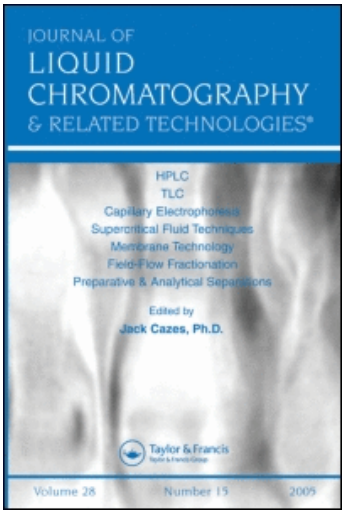

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SEPARATIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY ZONE ELECTROPHORESIS: A COMPARATIVE STUDY

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ABSTRACT

In this comparative study we attempt to examine the similarities and differences between analytical high-pressure liquid chromatography and capillary zone electrophoresis with regards to mechanism of separation, instrumentation and fields of application. Based on careful reading of the recent literature it is safe to conclude that the two techniques are complimentary, especially for the separation and analysis of biomolecules. Both have points of strength and weakness. Capillary zone electrophoresis is superior whenever high peak capacity is required such as in the analysis of DNA fragments, while high pressure liquid chromatography is superior for small and neutral molecules and in its quantitative capabilities.

INTRODUCTION

High Performance Liquid Chromatography (HPLC) is a well established separation technique which is suited for micro as well as macro separations. Capillary Zone Electrophoresis (CZE) is a novel technique which is only suited, in its present form, for the analysis of micro samples. Basic differences exist between these two techniques, in mechanism of separation, solute migration, and instrumentation.

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In this review we wish to compare the two techniques as well as outline the advantages and disadvantages of each and show how they complement each other. With regards to HPLC the discussion will focus on the most popular mode (i.e. the conventional analytical column with 1-5 mm i.d.) and not the more recently introduced and much less frequently used techniques of micro and open-tubular columns. Electrophoresis is the main separation technique used by biochemists even though chromatographers have only been recently introduced to it when it was demonstrated that electrophoresis could be performed in a capillary tube with an unprecedented degree of column efficiency (1). Chromatographers, on the other hand, have been involved in biomedical analysis ever since reversed phase, hydrophobic interactions, affinity, ion-exchange and size-exclusion modes of HPLC were developed whereby water soluble solutes could be analyzed. The recent development of CZE in the 1980's presented the technique as complementary and in some instances a better substitute for HPLC.

This article is not intended to be a comprehensive review and only representative and illustrative examples will be given. HPLC was recently reviewed by P.R. Brown (2) who gave a critical assessment of the past, present and a projection of the trends in the near future. Ewing and co-authors provided comprehensive reviews of capillary electrophoresis (3,4). Other reviews (5-8) covered various aspects of the technique.

THEORY

The mechanisms of separation in all chromatographic techniques including HPLC is based on the differential partitioning of the solutes between two immiscible solvents. The theory has been outlined in many excellent books and reviews and will not be discussed further in this article. Although the theory of electrophoresis is complex yet its analytical aspects, to a first degree of approximation, is simple and easy to comprehend. Separations in CZE are due to differences in the electrophoretic mobilities of charged solutes in a voltage gradient inside a buffer filled capillary. The buffer anions and cations, pH and concentration are important factors in determining not only the electrophoretic and osmotic flow velocities but also the amount of heat

generated inside the capillary. Electroosmotic flow velocity in turn affects separation efficiency as well as resolution (1). In what follows the salient features of CE theory as it relates to solute migration, column efficiency and resolution will be briefly outlined.

When a charged particle is placed in an electric field (E) it experiences a force which is proportional to its effective charge (q) and the electric field strength. The translational movement of the particle is opposed by a viscous drag force which is proportional to the particle velocity (V), hydrodynamic radius (r) and medium viscocity (η). When the two forces are counterbalanced the particle moves with a steady state velocity (9):

$$V_{ef} = \mu_{ef} E \qquad \text{(I)}$$

where μ_{ef}, the electrophoretic mobility, is given by:

$$\mu_{ef} = \frac{q}{6\pi\eta r} \qquad \text{(II)}$$

According to Huckel (10) the electrophoretic mobility is related to the Zeta potential (the potential at the plane of shear i.e. at the effective radius of the charged particle) by the following expression:

$$\mu_{ef} = \frac{D\,\zeta_{ef}}{6\pi\eta} \qquad \text{(III)}$$

where ζ_{ef} is the electrophoretic Zeta potential and D is the dielectric constant of the medium.

Equation III differs only by a constant factor of 2/3 from the Helmholtz von Smoluchowski equation (11):

$$\mu_{ef} = \frac{D\,\zeta_{ef}}{4\pi\eta} \qquad \text{(IV)}$$

which according to Henry (12) is a better representation of the electrophoretic mobility. Moreover, the Zeta potential is directly proportional to the thickness of the double layer (δ, The Debye radius) which, in turn, is related to the concentration of the buffer (13) according to the following approximate relationships:

$$\zeta_{ef} = \frac{4\pi\delta e}{D} \qquad \text{(V)}$$

$$\delta \simeq [3x10^{7}|Z|C^{1/2}]^{-1} cm \qquad \text{(VI)}$$

where e, Z and C are the total excess charge in solution per unit area, the number of valence electrons and the concentration of the buffer, respectively. Equations V and VI are valid for aqueous solutions under certain experimental restrictions outlined in reference 13.

Electroosmosis in capillary tubes, on the other hand, refers to the propulsion of the bulk solvent in the tube under the influence of an applied electric potential. The surface of silica consists of Si-OH groups which are ionized to SiO^{-} in alkaline and slightly acidic media (PH>2). The negatively charged surface is counterbalanced by positive ions from the buffer and a double layer is formed. Under the influence of an applied potential the positive ions in the diffuse region migrate towards the cathode and in so doing they entrain the water of hydration resulting in electroosmotic flow. The equations of electroosmotic flow are identical to those developed for electrophoretic migration since both phenomena are complementary. The electroosmotic velocity (V_{eo}) is given by:

$$V_{eo} = \mu_{eo} E \qquad \text{(VII)}$$

where μ_{eo} (the electroosmotic mobility) is represented by an expression similar to equation III:

$$\mu_{eo} = \frac{D\,\zeta_{eo}}{4\pi\eta} \qquad \text{(VIII)}$$

ζ_{eo} is the electroosmotic Zeta potential and all other terms are as given earlier.

