoyle, CMC deficiencies can lead to rejection of regulatory submissions. A company invests millions of dollars to bring a drug to Phase 1 clinical trials, and hundreds of millions to conduct the trials through Phase 3 and achieve licensing. This is all money wasted if applications are rejected or clinical trials fail because of inadequate or inappropriate assays.

2.1.2 The "Eight Points" Model of Analytical Development

The U.S. FDA current Good Manufacturing Procedures for drugs (CGMP, Title 21 of the Code of Federal Regulations, Parts 210 and 211) states that all pharmaceutical products must be analyzed for "identity, strength, quality, and purity." Many analytical development scientists in biotechnology consider these precepts too vague to serve as effective guidelines for biopharmaceutical analysis. When one of the authors (TJP) was working in the biopharmaceutical industry, he developed the following "eight points" model to guide analytical development for any new macromolecule he and his staff encountered. Several successful regulatory filings attest to the adequacy of the model.

According to the model, assays should be developed and validated to address each of the following points:

1) Identity
2) Quantity
3) Purity
4) Activity
5) Heterogeneity
6) Stability
7) Process Consistency
8) Safety

CE can be used directly for all the points except, perhaps, numbers 4 and 8, which are usually addressed using biological and microbiological assays (supplemental CE activity assays may, however, sometimes be validated against biological assays). In the following pages, details of the use of CE to assess the identity, quantity, purity, heterogeneity, stability, and process consistency of proteins and peptides will be discussed.

While the emphasis is on the role of CE in the analysis of biopharmaceutical proteins, many of the examples also pertain to the analysis of proteins in academic and clinical research laboratories. For more information on the role of analytical techniques in biotechnology with regard to quality control testing and FDA concerns, the reviews by Garnick *et al.*, (1988) and Avallone (1986) are a good starting point.

2.2 Identity

The purpose of an identity assay is to provide scientific proof that the contents of a container correspond, qualitatively, to what is claimed on that container's label. Until identity is established, all other analytical concerns are secondary. Because of this central role, proof of identity is usually approached by a summation of evidence from *several* assays. In addition, using multiple structural determination methods ensures that products are thoroughly characterized as well as adequately identified. For proteins, commonly used identity assays include specific activity, amino acid composition and sequence, and assessment of such physicochemical parameters as molecular weight and isoelectric point. A list of assays commonly used to establish the identity of a biopharmaceutical protein is presented in Table 2-1.

Table 2-1. Methods Commonly Used for Characterization of Proteins: Identity

- Specific activity assays
- Immunological methods
  - Immunoblots
  - ELISAs
  - RIAs
- Electrophoretic profile
- Chromatographic profile
- Peptide mapping
- Amino acid analysis
- Carbohydrate analysis
- N-terminal sequencing
- Mass spectrometry
- Colorimetric assay
- Optical rotatory dispersion
- Circular dichroism

Several CE techniques readily lend themselves to providing evidence of a protein's identity. These include:

- Peptide mapping by free-solution CE (CZE)
- SDS-capillary gel electrophoresis (SDS-CGE) for evaluation of relative molecular weight
- Capillary Isoelectric Focusing (CIEF) for evaluation of a protein's characteristic isoelectric point
- CE-Mass Spectrometry (CE-MS) for direct assignment of molecular weight

In the following section, examples of work in which these CE techniques are used for identity assays are discussed.

2.2.1 Peptide Mapping: Utility of CZE

In peptide mapping, a protein is enzymatically or chemically cleaved into specific peptide fragments which are then separated and detected. Based mainly on its primary structure and the specificity of the cleavage reagent, a given protein will exhibit a characteristic pattern of peptides called its "peptide map." Digestion of the protein into smaller peptide fragments allows subtle structural features of the protein to be detected. A peptide map thus provides a "fingerprint" of a protein, and evidence of its correct identity.

Additional applications of peptide mapping include primary structure determination (*i.e.*, peaks are collected and sequenced), detection of posttranslational amino acid modifications, identification of genetic variants, and the determination of glycosylation and/or disulfide sites. For these reasons, peptide mapping has found widespread use in the quality control (QC) and characterization of recombinant DNA derived products.

Traditionally, analysis of peptide fragments has been achieved using high-resolution techniques such as reversed-phase HPLC (RP-HPLC). Slab gel electrophoresis (SDS-PAGE) is sometimes used, although less frequently than HPLC. CZE peptide mapping provides not only an excellent high-resolution separation tool, but is eminently suitable to complement RP-HPLC in identification or proof of structure cases. This is because the separation mechanisms of RP-HPLC and CZE are different, being based on hydrophobicity and mass-to-charge ratio, respectively. Hence, whereas certain peptide fragments cannot be resolved by RP-HPLC (because of similar hydrophobic character), CZE readily provides baseline resolution; likewise, RP-HPLC may resolve peaks not separable by CZE (see Grossman *et al.*, 1989; Bullock, 1993).

Figure 2-1, from Rush *et al.* (1993), illustrates the power of CE peptide mapping in the analysis of recombinant human erythropoietin (rHuEPO). Here, the enzyme trypsin, which specifically cleaves at argenine and lysine residues, was used as the cleavage reagent. A pH-2.5, phosphate CE run buffer was used, and an ion pairing reagent, 100 mM heptanesulfonic acid, was included, similar to the example of Figure 1-7, Section 1.3.2.3. The tryptic maps of rHuEPO derived from two different expression systems are shown. The upper trace shows the map of rHuEPO expressed in *E. coli*, a prokaryotic system, while the lower trace shows the map of rHuEPO expressed in Chinese hamster ovary (CHO) cells, a eukaryotic system. The former (*E. coli*) protein is not glycosylated while the latter (CHO) originally contained carbohydrate groups typical of mammalian systems, but was treated with N-glycanase which cleaves the N-linked oligosaccharides, leaving an aspartic acid residue in place of the original asparagine residue. While 16 peaks have identical migration times in the two electropherograms, the peaks labeled a through f are only present in the *E. coli*-expressed rHuEPO material and can be attributed to structural differences between the two preparations (Rush *et al.*, 1993). The peptide maps also provide information on the heterogeneity associated with the three rHuEPO glycopeptides (see Section 2.5). The combination of CE with mass spectrometry (CE-MS) would, in principle, allow the unknown peaks in Figure 2-1 to be unequivocally identified (see Section 2.2.4).

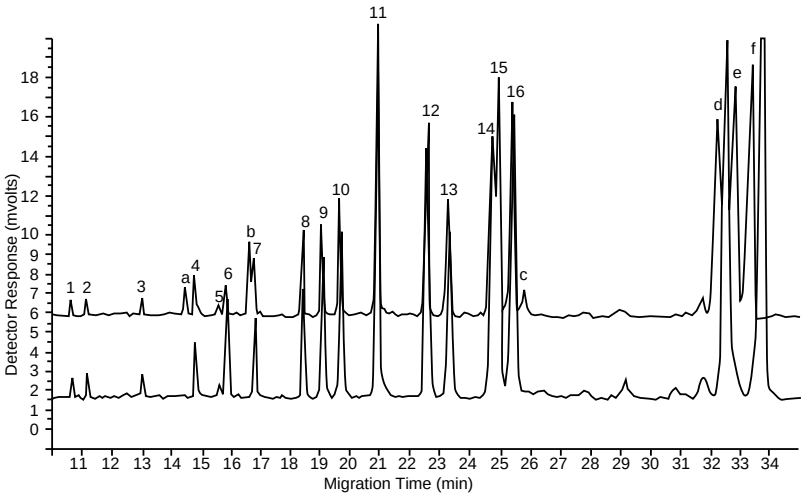


Figure 2-1. *Comparison of N-glycanase-treated CHO cell-expressed (lower trace) versus* E. coli *expressed (upper trace) rHuEPO tryptic maps by HPCE. Reproduced with permission from Rush* et al.*,* Anal. Chem. 65*, 1834 (1993).*

2.2.2 Molecular Weight Estimation: Utility of SDS-CGE

While accurate molecular weight (MW) determination of proteins usually involves ultracentrifugation or mass spectrometry, estimation of relative MW is typically performed using size-exclusion HPLC and SDS-PAGE. The capillary analog of SDS-PAGE (SDS-capillary gel electrophoresis) was described in Part 1 (Section 1.6) and involves the use of SDS-containing polymer solutions ("replaceable gels") which can be pumped in and out of the capillary. The capillary format offers fast and reproducible analyses, accurate quantitation, and direct, on-line UV detection of proteins without the need for staining or destaining. Beckman's Gold software allows rapid calibration and MW estimation of proteins.

Examples of SDS-capillary gel electrophoresis (SDS-CGE) can be seen in Figures 2-2 and 2-3 which are representative of the separation of relatively low and high MW proteins, respectively. The IgG monoclonal antibody (Figure 2-3) was also run under reduced conditions—the sample was treated with

2-mercaptoethanol—resulting in the appearance of light and heavy chains (MW 25,000 and 50,000).

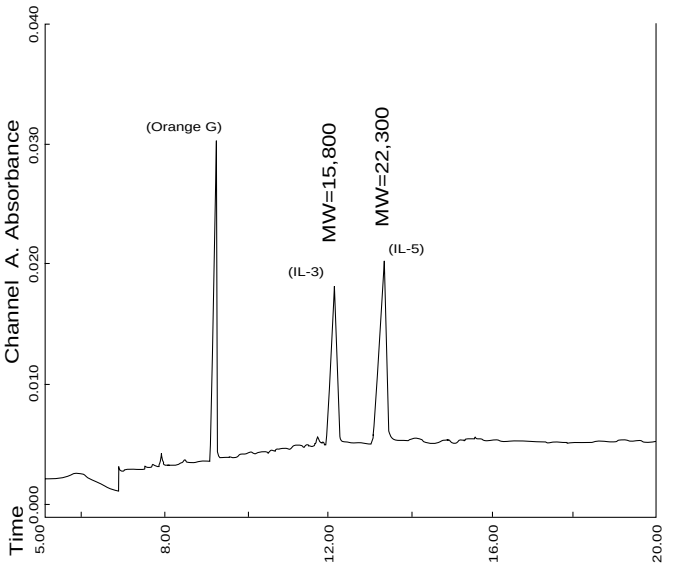


Figure 2-2. *SDS-CGE Separation of interleukins 3 and 6. Orange G was used as a reference standard.*

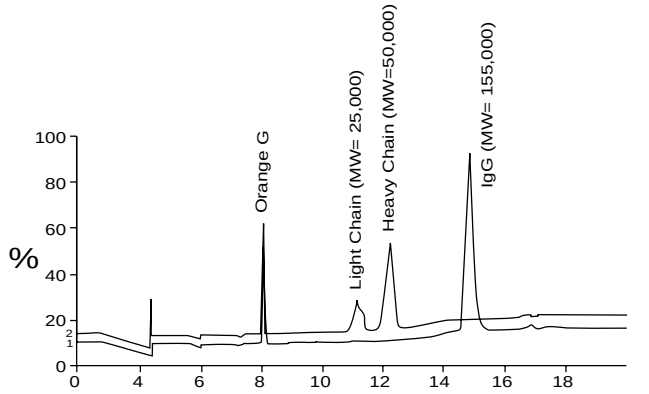


Figure 2-3. *Purity check of IgG monoclonal antibody by SDS-CGE. Trace 2 was run under reduced conditions.*

SDS-CGE, as well as CZE, can also be very useful in monitoring dimer–oligomerization processes (Palmieri and Nolan, 1993; Tsuji, 1993). Figure 2-4 shows the SDS-CGE electropherogram of a dimer-enriched recombinant bovine somatotropin (rbSt) preparation. Baseline resolution of the monomer, dimer, trimer, and tetramer peaks was obtained, demonstrating the resolving power of the method.