Wieme (9) predicted that the electrophoretic mobility and its complementary electroosmotic mobility should be directly proportional to the reciprocal of the square root of ionic strength. A combination of equations IV, V and VI results in the following expression for electrophoretic mobility which clearly agrees with Wieme's prediction:

$$\mu_{ef} \propto \frac{e}{3x10^{7}|Z|\eta\sqrt{C}} \qquad \text{(IX)}$$

The migration time (t_m) which is measured directly from the electropherogram is related to the mobility by the following:

$$t_m = \frac{l}{v} = \frac{l}{\mu E} \qquad \text{(X)}$$

where l is the column length from injector to detector and μ is the net mobility ($\mu_{ef} + \mu_{eo}$).

Column efficiency in CZE is gauged by the number of theoretical plates (N) generated by the column. The expression for N has its basis in chromatographic theory and is defined as (1).

$$N = \frac{\mu V}{2D} \qquad \text{(XI)}$$

The assumption inherent in this expression is that solute diffusion is the only zone broadening mechanism. According to equation XI the number of

theoretical plates increases with increasing voltage. Maximum column efficiency is attained at the highest possible applied voltage. Unfortunately, the buffer cooling techniques used in CZE instruments are not efficient enough to dissipate the heat generated at very high voltages. Consequently, buffer temperature is increased and a radial temperature gradient is established inside the column. As a result N approaches a maximum as the applied voltage is increased then drops as the voltage is increased further. The magnitude of the applied voltage at which N plateaus depends on the buffer concentration (ionic strength), column length and radius. Thus the limit of efficiency in CE is Joule heating, while in HPLC the limit is the rate of solute mass transfer between the stationary and the mobile phases.

Finally, the resolution (R_s) equation in CZE is given by (14):

$$R_s = \frac{N^{1/2}}{4} \frac{\Delta\mu}{\bar{\mu}} \qquad \text{(XII)}$$

where $\Delta\mu/\mu$ is the relative mobility difference of the two solutes being separated.

While the effect of applied voltage on column efficiency and resolution has attracted the attention of many workers (13,15-21) the effect of buffer type and buffer concentration has been largely ignored. The latter effect has been the subject of detailed studies in our laboratories (22-25). According to the theoretical equations that have been qualitatively verified by our results, the solute migration time is increased by about 30% if the buffer concentration is doubled while it is cut in half if the applied voltage is doubled. Furthermore, doubling the applied voltage results in a 4-fold increase in the amount of heat generated while doubling the buffer concentration cause a 2-fold increase only. Reduction in analysis time is not necessarily advantageous since column selectivity (as measured by the separation factor) will be adversely affected. In contrast increasing the buffer concentration results in improved column selectivity. Hence, resolution is better enhanced by increasing buffer concentration at a moderate applied voltage.

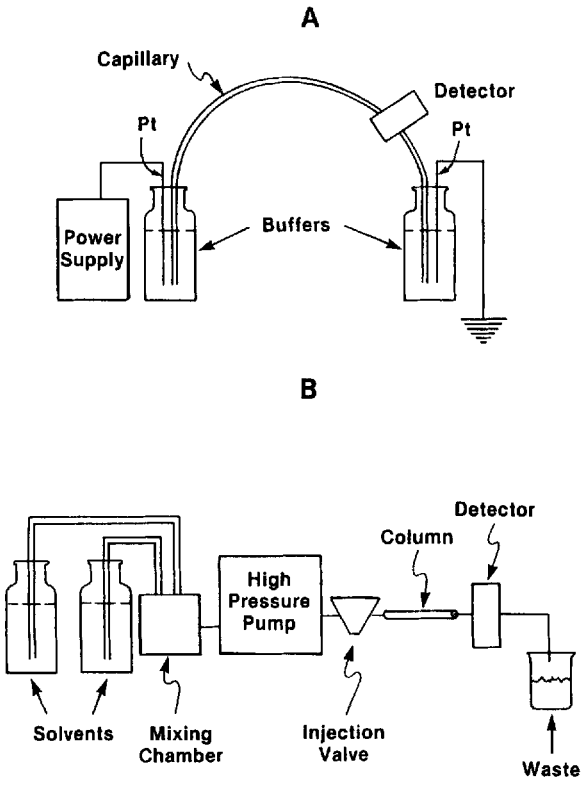

Figure 1. Schematic diagram of CZE (A) and HPLC (B) instrumentation.

INSTRUMENTATION

The instrumentation for HPLC and CZE are similar in some respects but different in others. It is simpler for CZE due to the absence of an injector, a pump and a solvent mixer (or proportionating value) and a special detection cell. In CZE, on-column detection is performed. Figure 1 is a schematic diagram of HPLC and CZE instrumentation. The finer difference and similarities between both techniques will be discussed below:

a) Injection (Sample Introduction)

The sample solution in HPLC is introduced into the column by placing a known volume, with the aid of a syringe in the injection valve's cavity, after

which it is swept into the head of the column by the mobile phase. The volume of the injected solution is exactly measured, quantitatively known, and reproducible.

In CZE, there is no injection valve as in HPLC. The sample solution is introduced into the capillary by one of two modes:

I) Electrokinetic (26)

II) Hydrodynamic (27)

The hydrodynamic injection can be any one of three: i) pressure; ii) vacuum; or iii) gravity. Unlike HPLC the amount injected in CZE is not immediately known and has to be calculated depending on the mode of sample introduction employed. The injection amount using electrokinetic mode is calculated from the following equation:

$$amount\ injected = (\mu_{ef}+\mu_{eo})\frac{\pi r^2 VtC}{L} \qquad \text{(XIII)}$$

if $\mu_{eo}>>\mu_{ef}$ (at high buffer pH) therefore:

$$amount\ injected \cong \frac{\mu_{eo}\pi r^2 VtC}{L} \qquad \text{(XIV)}$$

where L = total length of column

r = radius of the column

V = applied injection voltage

t = time of applied voltage

C = solute concentration

For pressure injection the following equation is used:

$$amount\ injected = \frac{\Delta P\pi r^4 tC}{8\eta L} \qquad \text{(XV)}$$

where ΔP = pressure differential across the column

η = viscocity of the medium (buffer)

t = injection time

It is clear from the equations that the parameters which will affect the reproducibility of sample introduction are t and V in the electrokinetic mode and t and ΔP in the hydrodynamic mode. It is also important to know the exact measurements of the column dimensions (r and L) in order to calculate the amount injected.

It has been observed (28,29) that in electrokinetic injection the sample components are preferentially migrated into the capillary depending on their charge to size distribution (electrophoretic mobility) which means that the amount of each component should be determined separately using eq. (XIII). This problem does not exist in HPLC. Other types of injection devices were developed (30-33), however, they have not been commercialized. Olefirowicz and Ewing (33) reported the use of a modified microinjector for the direct sampling of single cell cytoplasm without the need for extensive sample handling. This technique allowed the study of easily oxidized neurotransmitters and metabolites in the cytoplasm of single intact neurons. Conventional HPLC cannot be used for such an application because of the very minute sample size. Open tubular liquid chromatography has been used with limited success because of the extensive sample preparation required, and the band broadening effects introduced by laminar flow during microsyringe injection (33).

Since CZE is a micro separation technique the volume introduced into the column is in nanoliters, in HPLC it is in microliters. The volume of sample required to perform an analysis is 1-5 μl for CZE and 5-25 μl in HPLC depending on the solute's concentration and mode of detection.

Collection of sample fractions in HPLC is much easier than that for CZE, since the two ends of the capillary has to be at all times in the buffer. Wellingford and Ewing (34) were able to collect fractions of analyzed samples by using a porous glass junction which will not interrupt the flow of current.

Huang and Zare (35) used an on-column frit structure that allows the flow of current and would not interrupt neither the electrophoretic process nor dilutes the zones collected. Other fraction collectors were used but lack the advantages of those mentioned above (36,37).

b) Flow Characteristics:

The sample in HPLC is swept from the injector to the head of the column with the mobile phase which is being driven by a pump at a predetermined constant flow rate. The speed of analysis is a function of the mobile phase composition and flow rate. In free solution CZE solute migration is influenced by the mobile phase (buffer) velocity. This in turn is determined by the electroosmotic flow which is a function of: (a) buffer type; (b) concentration (ionic strength); (c) pH, and (d) applied voltage among others. The buffer's cation and anion affect the electoosmotic flow, the larger the cation the slower μ_{eo}. Also, V_{eo} increases with increase in the applied voltage and decreases with increasing the buffer's concentration and viscosity. The flow is therefore the fastest under conditions that maximizes the zeta potential (see theoretical section for details).

Since the flow of the mobile phase is controlled differently in HPLC (a pump) and CZE (electrically) the flow profiles are different. In HPLC the profile is parabolic while in CZE, where mainly longitudinal diffusion is present, is flat (see figure 2). This flat profile of CZE results in narrower peaks and better resolution.

c) The Buffer in CZE:

The buffer plays an important part in CZE (22-24). As mentioned above the buffer type, concentration (ionic strength), pH and viscosity affect not only the electroosmotic flow but efficiency, selectivity, and resolution.

The type of buffer used will not only affect the CZE selectivity but will also affect the amount of Joule heat generated in the capillary (22-24). For example, it was found that at an applied voltage of 20 kV a 0.1 M LiAc gave a current of 111 μA, while KAc at the same concentration and pH gave a current of 195 μA.

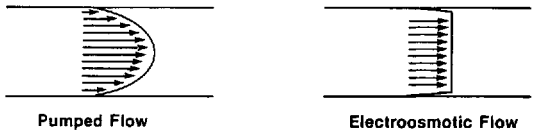

Figure 2. Flow profiles in HPLC (left) and CZE.

Also, small changes in buffer composition and pH can affect CZE separations (22-24, 38,39). It is recommended that the buffer be replaced after 4-6 runs with fresh buffer, while replacement of the mobile phase is not a requirement in HPLC.

Care should be taken not only in selecting the buffer that will give a low current but in its preparation. For example, a 0.1 M sodium phosphate buffer (pH 6.5) can be prepared by any one of the following procedures: a) titrating the acidic salt (NaH_2PO_4) with NaOH; b) titrating the acidic salt with the basic salt (Na_2HPO_4); c) titrating the basic salt with H_3PO_4; and d) titrating H_3PO_4 with NaOH. These four procedures should yield the same buffer if it is carefully prepared. The difference in the results reported elsewhere (22) is due to differences in buffer concentration as a result of the procedure used for the preparation of the buffer.

d) The Column:

HPLC is a micro as well as a macro separation technique where the column diameter can vary considerably while the column diameter in CZE is limited by the efficiency of heat dissipation. Since the heat gradient between the center of the capillary and the walls is proportional to the square of the radius, the more efficient the system in heat dissipation the wider is the diameter of the column that may be used (16). So far, capillaries of 2-200 μm diameter and 10-100 cm length have been used. The longer the column the less the resistance (less heat generated) so higher voltages can be applied which will result in higher column efficiency. Assuming that the buffer concentration, type and pH are the same, resolution and efficiency are a function of applied voltage and not column length (14).

The heat generated in CZE is the limiting factor and it is directly proportional not only to the column diameter but the buffer concentration (C), applied voltage (V) and molar conductance (K). The power generated (W) can be evaluated from the following equation:

$$W = \frac{KCr^2V^2}{L} \qquad \text{(XVI)}$$

when K, C and V are constant. The factors which will affect heat generation are the column's radius and length.