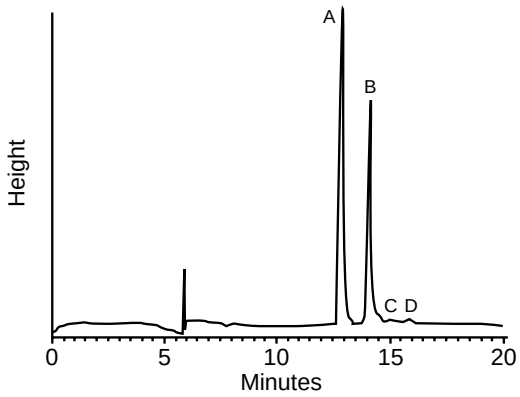


Figure 2-4. *SDS gel-filled capillary electropherogram of a dimer-enriched rbSt sample indicating a baseline resolution of monomer, dimer, trimer, and tetramer peaks. Conditions, 300 V/cm (24 μA); detector: 214 nm; column temperature: 20°C; migration distance: 40 cm; coated capillary: 100 μm i.d.; Peaks: A = monomer; B = dimer; C = trimer; D = tetramer. Reproduced with permission from Tsuji,* J. Chromatogr. 652, *139 (1993).*

2.2.3 Identity of Monoclonal Antibodies (MAbs): Utility of Capillary Isoelectric Focusing (CIEF)

Monoclonal antibodies (MAbs) are increasingly used in a variety of diagnostic and therapeutic applications. According to a 1993 survey by the Pharmaceutical Manufacturers Association, 35% of biotechnology medicines in clinical trials in the United States were MAbs. Monoclonal antibodies are also used as highly selective agents for the affinity purification of recombinant proteins, allowing the recovery of the protein antigen from crude mixtures and the removal of contaminants from a preparation. According to FDA regulations, MAbs used in the purification process must have quality control procedures essentially equivalent to those for the drugs they are being used to purify.

As is the case for most of the proteins used in biotechnology, the composition of MAbs is often heterogeneous (that is the product consists of a population of related molecules rather than a singular molecular species). For MAbs, this heterogeneity is due mainly to differing post-translational modifications. Such heterogeneity makes high-resolution analytical techniques particularly important. Traditionally, analytical biotechnologists have turned to HPLC, in particular RP-HPLC, to provide high-resolution analysis. Developing high-resolution chromatographic techniques for MAbs, however, has proven extremely difficult. For example, with RP-HPLC, poor peak shapes and extremely low recoveries are often obtained due to adsorption of the MAb on the reversed-phase packing material.

CE—in the free solution CZE, gel, or IEF modes—provides the analyst with high resolution tools to study MAbs. CE offers all of the advantages of classical, HPLC techniques in terms of automation, analysis time, direct, on-line detection and computerized data storage. CE, however, offers a significant advantage over RP-HPLC. Newly developed coated capillaries such as Beckman's Neutral Capillary minimize MAb interactions to the capillary wall, resulting in high-resolution separations. Finally, with the new coated capillaries methods development time is greatly reduced, as there is no longer a need to try numerous additives and dynamic coatings to prevent with minimal methods development time. Following is an example of high-resolution MAb analysis using capillary isoelectric focusing in a coated capillary.

One important application of IEF is the determination of a protein's isoelectric point (pI). As in classical IEF, the CIEF method involves calibration with standard proteins whose pIs are known. A plot of the mobilization time vs. the pI should exhibit linearity within a defined pH range. In Figure 2-5 (Pritchett, 1994), such a calibration plot is shown for the determination of the pIs of components of anti-carcinoembryonic antigen (CEA) MAb. The CIEF method described in Section 1.7 (Figure 1-39) was used along with a coated capillary (eCAP Neutral, P/N 477441). As shown in the electropherogram, the sample was spiked with internal standards and, from the calibration plot, it was estimated that the isoelectric points of the anti-CEA MAb varied from 6.73 to 7.46. Figure 2-6 shows a separate run of anti-CEA MAb. The difference in the migration times between the two runs is due to differences in sample concentrations and matrix compositions, and highlights the importance of running internal standards for pI determinations.

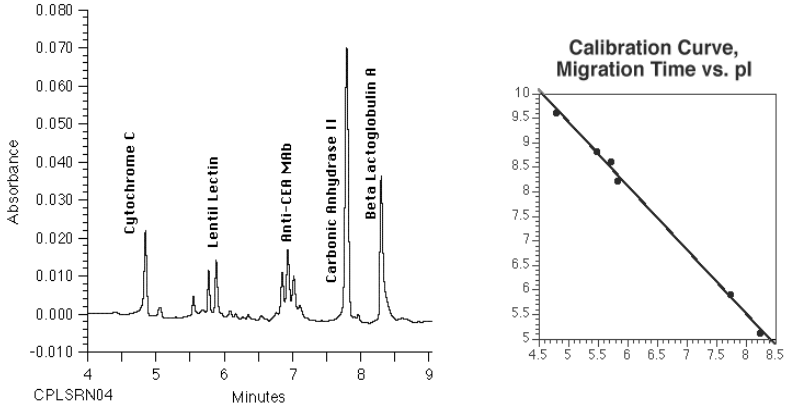


Figure 2-5. *CIEF Analysis of anti-CEA MAb. The method of Figure 1-39 was used with an eCAP Neutral coated capillary. The pI was calculated by comparison with standards.*

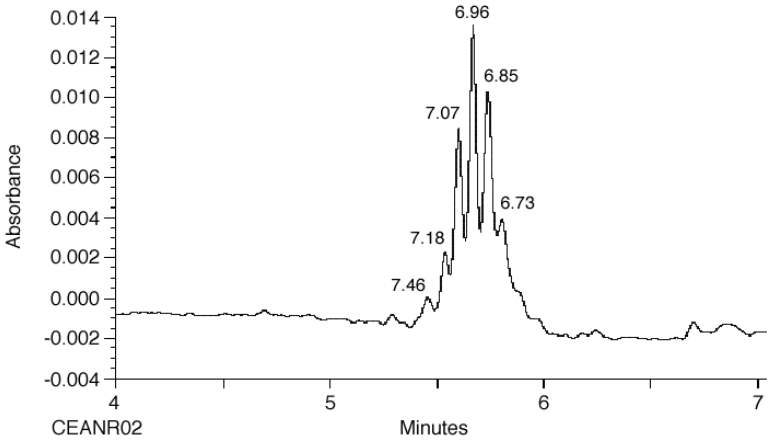


Figure 2-6. *CIEF analysis of anti-CEA MAb without internal standards. The estimated pIs of the individual zones are indicated.*

2.2.3.1 Immunotoxins: Utility of MECC with P/ACE-LIF Detection

Immunotoxins are biotechnology-derived drugs that consist of cytotoxins conjugated to monoclonal antibodies. They deliver cytotoxins (*e.g.*, doxorubicin) to the site of cancerous cells at relatively high local drug concentrations, while allowing the total systemic dose to be relatively low. The specificity of the antibody avoids the toxicity to normal cells (particularly those of the bone marrow and gastrointestinal tract) characteristic of conventional anticancer drug therapy.

Hughes and Richberg (1993, 1994) have shown that CE is an excellent technique to examine doxorubucin-linked chimeric antibodies. While UV detection can be used, P/ACE with laser-induced fluorescence (LIF) detection provides both high sensitivity and selectivity for the analysis of antibody-conjugated anthracyclines: only those protein species that are conjugated with the fluorescent drug are detected. Detectability with LIF is typically several orders of magnitude better than that with UV absorbance (for a review of CE-LIF applications, see Schwartz *et al.*, 1994). With drug-labeled antibodies, different drug–antibody ratios and conformations are possible. In an SDS-containing run buffer (*i.e.*, MECC conditions were used), the doxorubicin-conjugated antibody was separated into three species, as shown in the electropherogram of Figure 2-7 (peaks 1, 2, 3). These peaks are well separated from the unconjugated drug (peak 6) which separates on the basis of micellar partitioning (see Sections 1.3.2.1 and 1.4.3.1 for MECC principles). Also appearing in the electropherogram are the conjugated light and heavy chains (peaks 4, 5). Thus CE-LIF permits sensitive detection of doxorubicin and its conjugated antibody species. Examination of the antibody conjugate at picomolar levels would be difficult, if not impossible, by other separation/detection techniques.

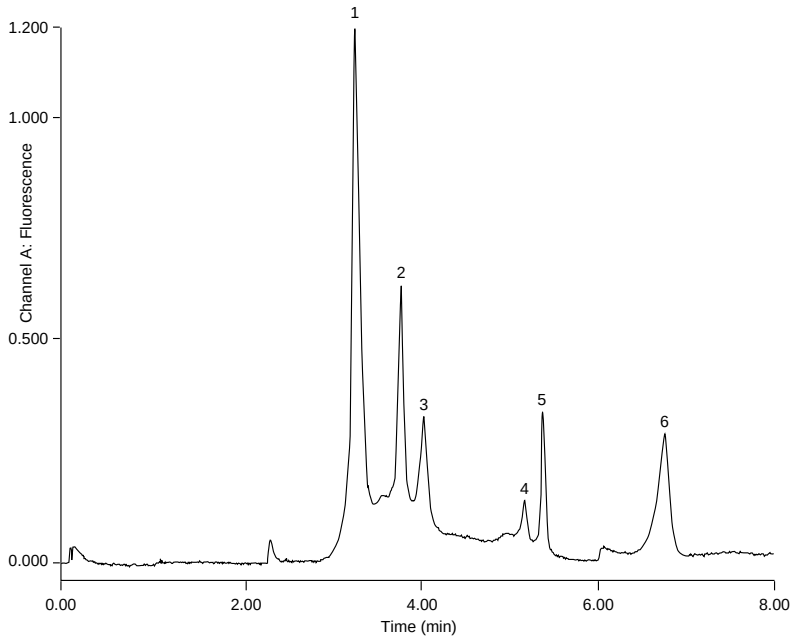


Figure 2-7. *CE-LIF electropherogram of a 420-pmol solution of IgG antibody conjugated with doxorubicin. Peak identification: 1, 2, 3 = antibody conjugate; 4, 5 = conjugated heavy and light chains; 6 = unconjugated doxorubicin. From Hughes and Richberg,* Beckman Application Information Bulletin A-1763.

2.2.4 Confirmation of Peak Identity by CE-Mass Spectrometry (CE-MS)

In principle, the combination of CE with a mass spectrometer would permit direct identification of unknown peaks (*e.g.*, peaks a to f in Figure 2-1). While CE-MS is not routine yet, significant progress has been made lately in protein and peptide applications (see reviews by Smith *et al*., 1994; Pleasance and Thibault, 1993).

Electrospray MS has revolutionized the way in which large—and sometimes labile—biomolecules are analyzed by MS. Electrospray is a "soft" ionization technique which produces an abundance of molecular ions relative to

ion fragments. Multiply charged protein or peptide molecular ions permit MW measurements on single quadropole MS instruments. A CE-MS electrospray interface is commercially available from Beckman (P/N's 727616 and 727617 for interfacing with the Finnigan and PE Sciex mass spectrometers, respectively). In contrast to HPLC-MS, with CE-MS the effluent flow from the capillary is not sufficient to generate an effective electrospray. The interface has a coaxial flow design in which a sheath liquid flow ("make-up buffer") is used to effect the spray of capillary effluent into the ion source. Figure 2-8 shows a schematic drawing of the P/ACE CE-MS interface. The capillary extends to the very tip of the needle assembly where the electrospray is produced with the aid of a dry nitrogen gas. This type of coaxial flow design produces minimal zone broadening; hence, the integrity of the electrophoretic profile is completely maintained.

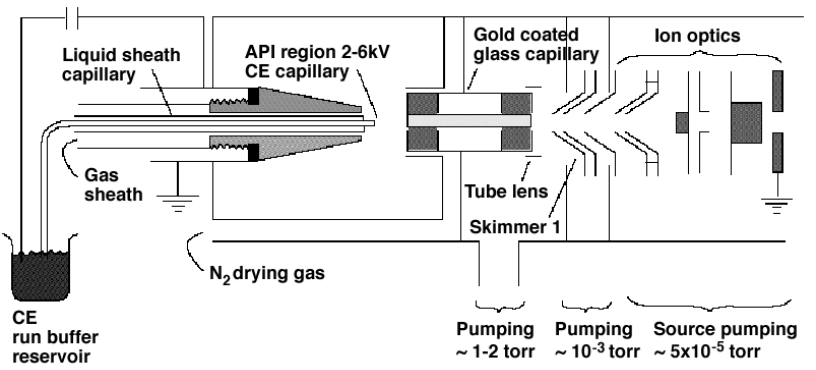


Figure 2-8. *Schematic of the CE-MS interface. Reproduced with permission from Tomlinson* et al., Electrophoresis 15*, 62 (1994).*

As new and even more sensitive MS methods are being developed, it is expected that CE-MS will become an extremely powerful tool for protein characterization. With the electrospray MS, proteins with MWs exceeding 100,000 Daltons have been analyzed with excellent accuracy. The added dimension of CE-MS requires some restrictions in the choice of (make-up) buffers and capillaries (Smith *et al.,* 1994) as volatile buffers such as ammonium acetate must be used.

An example of the utility of CE-MS for MW determination is shown in Figure 2-9 from Tsuji *et al.* (1992). Recombinant porcine somatotropin

(rpSt) was analyzed using P/ACE coupled to a Vestec electrospray MS. A characteristic pattern of multiply charged ion clusters can be seen ranging from *m/z* 1363.2 (16 charges) to *m/z* 1982.5 (11 charges). Software deconvolution of the mass spectrum resulted in an average MW of 21798.3 ± 3.6, close to the theoretical value of 21797.9. The CE-MS method allowed identification of both mono- and dioxidized homologs in samples of rbSt and rpSt.

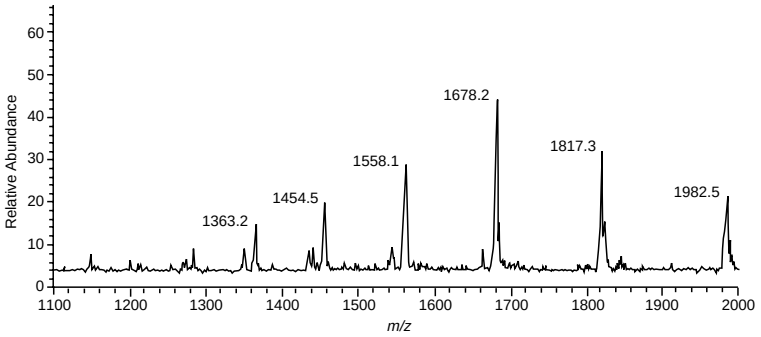


Figure 2-9*. Electrospray CE-MS of the major rpSt peak showing multiply charged ion clusters (*m/z*). Conditions: electrolyte buffer, 20% acetonitrile in 20 mM ammonium acetate, pH 9.0; makeup solution, 5% acetic acid in 50% methanol. Reproduced with permission from Tsuji,* Anal. Chem. 64*, 1868 (1992).*

Fragmentation patterns of molecular ions, needed for sequence information, can be obtained with triple quadropole MS units. For example, using online CE-MS, Tomlinson *et al.* (1994) have shown preliminary results on the sequencing of peptides which were shown to bind class I major histocompatibility complex molecules. The CE method involved isotachophoresis (ITP) preconcentration of sample components to improve peptide detectability, as discussed in Section 1.5.

ITP preconcentration of proteins was also utilized by Thompson *et al.* (1993); full scan CE-MS data of proteins were obtained at the 10^{-7} M level. Finally, Kelly *et al.* (1993) demonstrated the utility of CE-MS for the identification of protein glycoforms using a P/ACE system combined with a Perkin Elmer-Sciex triple quadropole mass spectrometer. Contour plots (intensity vs. *m/z* vs. time) were used to identify closely related glycoforms present at a given attachment site. It was possible to identify unambiguously oligosaccharides in digests of complex-type glycoproteins (*e.g.*, horseradish peroxidase).

2.3 Quantity

Providing information on the mass or concentration of a given substance in a variety of matrices is the goal of quantity assays. Knowledge of concentration is essential for every aspect of drug development and manufacturing, including process control (*e.g.*, calculation of yield), pre-clinical studies (*e.g.*, animal toxicity), and clinical trials (*e.g.*, for dose escalation studies and to monitor serum concentration). The crucial importance of quantitative assays, even early in the development cycle, is underscored in the FDA's *Guideline for Submitting Documentation for the Manufacture of and Controls for Drug Products*, Section F.1, which states, "The product tests and specifications appropriate to investigational drug products are, understandably, not as well developed as when an NDA is submitted . . .. Information should also be submitted to support the specificity, linearity, precision, and accuracy applicable to specific quantitative methods used to test the dosage form."

Table 2-2 lists commonly used, traditional methods for protein quantitation. As detailed in the following sections, several modes of CE can provide solutions to protein quantitation problems: CZE, MECC, CIEF, and SDS-CGE. Affinity capillary electrophoresis (ACE) appears very promising for the study of receptor–ligand reaction kinetics and to quantitate protein binding (*e.g.*, with drugs, DNA, antigens), *e.g.*, in immunoassays.

Table 2-2. Methods Commonly Used for Characterization of Proteins: Quantity

- Amino acid analysis
- Spectroscopy
  - UV extinction coefficient
  - Tyrosine titration difference spectroscopy
  - Colorimetric methods
- HPLC
- Electrophoresis (SDS-PAGE)
- Immunoassays
  - ELISA
  - RIA

2.3.1 Accuracy and Precision in Quantitative CE Analysis

As pointed out in Section 1.4, CE protein analysis, when performed in bare silica capillaries, often requires special capillary pretreatment procedures and additives to prevent protein interactions with and adsorption to the capillary wall. In addition, special rinsing protocols are often necessary between runs to remove adsorbed protein. These steps are needed because proteins fouling the capillary wall can cause severe decreases in quantitative accuracy and precision. Further evidence for the importance of certain buffer additives on the CZE analysis of MAb preparations in uncoated capillaries was presented by Guzman *et al.* (1992). They found that improper separation conditions may lead to significant errors in the quantitation of proteins in pharmaceutical preparations. Addition of alkylamines and/or zwitterions (see also Section 1.4.2.3) was recommended to improve the performance of separation and to enhance the resolution and reproducibility of the protein analytes.

The new Beckman coated capillaries for CZE and SDS-CGE are designed to minimize protein–capillary wall interactions. They often give superior protein separations compared to untreated capillaries, even when the bare silica capillaries are used in conjunction with buffer additives (see examples in following sections). In addition, the time formerly spent finding the right pretreatments, additives, and rinses is eliminated, greatly reducing method development time.

The complexity and/or variability of the sample matrix also plays an important role with regard to quantitative precision and accuracy. Many first-time users of CE equipment have the experience that results with “clean” samples are often superior to those obtained with “dirty” or complex samples. Many such problems are the result of adsorption of proteins to the capillary wall and can be avoided by using the aforementioned coated capillaries, or going through the process of finding the right additives.

The quality of the separation also affects the analytical accuracy and precision. With well-defined, baseline-separated peaks, precision is generally excellent, *i.e.*, typically < 0.5% RSD for migration times and ≈ 0.5 to 3.0% RSD for peak areas.