The column in CZE is made of fused silica, glass or teflon and filled with a buffer or a gel (not available commercially). In HPLC the column is made of a stainless steel tube which is filled with spherical or irregular shaped silica particles to which may or may not be bonded an aliphatic, aromatic, ionic or other groups depending on the mode of separation used (normal phase, reversed phase, ion exchange, chiral or mixed mode, etc). In HPLC the silanol groups are derivatized with a short chain aliphatic silane. In CZE the silanol groups contribute to the generation of electroosmotic flow which may be advantageous for the separation of small molecules, however, they are a disadvantage when large molecules (proteins) are to be separated. Another disadvantage is that the charged interior surface may attract unwanted charged species to the surface which will alter the chemistry of the column and result in irreproducible results. In this case the column has to be washed with 0.1 M NaOH or KOH solution. The interior surface chemistry of the fused silica capillary may be modified chemically or physically to achieve the required separation mechanism. This is achieved by derivatizing the silanol groups (as mentioned above) or by the addition of a modifier to the buffer solution. One has to remember that derivatization of the silanol groups and the generation of a neutral surface will eliminate the electroosmotic flow which will lead to longer migration times of the solutes but better separation, especially of biomolecules, because solutes are resolved by the differences in their electrophoretic mobilities.

e) Detection:

Modes of detection in both HPLC and CZE are similar (uv, fluorescence, electrochemical, mass spectrometry, etc.). However, since in HPLC the column diameter is wider and allows the injection of 100-1000 times larger samples, off column detection is feasible and much easier to achieve.

In CZE, where the injected volumes are in nanoliters and the separated zones are extremely narrow, off-column detection is not, at this time, the method of choice, as in HPLC. Since fused silica capillaries are transparent to uv and fluorescence radiation, the method of choice for CZE is on-column detection. By doing so, the analyst can detect the narrowest of zones and also eliminates any zone dispersions, which may affect the resolution of closely emerging peaks (zones). In addition to uv absorption and fluorescence especially laser induced fluorescence, the following detection methods were employed, raman (40), mass spectrometry (41), electrochemical (42), conductivity (43) and radioisotope detection (44). For detailed discussion see refs. (45) and (4). The sensitivity factor in CZE is 20-100 times higher than in HPLC (45) while the concentration sensitivity in HPLC is at least 10 times better than that of CZE (46). This is due to the fact that the cell path length (capillary width) in CZE is much smaller than that in HPLC. Also, the amount injected in HPLC (μl) is much larger than that in CZE (nl). Erni et al (46) also found that a 100 fold increase in sample concentration resulted in 4 fold decrease in CZE column efficiency which leads to unacceptable loss of resolution. Weinberger and Albin (this issue, figure 7) found that a 4 fold increase in solute concentration leads to loss of resolution. However, increase in loading capacity can be achieved by increasing the ionic strength but this may lead to the generation of excess Joule heat. This may be eliminated by decreasing the column radius, which will result in loss of sensitivity. Decreasing the column's diameter by half would cut the cell size in half and pass only one-fourth the current.

COLUMN EFFICIENCY AND RESOLUTION

Peak capacity in CZE is much higher than that in HPLC. This is due to the flat flow profile of CZE compared to the parabolic flow profile of HPLC,

as mentioned earlier. Due to this fact column efficiencies in CZE of 500,000 to 1.5 million theoretical plates have been reported. These high column efficiencies allow the resolution of closely eluting peaks and the separation of a large number of components in a mixture. Karger et al (37) were able to resolve a mixture of oligonucleotides, which would have been difficult to do by HPLC.

Another example of the high efficiency of CZE is the separation of dansylated methylamine from dansylated methyl-d_3-amine using micellar electrokinetic capillary chromatography with 25 mM SDS, 20% methanol modified phosphate-borate buffer (47). The separation factor (α) obtained for this almost baseline separation was 1.009. The same two compounds were also resolved by reversed phase micro (5 μm) column chromatography giving an α of 1.025 (48).

MODIFIERS

In HPLC the use of mobile phase modifiers to improve resolution and selectivity is very common. As a matter of fact whenever a mobile phase consists of more than one pure solvent, the second one is a modifier. For example, in reversed phase HPLC polar organic solvents are used as modifiers. Ion pair reagents are added to the mobile phase to suppress certain interactions, organic molecules such as triethylammonium acetate are added to suppress the interaction of the silanol groups with the solutes to be separated.

In CZE modifiers are added to effect a separation and enhance selectivity by suppressing the electroosmotic flow, which as a result decreases the migration time and increases the resolution. Cyclodextrins and micelles such as sodium dodecyl sulfate (SDS), in addition, methanol and acetonitrile have been used as mobile phase additives. Terabe et al (49) improved the selectivity of the buffer by adding surfactants, such as SDS, above their critical micelle concentration, to improve the resolution of charged compounds and to effect the separation of neutral molecules. This technique, known as micellar electrokinetic capillary chromatography (MECC), is widely used and well

accepted. A good review of the subject is given in this issue by Weinberger and Albin.

Gradient elution is another way of modifying, with time, the mobile phase. It is automatically and easily done in HPLC employing both continuous or step gradient. In CZE gradient elution is not commonly utilized. Few attempts have been described whereby solvent and voltage gradients have been used. Balachunas and Sepaniak (50) utilized a mechanical setup to achieve step gradient elution. The inlet reservoir was placed on a magnetic stirrer to which was added 0.5 ml of 2-propanol every five minutes. A small magnetic stirring bar ensured thorough mixing. Bocek et al (51) performed CZE in a mobile pH gradient which is dynamically programmed, whereby a moving pH profile along the separation path is generated. In a recently published study (52) an ionic matrix pulse was used to effect the separation. Pulses of pH and counter ions were used to quantitatively control the selective effects of the pulse by controlling its composition, its length and direction of migration. The introduction of mobile phase plugs (pulses) in HPLC to achieve the separation of coeluting solutes was first introduced by Berry in 1984 (53). Rose and Jorgenson (54) applied, after injection, voltages of 5, 10 and 15 kV for 60 seconds each before applying the 30 kV run potential. McCormick (55) reported that a voltage gradient after injection improved the column's efficiency.

FIELDS OF APPLICATION

Both HPLC and CZE can resolve large as well as small molecules, charged and neutral compounds. However, HPLC is more suited for the separation of small neutral molecules, while CZE is more suited for the separation of large biomolecules and charged compounds.

Due to the high resolving power of CZE columns (>500,000 plate counts and >1.5 million plate counts in some gel filled columns) the separation of complex mixtures (large number of solutes, or a mixture of neutral and charged compounds) is more suited to CZE than HPLC. Also, CZE is better suited for purity analysis where small sample volumes are available, and where the

impurities are a small percentage of the sample. It is easier to detect a small peak by CZE due to the sharpness of the peak than by HPLC. Such an application will be important in the pharmaceutical industry where impurities in a drug should not exceed 1% each.

This section gives a brief account of selected areas of CZE applications. Since HPLC is much more developed compared to the more recent CZE technique the emphasis will be on the complimentary nature of CZE in areas of application where HPLC methods have been routinely used.

a) Amino Acids and Peptides:

When capillary zone electrophoresis was first introduced (1) the model compounds chosen to illustrate the utility of this novel technique were amino acids and peptides. Conventional gel electrophoresis is a well established technique for the analysis of proteins (56). However, because of problems in separation, quantitation and reproducibility this method has not been particularly suited for the analysis of small peptides (molar mass <5,000). Among other available analytical techniques, reversed phase HPLC is the most widely used. One of the most powerful procedures involved in the confirmation of protein structure is to use reversed phase HPLC for "fingerprinting" of peptide fragments from proteolytic enzyme digests of protein samples (56-58). Despite the great advances achieved by this technique, in the analysis of small peptides and protein triptic maps, it too suffers from low resolution and limited peak capacity. CZE is an alternative technique that offer higher resolving power and a completely different separation mechanism (4,7,55,59-61).

In HPLC the primary mechanism of separation of peptides is hydrophobic interaction while in CZE the molecules are separated based on differences in charge, size and shape. The results of the two techniques are orthogonal and complimentary. Several workers have used CZE for the separation of peptides. The high resolving power of CZE allows the separation of peptides that differ only slightly in the net charge. Furthermore, depending on the peptide mixture at hand the pH of the buffer can be adjusted to maximize differences in net charge and hence improve the separation (55,61).

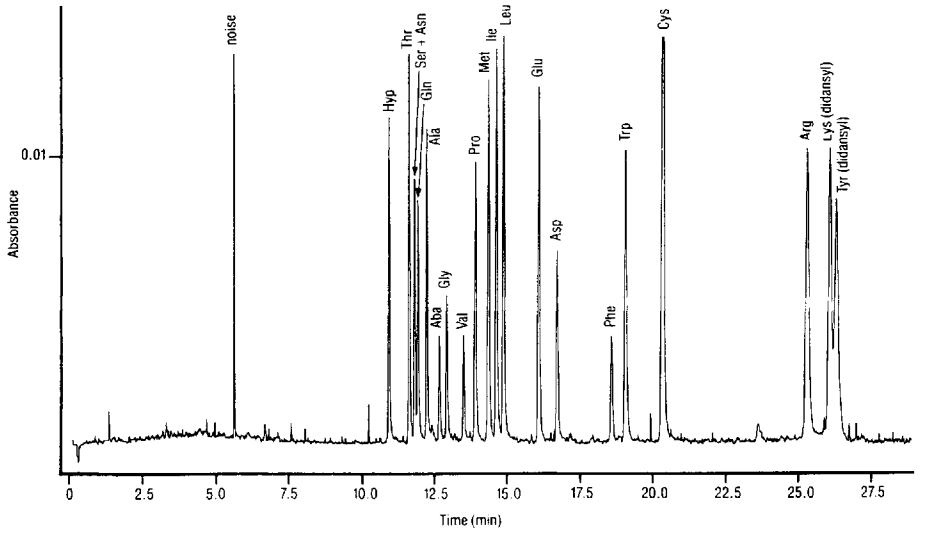

Figure 3. Analysis of 21 DNS-amino acids by CZE. Separation was performed at 15 kV (52 μA), capillary temperature of 20°C, and detection wavelength of 200 nm. (Reprinted from ref. 62).

The separation of solutes with similar electrophoretic mobilities can be affected by MECC, and chiral separations can be achieved if cyclodextrin is added to the micellar solution. To illustrate, Figure 3 shows the separation of 21 DNS amino acids by MECC with a 100 mM sodium dodecyl sulphate (SDS) solution (62). Separation of the same set of solutes by HPLC requires gradient elution and longer analysis time. Chiral separations are represented in Figure 4 which shows the separation of five DNS-DL-amino acids, by MECC with γ cyclodextrin modifiers (62). Compared to HPLC only small quantities of chiral selectors are needed to afford a separation. The complimentary nature of CZE is clearly illustrated in figure 5 which gives a comparison of HPLC and CZE profiles (fingerprints) for a trypsin digest of human growth hormone (7). The figure clearly shows that the fragments that are not separated by HPLC are separated by CZE and vice-versa. Furthermore, since the mechanisms of separation are different, no correlation is found between the elution order in CZE with that in HPLC. Also, it is clear from the figure that the resolution

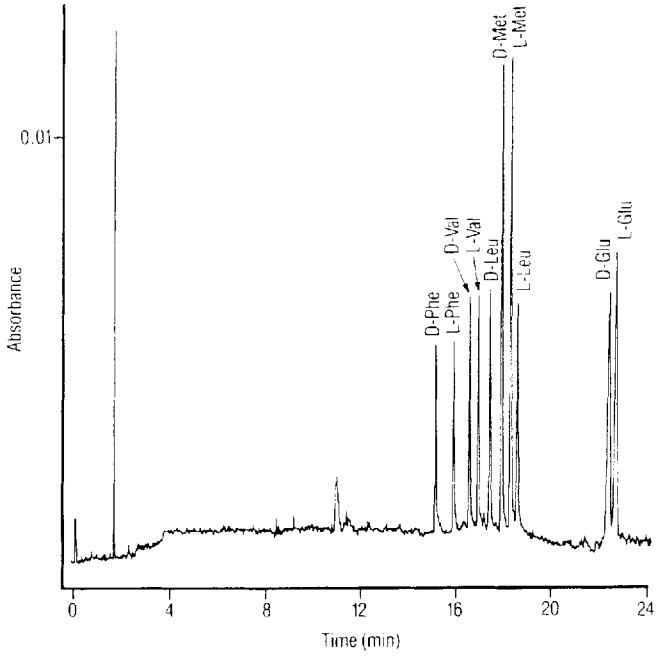

Figure 4. Chiral separation of five DNS-DL-amino acids by CZE. Separation was performed with 60 mM γ-CD in a 100 mM SDS solution (pH 8.3) at 12 kV (47 μA). Other conditions were the same as given in Figure 3. (Reprinted from ref. 62).