When low-level impurities are quantitated, precision decreases as not only resolution but also peak detectability (signal-to-noise ratio) becomes an issue. For example, Bullock (1993) quantified with CZE low-level impurities in recombinant human interleukin-4 (rhIL-4) preparations with RSDs varying

from 5 to 26% (Table 2-3). However, the precision in the overall purity determination of rhIL-4 was excellent: 0.843% (n = 8). The use of on-capillary sample concentration techniques, discussed in Section 1.5, provides a possible way to improve detectability (with concomitant improvement of precision) of low-level impurities.

Table 2-3. Precision Data for the Purity Determination of rhIL-4 by CZE

	rhIL-4	Impurity 1	Impurity 2	Impurity 3	Impurity 4
Mean (n = 8) (peak area%)	79.75%	1.16%	1.72%	11.95%	5.42%
S.D. (peak area%)	0.6724	0.132	0.457	0.651	0.815
RSD. (%)	0.843	11.4	26.6	5.45	15.0

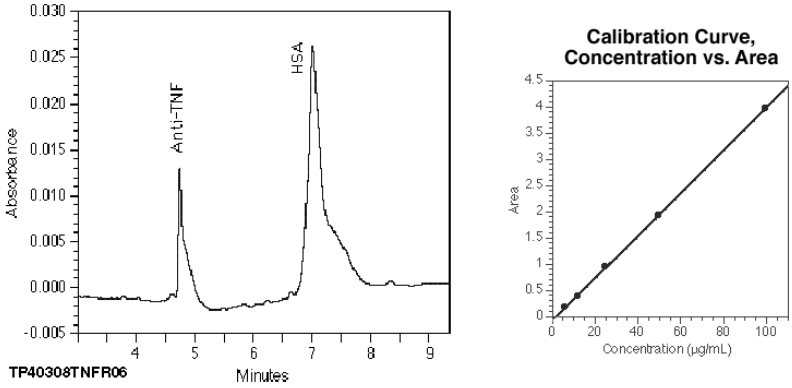
From: Bullock, *J. Chromatogr. 633*, 235 (1993)

Finally, automated data systems with suitable peak-defining algorithms for CE such as Beckman's Gold are imperative to achieve optimum quantitative precision and accuracy from CE runs. Gold software is designed for quantitation with both HPLC and CE. CE and HPLC systems can be simultaneously operated and controlled from one computer. Gold allows for internal and external standardization and the detector response can be tracked using single- or multilevel concentration calibrations (see, for example, the insets in Figures 2-10, 2-12, 2-13). In electropherograms, up to three reference peaks may be selected for precise alignment and comparison of data. Examples of the utility of the various CE modes for quantitation purposes are described next. They show that CE can provide rapid, accurate, and precise protein assays in often complex media.

2.3.2 Quantitation of Dosage Forms by CIEF

Pharmaceutical and diagnostic products typically do not consist of pure drug substances, but rather of formulated mixtures. Excipients are added for several reasons (for example to enhance stability during storage, or to provide protection during the manufacturing process). Many of the excipients used in biopharmaceutical products are themselves proteins (for instance, human serum albumin), which adds significantly to the challenges of developing a good quantitative method. In addition to its speed and high resolution, a decided advantage of CIEF for dosage form analysis is that, since detection is accomplished at 280 nm, many small molecule excipients are transparent and thus do not interfere with the analysis.

MAbs were quantitated in dosage forms by Pritchett *et al.* (1994) using the recently introduced eCAP Neutral coated capillary. Figure 2-10 shows the CIEF analysis (method of Figure 1-39, Section 1.7.3.2) of anti-tumor necrosis factor (TNF) MAb. For these types of assays, the method should have sufficient sensitivity and range to measure low and high levels of the drug in various investigational and licensed dosage forms. In addition, resolution should be adequate to separate the active drug from the excipients. The electropherogram shows two protein peaks, the MAb, and human serum albumin (HSA) which was included in this experimental formulation. The CIEF method's dynamic range was from ≈ 5 to 250 mg/mL, as shown in the insert of Figure 2-10. Table 2-4 lists the results of the CIEF assay as percent of label along with the precision of three measurements. It is our experience that the peak area precision with the above CIEF method is quite good (≈ 0.5 to 2% RSD) and even slightly better than with other CE methods. This may be due to the fact that, with the CIEF method, the entire capillary is filled with sample. In contrast, only a small fraction of the capillary is filled with sample in the CZE or SDS-CGE methods.



***Figures 2-10**. Quantitative CIEF analysis of anti-TNF dosage form. Conditions as in Figure 2-5.*

Table 2-4. Quantitative CIEF Analysis of Anti-TNF MAb: Assay Percent of Two Dosage Forms

Dosage Form	% of Label
0.5 mg vial	101.0
	101.8
	102.2
Mean	101.7
RSD (%)	0.6
1.0 mg vial	99.2
	99.2
	98.6
Mean	99.0
RSD (%)	0.35

In a similar application (involving the same CIEF method and also performed on a P/ACE instrument), Yowell *et al.* (1993) determined granulocyte macrophage colony stimulating factor (GM-CSF) in the presence of HSA (Figure 2-11). The results of three different dosage forms are shown in Table 2-5 along with results obtained by CZE and reversed-phase HPLC. Dosage forms of GM-CSF showed 5 to 6 major components in the electropherogram which were well separated from GM-CSF. The figure also shows a run of recombinant granulocyte colony stimulating factor (G-CSF), trade name Neupogen, which has a different pI. CIEF has excellent potential for routine QC testing. Compared to HPLC, it lacks the problems associated with clogged check valves, leaking seals, large solvent consumption (and waste production), and relatively long analysis times.

Table 2-5. Comparison of FSCE, CIEF, and HPLC Analyses for GM-CSF Dosage Forms

Sample	RP-HPLC	FSCE	CIEF
1-SFG-303	98.45	105.37	102.44
0-SFG-303	98.73	99.80	104.32
0-SFG-304	104.63	102.22	105.21

From Yowell, *et al., J. Chromatogr. 652,* 215 (1993).

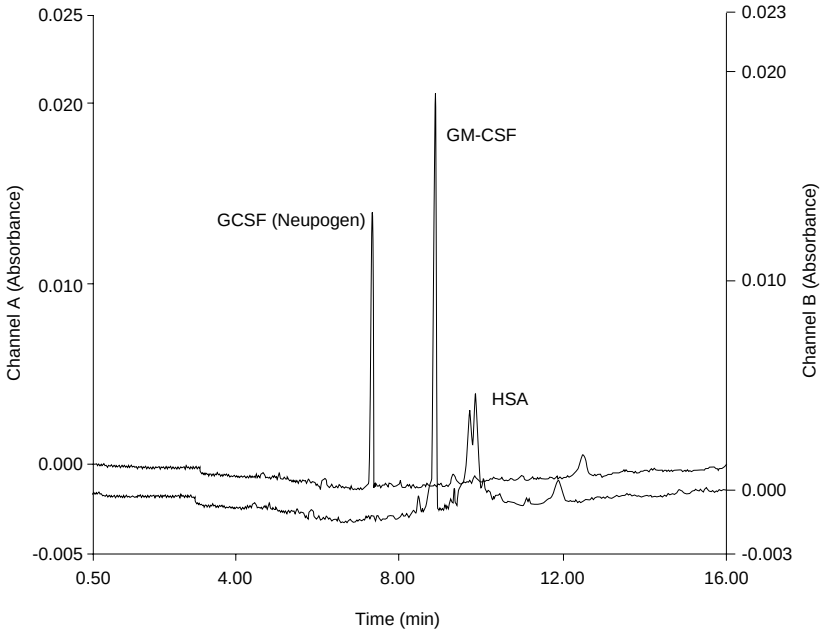


Figure 2-11. *A CIEF electropherogram of GM-CSF and G-CSF (Neupogen) showing different pI values as a method for product identification. From Yowell* et al., Beckman Application Information Bulletin A-1744.

2.3.3 Quantitation with CZE and SDS-CGE

Figure 2-12 shows the CZE assay of a MAb, anti-CEA, in serum-free tissue culture medium (Pritchett *et al.*, 1994). A 20-mM citrate, pH 3, run buffer and eCAP Neutral capillary were used. The method had a linear dynamic range of 5 to 1000 mg/mL and the analysis time was less than 10 minutes. In our experience, and that of others (Yowell *et al.*, 1993), the linear dynamic range with CZE is typically higher (5 to 10 times) than with CIEF; since the latter method is more prone to protein precipitation at higher concentrations. The peak area precision from a variety of studies with sample sizes ranging from 3 to 9 was between 2.5% and 3% RSD. To determine the recovery, purified MAb was spiked into Hybrimax (Sigma) medium at the levels of 25 and 50 μg/mL. The percent recovery found at these levels was 97.2 and 98.6%, respectively.

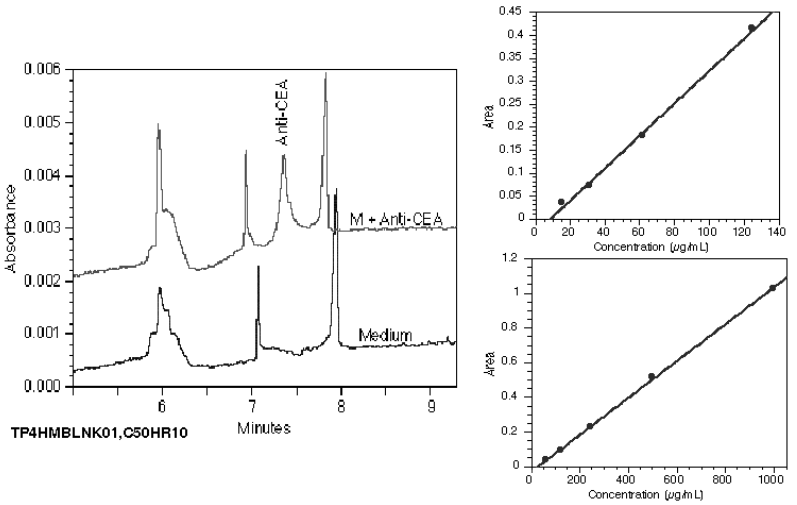


Figure 2-12. *CZE analysis of anti-CEA MAb in tissue culture. Capillary, eCAP Neutral coated, 37 (30) cm × 50 µm; run buffer, 20 mM citrate pH 3; run temperature, 20°C; field strength, 400 V/cm; detection, 214 nm.*

Evidence that SDS-CGE can also be used as a quantitative method was presented by Pritchett *et al.* (1994) and Tsuji (1993). In the former, the same MAb as that of Figure 2-12, anti-CEA, was analyzed by in a serum-containing tissue culture medium (10% newborn calf serum in QBSF-52 [Sigma]) using the Beckman eCAP SDS 14-200 kit (see Section 1.6 for more information on this method development kit). Figure 2-13 shows the electropherograms of anti-CEA MAb in the serum-containing medium along with the blank run. At the 25 mg/mL level, the recovery was 98% (RSD 1.4%). Using the same coated capillary and gel buffer, Tsuji (1993) determined the percent monomer, dimer, trimer, and tetramer in recombinant bovine somatotropin (rbSt) samples. The SDS-CGE electropherogram of a dimer-enriched rbSt sample was shown earlier in Figure 2-4. Composition of monomer and dimer in rbSt was 96 and 4% (tri- and tetramer < 1%) with RSDs of 0.2 and 4%, respectively ($n = 7$).