and peak capacity are much better in CZE compared to HPLC. Figure 6 illustrates this advantage in the assessment of purity of synthetic peptides. The HPLC chromatogram shows one peak while the CZE electropherogram of the same sample shows six peaks. Another advantage of CZE is analysis time which is much shorter in comparison to HPLC as clear from figures 5 and 6.

b) Proteins:

Significant advances have been accomplished lately for the adaptation of HPLC techniques for the analysis of proteins. New HPLC columns suitable for protein analysis have been introduced. Affinity chromatography makes use of biospecific interactions and size-exclusion columns separate proteins based on size differences. Moreover, hydrophobic interaction chromatography is used

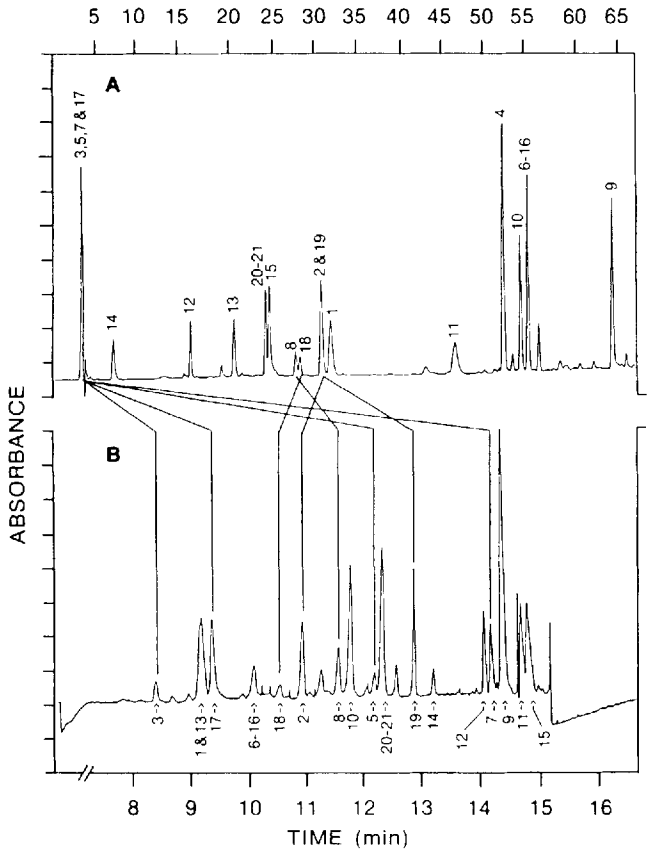

Figure 5. Comparison of RPHPLC (A) and CZE (B) tryptic maps. Peak correlations for selected digest fragments are illustrated, and peak assignments are indicated. Electrophoresis conditions: field, 316 V/cm; current, 20 μA; buffer, 0.01 M tricine, 0.045 M morpholine, 0.02 M NaCl, pH 8.0; detector, 200-nm wavelength. Chromatographic conditions: column, Aquapore RP-300 (4.6 x 250 mm, Brownlee Labs); flow rate, 1 mL/min; detector, 214-nm wavelength; solvents, A = 0.1% trifluoroacetic acid (TFA) in water, B = 0.1% TFA in acetonitrile; gradient, 0-20% B in 20 min., 20-25% B in 20 min., 25-50% B in 25 min. (Reprinted from Ref. 7).

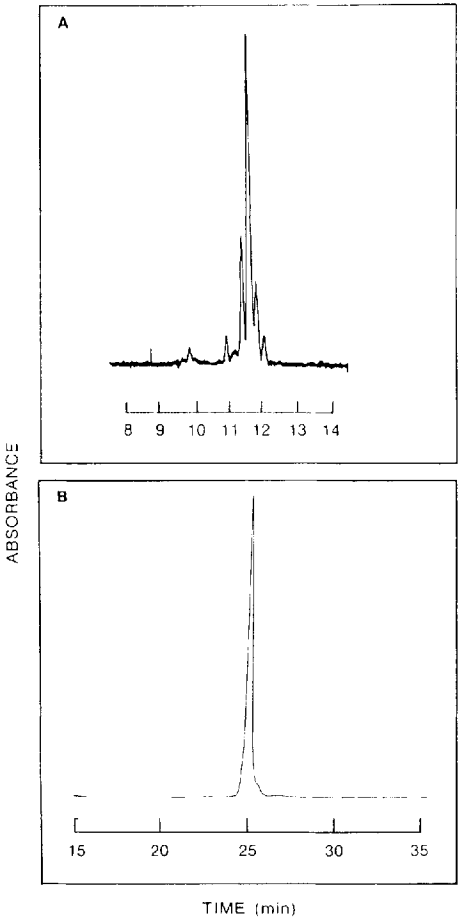

Figure 6. Qualitative comparison of the CZE profile (A) of a commercially produced synthetic peptide (proprietary sequence) with its RPHPLC profile (B). Electrophoresis conditions are the same as in Figure 5. Chromatographic conditions: column, Brownlee C8 (2.1 x 220 mm, Brownlee Labs); flow rate, 230 μL/min; detector, 214-nm wavelength; solvents, A = 0.1% trifluoracetic acid (TFA)in water, B = 0.08% TFA in acetonitrile; gradient, 0-60% B in 45 min. (Reprinted from Ref. 7).

for selective adsorption of proteins on materials such as calcium phosphate gels and reversed phase HPLC columns are commonly used for small proteins (63). The strength of HPLC lies not only in the variety of methods that could be used for protein separation but also in the fact that each method could be scaled-up for preparative purposes.

On the other hand, electrophoretic techniques, although not often used for preparative protein purification, are the most widely used analytical separation techniques in enzyme and protein chemistry. CZE is well suited for the analysis of large biomolecules, because the low diffusion coefficients for these molecules (which is a handicap in HPLC) is an advantage in CZE as it results in high separation efficiency. The advantages of capillary electrophoresis in terms of high resolving power and its limitation in terms of small sample capacity have already been discussed earlier.

A further limitation in the CZE analysis of proteins is the undesirable Coulombic interactions between the positively charged proteins and the negatively charged capillary wall which result in band broadening and sample loss. This handicap was investigated by several workers and remedies such as the proper choice of buffer pH, column deactivation, the use of buffer modifiers and gel filled columns were suggested (11,55,64-70). The cited literature offers many examples of protein separations using these techniques. The use of sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE)filled columns is perhaps the most promising. Cohen and Karger have developed efficient gel filled columns and used them for the separation of both proteins and oligonucleotides. Figure 7 is a representative example of their work (68).

c) Oligonucleotides, Nucleotides and Nucleosides:

The separation of oligonucleotides by HPLC has witnessed a significant improvement with the introduction of new types of stationary phases (71,72). Because of the nature of the mechanism of separation, the resolution becomes more difficult for larger nucleotides (>70 bases). The situation is not much better as far as free solution CZE is concerned (73), however, the resolution is significantly improved with the use of gel-filled columns (73,74). Figure 8 shows the resolution of DNA fragments up to 340 bases long in less than one

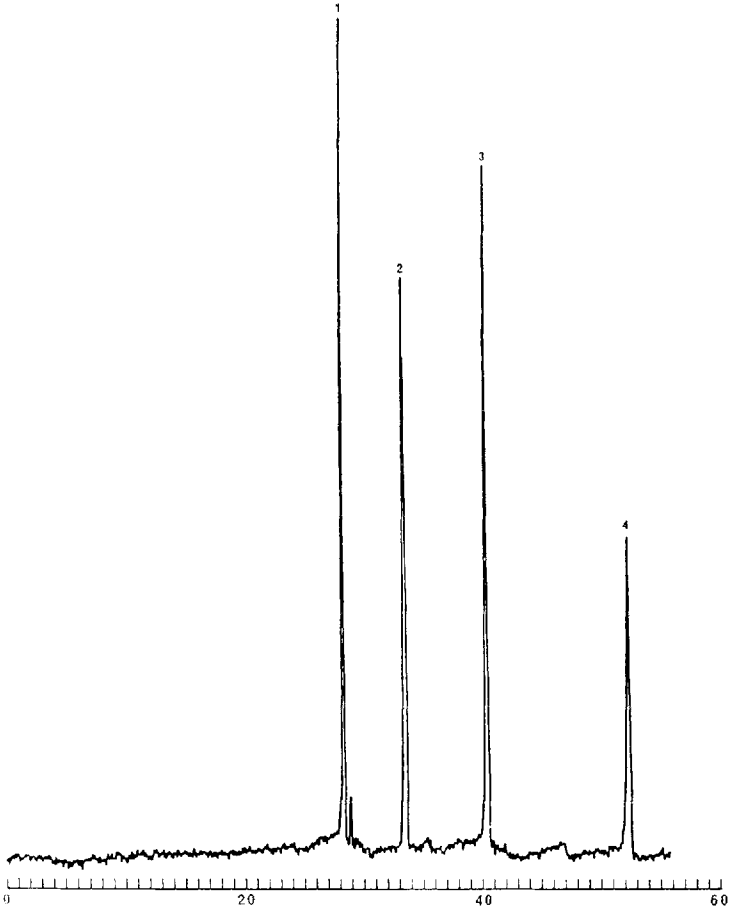

Figure 7. High-performance capillary SDS-PAGE separation of proteins. Conditions: 400 V/cm, 24 μA, 27°C, T = 10%, C = 3.3%. Buffer, 90 mM Tris-NaH_2PO_4 9pH = 8.6), 8 M urea, 0.1% SDS. Samples: 1 = α-lactalbumin; 2 = β-lactoglobulin; 3 = trypsinogen; 4 = pepsin. (Reprinted from Ref. 68).

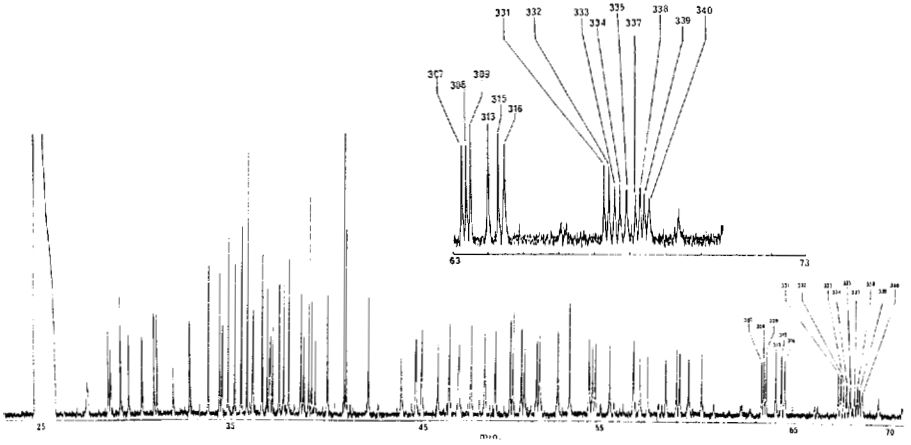

Figure 8 Electropherogram of chain-termination sequencing reaction products. Running conditions: L = 650, (B)/ = 750 mm, L = 920 mm, E = 310 V/cm, pH =8.), injection = 10 kV, 15s. (Reprinted from Ref. 74).