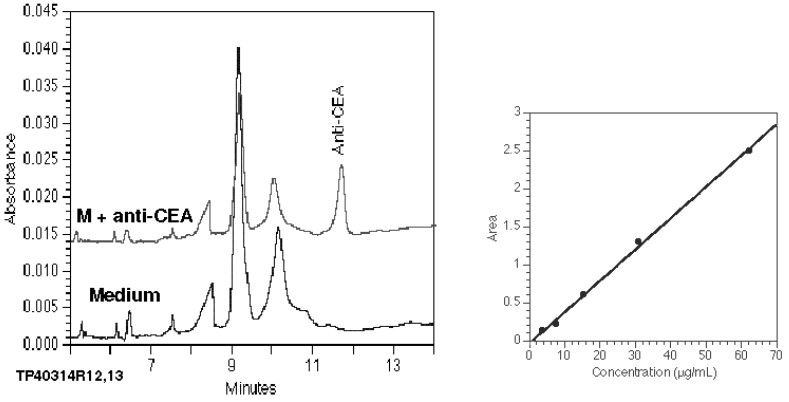


Figure 2-13. *Quantitative analysis of anti-CEA in serum-containing medium. Capillary, eCAP SDS 14-200, 27 (20) cm × 100 µm; field strength, 250 V/cm; run temperature, 20°C; detection, 200 nm.*

2.3.4 Immunoassays Using Affinity CE (ACE)

The principles of ACE were outlined in Section 1.9. Immunoassays are widely used for quantitative studies of proteins, particularly in clinical and diagnostic applications. However, the UV absorbance detection used in conjunction with CE is generally not sensitive enough for detection in biological media at very low protein levels, *i.e.*, concentrations < 10^{-6} M. Laser-induced fluorescence detection (LIF) provides sensitivity several orders of magnitude higher than UV absorbance and is now commercially available (P/N 477125). The P/ACE LIF detector permits coupling to various laser sources and can be used in a wide variety of bioapplications (Schwartz *et al.*, 1994).

Chen and Sternberg (1994) described a competitive immunoassay for determination of digoxin in human serum using the green He-Ne laser emitting at 543.5 nm. The antigen was conjugated with a dye-labeled oligonucleotide, $(dT)_{10}$. The laser dye used was tetramethylrhodamine (TMR). The oligonucleotide serves as a charge modifier so that the species of interest are well resolved with CE. The competitive immunoassay can be described by the equilibrium

$$Ag + Ag^* + Ab \rightleftharpoons Ag\text{-}Ab + Ag^*\text{-}Ab + Ag$$

where Ag = digoxin, Ag* = TMR-$(dT)_{10}$-digoxigenin, Ab = Fab fragment, and Ag-Ab and Ag*-Ab are the antigen–antibody complexes. Digoxin calibration with CE-LIF is demonstrated in Figure 2-14 with serum-based digoxin calibrators of 0.42, 2.72, and 5.13 ng/mL, respectively. As the digoxin concentration is increased in the sample, the amount of free Ag* also increases while Ag*-Ab decreases. Using the ratio of the areas of free Ag* and the total fluorescence signal in each electropherogram, a digoxin calibration curve can be constructed. In this immunoassay, both the free Ag* and the antibody-complexed Ag* can be measured simultaneously. This approach to immunoassay using ACE with LIF detection should be promising for a variety of other clinical and diagnostic applications.

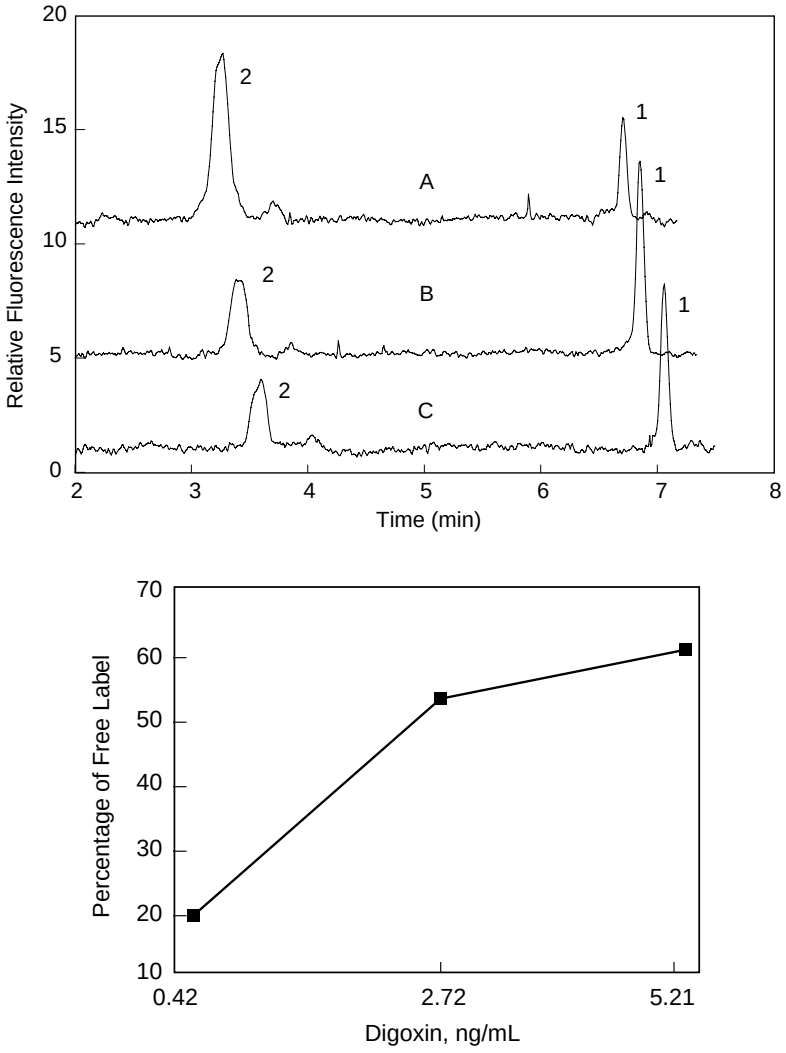


Figure 2-14. *(Top) Digoxin immunoassay with serum calibrator at (A) 0.42, (B) 2.72 and (C) 5.21 ng/mL, respectively. Peaks: 1, digoxigenin-3'-(dT)$_{10}$-TMR; 2, antigen–antibody complex of the Fab. (Bottom) Calibration curve. Percentage of free digoxigenin-3'-(dT)$_{10}$-TMR vs. digoxin calibrates. Reproduced with permission from Chen and Sternberg,* Electrophoresis 15, *13 (1994).*

2.4 Purity

Once it can be proven what is in the container (identity), and how much is there (quantity), the next question often asked is: How pure is the active component? In pharmaceutical biotechnology, the requirements are often such that minor amounts of impurities and degradation products must be determined in the presence of much larger quantities of the main component. In addition, these components may be structurally very similar to the main component. Here, the resolving power, sensitivity, quantitation capability, and speed of CE offer solutions to these analytical problems. Methods commonly used for purity determination are listed in Table 2-6. CE can be added to this list, either as a stand-alone technique or used in conjunction with these methods. The following examples illustrate the utility of CE for purity assays.

Table 2-6. Methods Commonly Used for Characterization of Proteins: Purity

- Chromatography
- Electrophoresis
- Residual DNA testing
- Microbiological testing
- Tests for process additives
- Immunological methods

2.4.1 Purity of Proteins

During the production of MAbs and rDNA proteins, process monitoring is required in order to assure that, among other things, the required purity levels are achieved and maintained. The recovery and purification of the product from a fermentation broth typically involve various procedures, including filtration, centrifugation, and chromatography. After each purification step, and especially after the final step, the intermediate or bulk product must be checked to see if the desired purity level has been achieved. In addition to its control function, this purity information is frequently used to further optimize the purification process. The CZE, CIEF, and SDS-CGE modes of CE are all well suited for such purity checks.

2.4.1.1 Purity Check by CZE and CIEF

Figure 2-15 shows an example of a purity check by CZE for recombinant hirudin, an inhibitor of α-thrombin. The top panel (A) shows the electropherogram of a sample extracted from a fermentation broth, showing more than

30 constituents. Hirudin-65 is a 65-amino-acid recombinant protein. Two process by-products, hirudin-64 and hirudin-63, are present in this sample and elute just after the major peak (migration time, 20.09 min). Even though these compounds are structurally similar to hirudin-65, they can be baseline separated from each other by CZE, thus showing its resolving power (Ludi *et al.* 1988). The bottom panel (B) of Figure 2-15 shows a purity check at a certain stage in the purification process. The two minor components eluting at 20.27 and 20.44 minutes are hirudin-64 and hirudin-63, respectively. Other, similar examples of the use of CZE and CIEF in the purity of recombinant proteins can be found in publications by Palmieri (1989), Arcelloni *et al.* (1993), Frenz *et al.* (1989), and Grossman *et al.* (1989)—dealing with the analysis of insulin and hGH by CZE and CIEF; Bullock (1993)—Analysis of rhIL-4 by CZE; Yowell *et al.* (1993)—Analysis of GM-CSF by CIEF.

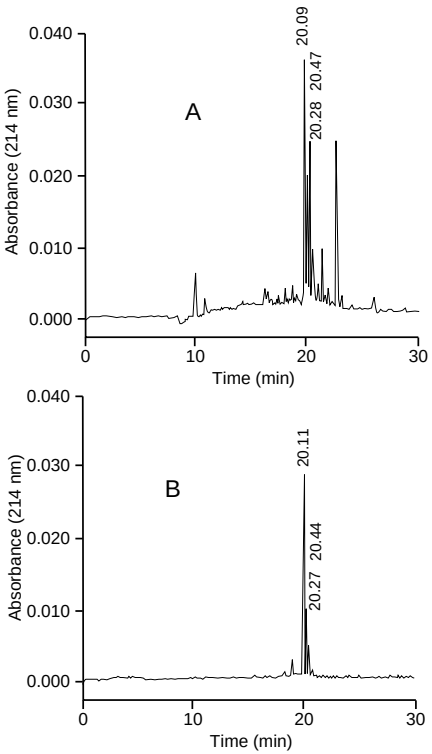


Figure 2-15. *(A) Separation by CZE of an r-hirudin-containing sample from a fermentation broth. (B) Purified r-hirudin sample. Run buffer: 20 mM tricine, 10 mM sodium borate, 1 mM EDTA, 0.2 mM diaminobutane, pH 8.4. From Paulus and Gassman,* Beckman Application Data Sheet DS-752.

2.4.1.2 Purity Check by SDS-CGE

A biotechnology company, Hemosol (Etobicoke, Ontario, Canada), used SDS-CGE to check the purity of a potential blood substitute product, Hemolink, during the manufacturing process.

Outside the red blood cell, hemoglobin does not readily release its oxygen to body tissue. It can break down and damage the kidneys during excretion. To be an effective, efficient, and safe oxygen carrier, hemoglobin must be stabilized to prevent its breakdown and have an oxygen-carrying capacity similar to that of whole red blood cells. Hemosol's proprietary crosslinked product, Hemolink, involves the stabilization of a highly purified hemoglobin through crosslinking with a reagent prepared from raffinose. The hemoglobin is modified to stabilize it against fragmentation into hemoglobin half-molecules, and to have oxygen binding and release properties similar to that of whole red blood cells. Figure 2-16 shows the electropherogram of a Hemolink sample.

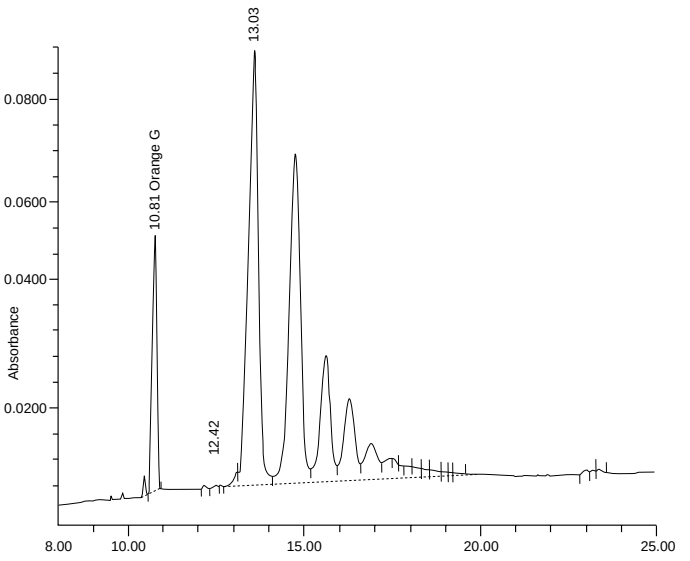


Figure 2-16. *SDS-CGE of Hemolink, a crosslinked and polymerized purified hemoglobin-based oxygen carrier. The standard procedure outlined in the eCAP SDS 200 kit was followed. Sample injection, 60 s, pressure. Orange G was used as a reference standard. Courtesy of Dr. David Wicks, Hemosol, Inc., Etobicoke, Ontario, Canada.*

At Biomira, Edmonton (Canada) SDS-CGE is used to monitor the purity of raw monoclonal antibody (IgG1). The latter is used in the manufacturing of immunoscintography kits for adenocarcinomas. The MAb is processed by a patented photoactivation method into a product, Tru-Scint AD, which is radiolabeled with Technetium (99m) at the clinic.

The MAb product is monitored for its consistency and purity by the Beckman SDS-200 kit on a routine basis (Figure 2-17). The percent of light and heavy chains generated during the manufacturing process, as well as the monomeric antibody content, can be quantified.

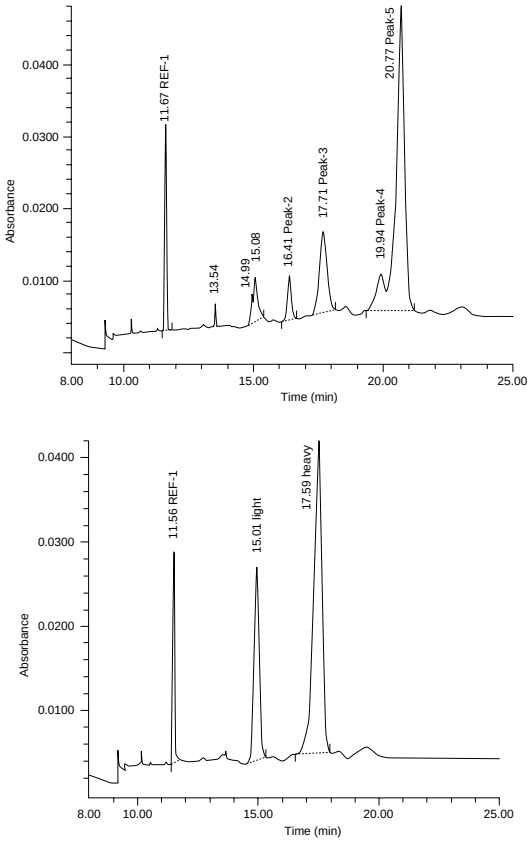


Figure 2-17. *Quality control of antibodies by SDS-CGE. Top: product; Bottom: reduced antibody. Courtesy of Dr. A. Abdul-Wajid, Biomira, Inc., Edmonton, Canada.*

2.4.2 Screening the Purity of Peptides

Purity checks of peptide products are needed in the biopharmaceutical industry for process control and QC of bulk peptides and final peptide products. The same criteria as discussed previously for protein products (Section 2.4.1) also apply to peptides. On a laboratory bench scale, purity checks of newly synthesized compounds generated on a peptide synthesizer are usually required.

In protein characterization studies involving N-terminal sequencing and peptide mapping, it is highly desirable to screen the purity of peptide fractions collected from HPLC by another *(i.e.*, non-HPLC), complementary method. Peptides often co-elute on HPLC columns, especially when their hydrophobicity is similar (Strickland, 1991; Grossman *et al.*, 1989). Thus, a quick purity check with CE would save valuable sequencer run time (and sample) as impure fractions cannot be easily sequenced and interpreted.

CZE is ideally suited to quickly check the purity of collected peptide fractions for two reasons: 1) very little sample (nL) is consumed so that almost all of the sample can be used for subsequent sequence analysis; 2) the separation mechanism is different from reversed-phase HPLC so that peptides similar in hydrophobicity can be resolved based on differences in charge-to-mass ratio. Thus, peaks appearing pure after HPLC analysis are often resolved in multiple peaks with a CE check (Strickland, 1991). In that case, a further purification step of the fraction might be considered. For further information on peptide separation conditions using CZE, see Section 1.3, and the papers by McCormick (1994), Langenhuizen and Janssen (1993), and Grossman *et al.* (1989).

2.5 Heterogeneity

Most purified proteins, even in their native state, consist not of a single molecular species, but rather are composed of a population of related molecules. One source of this heterogeneity is differences in amino acid sequence, due to mutant amino acids and modification of amino acid side chains (*e.g.*, deamidation of Asp or Gln; oxidation of Met). Differential, post-translational modifications provide additional heterogeneity. Common, post-translationally induced heterogeneities include variable polypeptide chain length resulting from differential cleavage by maturation enzymes (*e.g.*, some of the pro-protein species may be present in the population) and differential glycosylation of glycopro-

teins. The latter can exist at several levels of carbohydrate structure: 1) there may be different numbers of oligosaccharide chains; 2) the oligosaccharide chains may have different monosaccharide composition (*e.g.*, variable sialylation and fucosylation); 3) the oligosaccharides may have different monosaccharide sequences; 4) the oligosaccharides may have differing anomeric linkages among their monosaccharides.

CE offers valuable solutions to the analytical challenge of protein heterogeneity. Especially useful are CZE (*e.g.*, peptide mapping), MECC, and CIEF modes, while CE-MS provides unique capabilities for identification of isoforms.

2.5.1 Monoclonal Antibodies

Preparations of monoclonal antibodies often show considerable heterogeneity even though the amino acid sequence of the various forms observed is the same. This may be due to post-translational modifications involving glycosylation. It is important to monitor changes in glycosylation patterns because such parameters as serum half-life and bioactivity of the product may be adversely affected. The earlier-discussed example of Figure 2-6 (anti-CEA MAb using CIEF) shows a typical heterogenous distribution of glycosylated antibody isoforms with pIs ranging from 6.7 to 7.5.

2.5.2 Glycoforms of Recombinant Proteins.

Recombinant human tissue plasminogen activator (rtPA) is a fibrin-specific plasminogen activator used in the treatment of heart disease. The protein consists of 527 amino acids (MW ≈ 60,000) with 4 possible glycosylation sites. Yim (1991) examined glycoforms of rtPA with both CZE and CIEF. Two glycosylation variants of rtPA, named Type I and Type 2, were examined. Even though these variants contained the same amino acid composition, more than 20 peaks were visible in the electropherograms, reflecting the heterogeneity present in the two preparations. The number of potential glycoforms in proteins increases with the number of glycosylation sites. In addition, only some changes in glycosylation effect the protein's pI. Each peak may, therefore, represent a combination of glycoforms with the same pI. Resolution obtained with Yim's CIEF method was better than that with CZE. Treatment of the samples with neuramidase (which removes the sialic acids) dramatically simplified the electropherogram patterns. This would indicate that different levels of sialylation are largely responsible for the heterogeneity.

The heterogeneity of recombinant human erythropoietin (rHuEPO) has been characterized by various researchers (Watson and Yao, 1993 and 1994; Rush *et al.*, 1993; Tran *et al.*, 1991) using CZE and CE-based tryptic mapping. This 165-amino-acid glycoprotein has a MW of 34,000 to 38,000 while 40% of its weight consists of carbohydrate. Figure 2-18 shows the separation of rHuEPO glycoforms with the Beckman eCAP Amine coated capillary. The migration of the glycoforms is in the order of decreasing number of sialic acids (Watson *et al.*, 1994). The CZE method, while yielding slightly less resolution then slab gel IEF, offers the potential for quantitation in much shorter analysis times than with IEF.