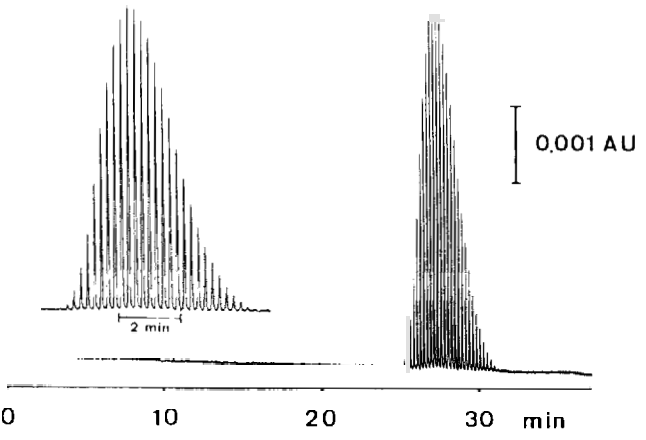

Figure 9 CZE separation of polydeoxyadenosine $PD(A)_{40-60}$. Capillary: 40 cm effective length; 100 μm i.d., surface pretreatment before filling with gel. Gel: polyacrylamide, 6% T, 5% C. Buffer: 0.1 M Tris; 0.25 M boric acid; 7 M urea. Separation conditions: 300 V/cm, 12 μA; injection: 5000 V, 2s; detection: UV/260 nm. (Reprinted from Ref. 78).

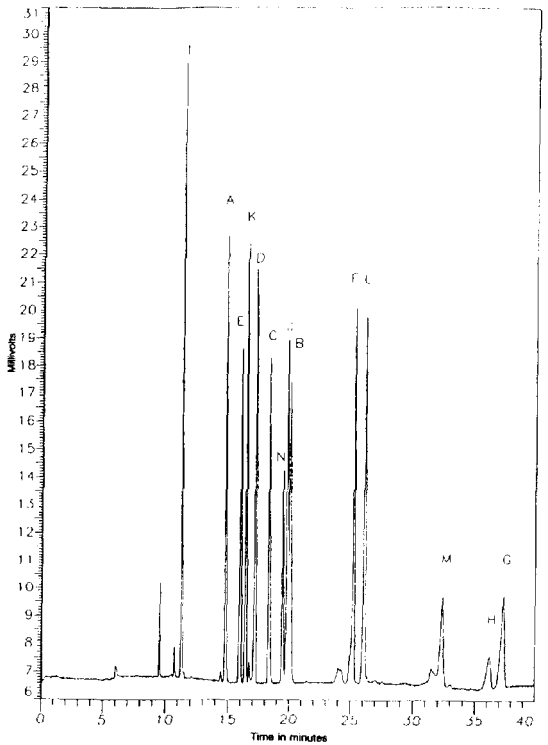

Figure 10. Separation by MECC: A) 5'-Deoxyadenosine (DA). B) 2',3'-Dideoxyadenosine (DDA). C) N^6-Methyl-2'-deoxyadenosine (MDA). D) 2'-Deoxyadenosine 5'-monophosphate (DAMP). E) N^6-Methyladenosine 5'-monophosphate (MAMP). F) Adenosine 5'-diphosphate (ADP). G) 2'-Deoxyadenosine 5'-triphosphate (DATP). HO 2',3'-Dideoxyadenosine t'-triphosphate (DDATP). I) w'-Deoxyguanosine (DG). J) 2',3'-Dideoxyguanosine (DDG). K) 2'-Deoxyguanosine 5'-monophosphate (DGMP). L) 2'-Deoxyguanosine 5'-diphosphate (DGDP). M) 2',3'-Dideoxyguanosine 5'-triphosphate (DDGTP). N) 3'-Azido-3'-deoxythymidine (AZT). (Reprinted from Ref. 82).

hour (74). The advantages of CZE for this application are: compatibility with laser-induced fluorescence detection, very high resolution, short analysis time, and automation multiple injections on a single column. The main disadvantage of using gel-filled columns is that the gel has to be chemically bonded to the capillary wall (75). However it appears that these columns were difficult to reproduce by other workers (76,77) according to the

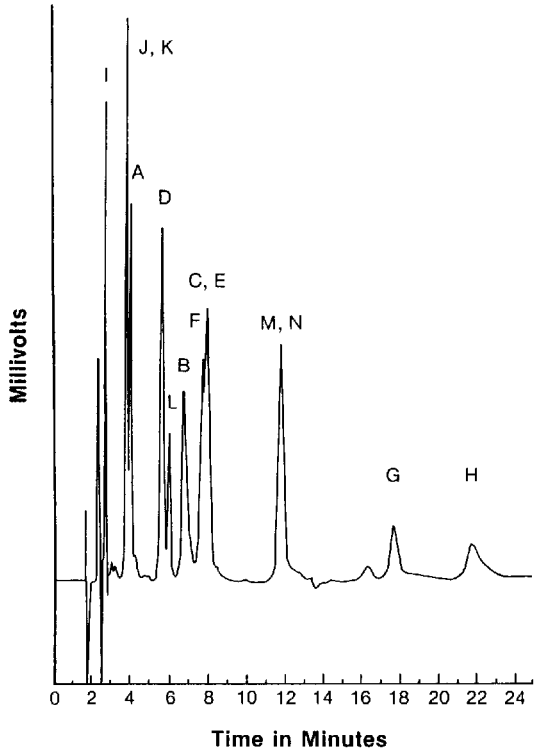

Figure 11. Separation by ion-pair liquid chromatography. For key to compounds refer to Figure 10 caption. (Reprinted from Ref. 82).

procedure in ref. 75. More recently, Schomburg et al. described a simple procedure for the preparation of gel-filled capillaries, which are stable for several hundred routine separations (78). A typical separation using this column is shown in figure 9.

Nucleosides and nucleotides are usually separated by ion (79) and reversed phase (80) chromatography. Separation of nucleotides from nucleosides usually require gradient elution which results in troublesome baseline fluctuations and long reequilibration times. Recently, MECC has been advanced as an alternative method (73,81,82). Lahey and St. Claire III (82) compared MECC with ion-pairing liquid chromatography for the analysis of this

class of compounds. All 14 compounds studied including six neutrals, three monophosphates, two diphosphates and three triphosphates were separated by MECC (figure 10), while an optimized isocratic HPLC procedure (figure 11) failed to adequately resolve the compounds studied.

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