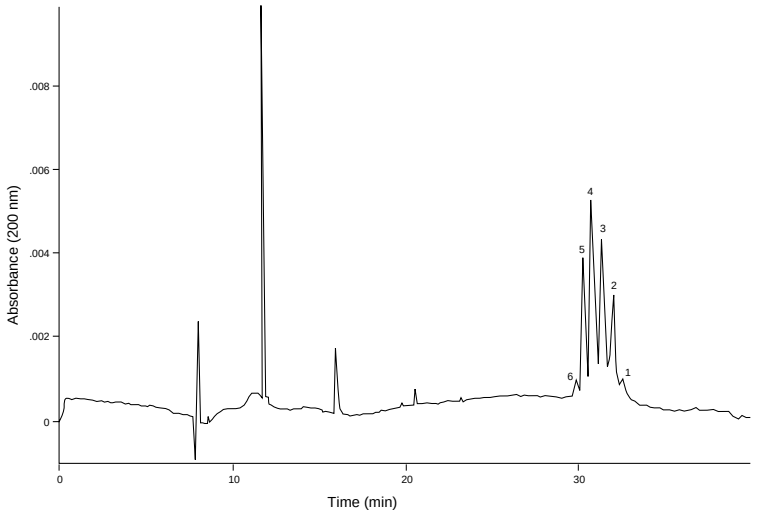


Figure 2-18. *CZE of r-HuEPO into six glycoforms on a eCAP Amine coated capillary. Voltage, 15 kV; sample concentration, 0.3 mg/mL; run buffer, 200 mM sodium phosphate, pH 4.0. Reproduced with permission from Watson* et al. *(1994).*

The separation of recombinant human interferon-γ (IFN-γ) under MECC conditions was described by James *et al.* (1994). With this glycoprotein, N-linked oligosaccharides may be attached to the 17-kDa protein backbone at one or two glycosylation sites, or may be absent. The electropherogram shown in Figure 2-19 reveals the difference in glycoform variants at these sites. It should be noted that, prior to the MECC analysis, the protein was denatured with SDS to prevent dimer formation. Further work by this group demon-

strated that CE can be used for real-time monitoring of glycosidase digestion. Other publications in which CZE is used for glycoprotein microheterogeneity analysis are Rudd *et al.* (1992) and Grossman *et al.* (1989)—ribonucleases; and Landers *et al.* (1992)—ovalbumin. Kelly *et al.* (1993) used the powerful combination of CE and electrospray MS to characterize ribonuclease, ovalbumin, and horseradish peroxidase (see Section 2.2.4).

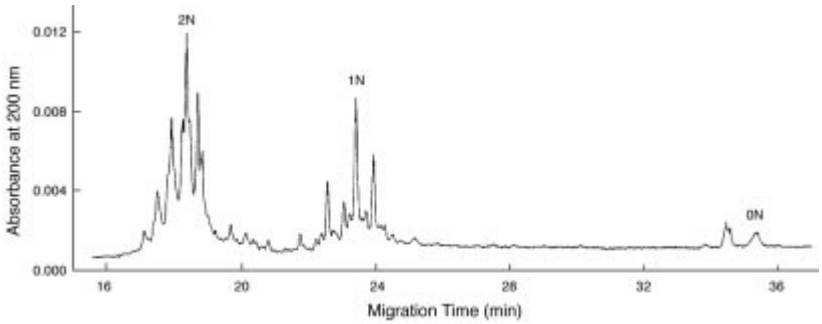


Figure 2-19. *Separation of IFN-λ glycoforms by MECC. Recombinant HuIFN-λ (1 mg/mL in 50 mM borate, 50 mM SDS, pH 8.5) was injected for 5 s prior to electrophoresis at 22 kV (120 μA) in 400 mM borate, 100 mM SDS, pH 8.5. Peak groups are designated as: 2N, two glycosylation sites occupied; 1N, one glycosylation site occupied: 0N, no glycosylation sites occupied. From James,* et al., Beckman Application Information Bulletin A-1761.

2.5.3 Heterogeneity in Proteins Relevant to Clinical/Diagnostic Applications

The utility of CE in clinical/diagnostic research is reviewed in several papers (see, for example, the special issue (Volume 15) of *Electrophoresis*, January 1994) and the book chapter by Klein and Jolliff (1994). Full discussion of this topic is beyond the scope of this book. The following papers deal with protein heterogeneity analysis by CE:

- Human transferrins. The heterogeneity results from differences in iron content, genetic polymorphism, and differences in glycosylation. Kilar and Hjerten (1989) used CIEF and CZE to study the glycosylation patterns in transferrin samples. Detection at 280 nm (for protein) and 460 nm (for iron) was used to identify the different transferrin isoforms (iron-free, monoferric, and diferric complexes).

- Hemoglobin variants. Zhu *et al.* (1992,1993) and Molteni *et al.* (1994) describe the utility of CZE and CIEF.
- Isoenzyme analysis. Klein and Jolliff (1994) proposed a new concept for lactate dehydrogenase isoenzyme analysis.
- Metallothionein isoforms. Beattie *et al.* (1993, 1994) used CZE and MECC for this type of analysis.
- Ferritin analysis. Zhao *et al.* (1994) used CZE for study of the apo-, holo-, recombinant-, and subunit-dissociated ferritins.

2.6 Stability

Methods commonly used for control and characterization of the stability of protein products are listed in Table 2-7. Physical and chemical instabilities of proteins can be related to such parameters as:

1) **Chemical reactions of amino acids.** For example, deamidation of asparagine and glutamine residues is common, as is oxidation of methionine and isoaspartate formation. The high resolving power of CE can be used to monitor the stability of products during storage. For example, Palmieri (1989), Frenz *et al., (1989),* and Grossman *et al.* (1989) demonstrated that CZE readily resolves the deamidated forms of hGH from nondegraded hGH. Figure 2-20 shows the difference between preparations of natural, pituitary-derived hGH (A) and recombinant hGH (B). The former shows a larger percent of mono- and dideamidated by-products. The desamido derivatives have an increased net negative charge due to the replacement of the side chain amide group with an ionized carboxylate. CIEF is also a very useful tool for these types of studies, as was demonstrated by Frenz *et al.* (1989) for hGH characterization. Peptide mapping (either by HPLC or CE) is another sensitive technique to detect subtle changes in amino acid composition. It has been shown that single amino acid deletions in a polypeptide chains can be detected by peptide mapping (Garnick *et al.* 1988).

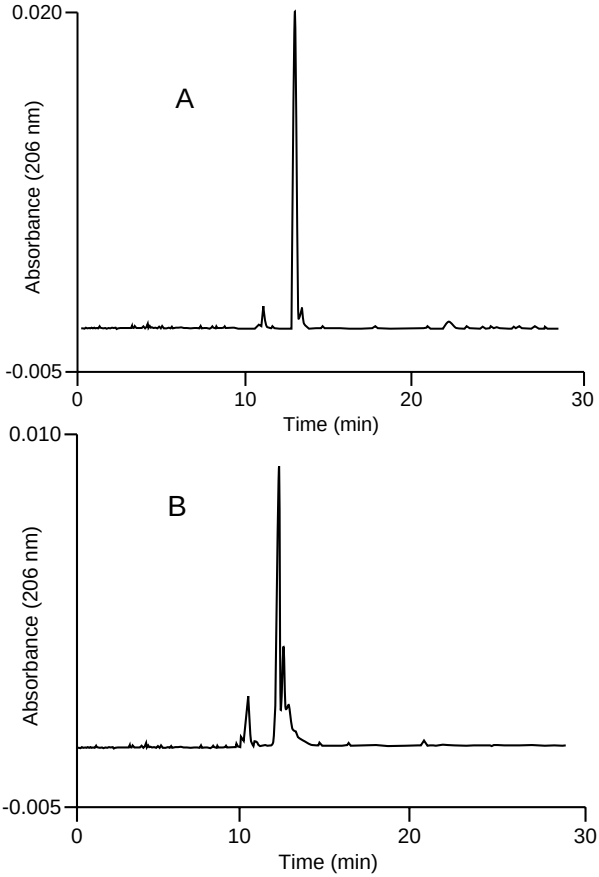


Figure 2-20. *(A) Separation of recombinant human growth hormone by CZE. (B) Separation of natural human growth hormone; run buffer, 100 mM borate pH 8.5; 7.5 kV; 20°C. From Palmieri,* Beckman Application Data Sheet DS-749.

2) **Deglycosylation of glycoproteins**. Glycoproteins from different manufacturing lots may differ in glycosylation pattern. Additionally, process-induced degradation may occur. Sialic acid groups, which have a bulky carboxyl group near the glycosidic bond, are particularly labile and prone to loss. CE can monitor deglycosylation, as shown in Figure 2-21. The enzymatically induced deglycosylation was followed over a 20-hour period. First an increase in singly glycosylated forms at the expense of dou-

bly glycosylated forms was observed. After 20 h, all of the N-linked oligosaccharides had been removed from the protein backbone.

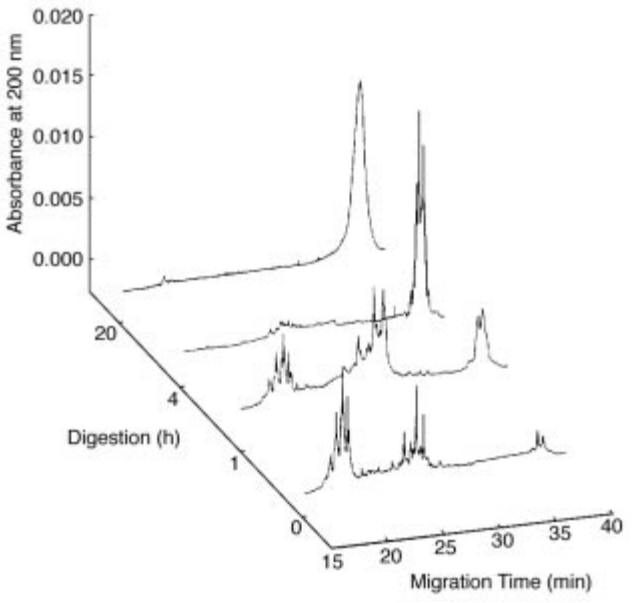


Figure 2-21. *Digestion of IFN-λ by PNGase F. Samples of the digest were injected for 4 s at the specified intervals and resolved by the electrophoretic conditions specified in Figure 2-19. From James* et al., Beckman Application Information Bulletin A-1761.

3) **Peptide bond cleavage.** In general, these types of instabilities are easily detected by chromatographic or electrophoretic techniques, as the molecular weight and also the hydrophobicity and/or the mass-to-charge ratio of the protein changes.

4) **Disulfide bond formation or reduction and scrambling**. Disulfide bonds are often required for biological activity. In cases where disulfide bond formation is undesirable, gentle oxidation can be carried out without dimerization which would occur under stronger conditions. CE can be utilized to monitor disulfide bond formation, as demonstrated by Landers *et al.*, (1993). Figure 2-22 shows the formation of homo- and heterodimers in the co-oxidation of synthetic peptides (termed Ntc and Ctc).

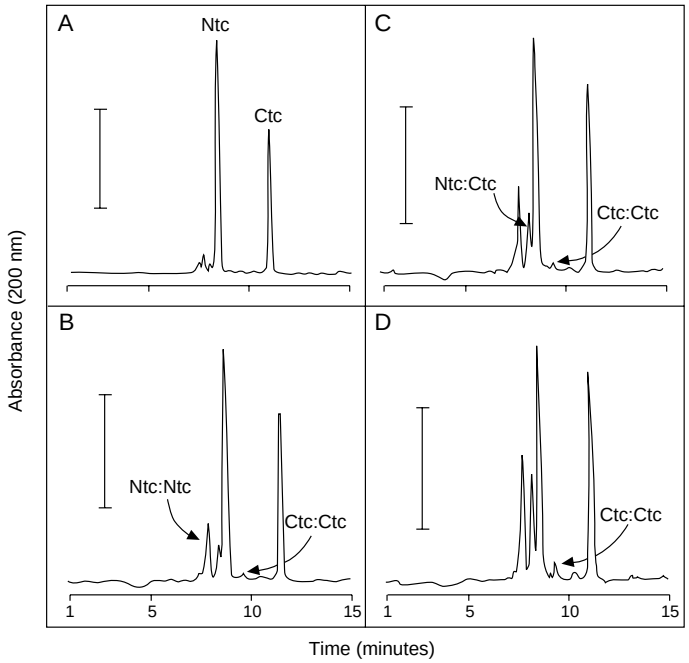


Figure 2-22. *Co-oxidization of the Ctc and Ntc peptides. A mixture of the purified Ntc and Ctc monomers was incubated at 27°C and analyzed at 0 min (Panel A), 2 h 46 min (Panel B), 5 h 31 min (Panel C), and 11 h 1 min (Panel D). Separation was carried out in 20 mM citrate buffer, pH 2.5. Bar represents 0.005 AU. From Oda* et al., Beckman Application Information Bulletin A-1757.

5) **Conformational changes (denaturation, aggregation)**. CE can monitor, for example, conformational changes with temperature variations, as shown by Rush *et al.*, (1991), protein folding (Strege and Lagu, 1993), and oligomerization processes (Tsuji, 1993). In Figure 2-23, the peak shape of α-lactalbumin (pI 4.2 to 4.5) is changing when the temperature control of the capillary is changed from 2 to 50°C. The broadened or multiple peaks (at 35 to 40°C) do not indicate that the protein sample is impure, but are a consequence of the fact that both the folded and conformationally altered species exist under the electrophoretic run conditions. Other temperature-related effects were observed with myoglobin (on-capillary reduction of the heme group).

Table 2-7. Methods Commonly Used for Characterization of Proteins: Stability

- Electrophoresis
  - SDS-PAGE
  - CE
  - IEF
- Chromatography
  - Size exclusion
  - Reversed phase
- Specific activity
- Differential scanning colorimetry
- Spectroscopy
- Mass spectrometry
- Analytical ultracentrifugation

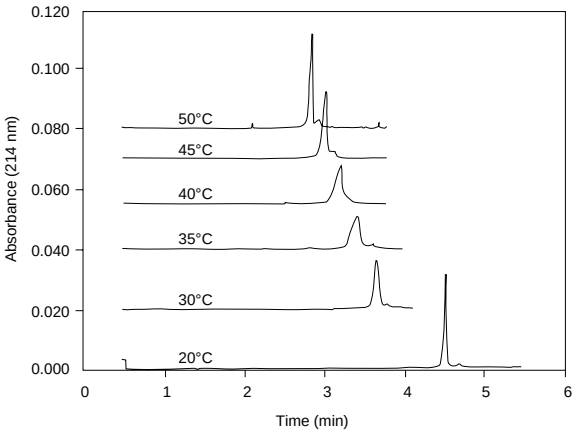


Figure 2-23. *Effect of temperature on the electrophoretic behavior of α-lactalbumin. Reproduced with permission from Rush* et al., Anal. Chem. 63, *1346 (1991).*

2.7 Process Consistency

When results from the analysis of multiple lots are compared, all of the above methods give information about process consistency. An additional example can be found the discussion of the CE analysis of Eminase in Section 2.8, following. From this information, control limits are established. Such information is also very valuable in pointing out areas in need of process optimization.

2.8 Method Validation

As required by the demands of good laboratory practice, as well as regulatory agencies, analytical procedures are typically validated for accuracy, precision, specificity, detection limit, quantitation limit, linearity, and dynamic range. This is particularly important in pharmaceutical biotechnology when the method is to be used for process or quality control purposes. With the advent of commercial instrumentation (including software allowing for reference peaks, internal and external standardization) and several years experience in hundreds of laboratories, it has become clear that optimized CE methods can be validated for process and quality control applications in biotechnology. Such CE methods are just as precise and accurate as methods based on chromatography. Support for this statement can be found in many recent papers on the CE of rDNA proteins and MAbs (see for example: Guzman *et al.*, 1992—CZE of MAbs and rDNA proteins; Arcelloni *et al.*, 1993—CZE of hGH; Pascual *et al.*, 1992—CZE of glutathione peroxidase; Harrington *et al.*, 1991—CZE of enzyme labeled IgG; Pande *et al.*, 1992—CZE of bovine serum albumin; Phillips, 1993—CZE of tissue cytokines; Moring, 1992, and Grossman *et al.*, 1989—quantitative aspects of CE; Bullock, 1993—analysis of rhIL-4; Tsuji, 1993—SDS-CGE of rbSt; Yowell *et al.*, 1993—CIEF and CZE of GM-CSF; Strege and Lagu, 1993—MECC of rDNA proteins in fermentation broths.

In many cases in the above papers, the CE method was validated by comparison with an already existing non-CE method. A case in point is the in-process assay for Eminase, a freeze-dried SmithKline-Beecham product used in the treatment of myocardial infarction. It is composed of an activator complex (lys-plasminogen:streptokinase) and human serum albumin which is added as a stabilizer. For the manufacturer of eminase, it was important to develop and validate a rapid, in-process assay which could result in considerable time savings in production runs (Figure 2-24). Eminase dissociates into its constituents under acidic conditions. Note that lys-plasminogen reveals two peaks, corresponding to the two glycosylated forms.

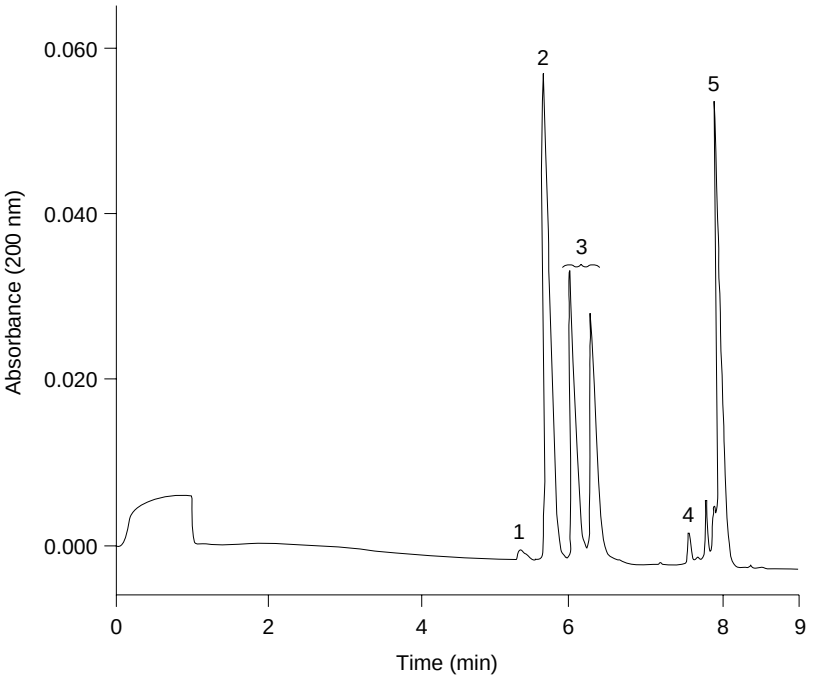


Figure 2-24. *In-process assay for Eminase. Buffer: 40 mM sodium phosphate, pH 2.5, with 0.01% HPMC added. Peak identification: (1) Eminase buffer component; (2) human serum albumin; (3) lys-plasminogen; (4) acylating agent; (5) streptokinase. From Birell and Brightwell, SmithKline-Beecham Pharmaceuticals, UK (1990).*

Four in-process samples, together with 9 different batches of freeze-dried eminase, were assayed for lys-plasminogen content relative to an in-house reference preparation of known potency.

The results of the CE method and the existing activity method are shown in Table 2-8. The CE method was also shown to be linear over the range tested (0 to 250% of the expected assay result) whereas precision with CE (2 to 3% RSD) was better than with the existing activity method (3 to 4% RSD).

Table 2-8. Comparison of CE with an Existing Activity Assay

In-process Samples	Activity Assay (mg/mL)	CE (mg/mL)	CE: Activity	Eminase	Activity Assay (mg/vial)	CE (mg/vial)	CE: Activity
1	9.32	9.54	0.982	1	29.0	29.4	1.01
2	6.14	6.07	0.989	2	31.7	29.1	0.31
3	5.38	5.78	0.983	3	31.3	32.3	1.03
4	6.66	6.58	0.988	4	31.5	30.9	0.98
				5	30.2	30.6	1.01
				6	32.2	31.0	0.96
				7	31.5	28.7	0.91
				8	31.5	29.1	0.92
				9	29.7	28.3	0.95

Four in-process samples together with 9 different batches of freeze-dried Eminase assayed for lys-plasminogen content relative to an in-house reference preparation of known potency. From Birell and Brightwell, SmithKline-Beecham Pharmaceuticals, UK.

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