Capillary Electrophoresis Analysis of Species Variations in the Tryptic Maps of Cytochrome C

Technical Bulletin

History of Electrophoresis

The history of electrophoresis can be traced back over 100 years (for review see Compton and Brownlee, 1988). At the heart of this separation mechanism is the movement of charged compounds within an electric field. Initial work in electrophoresis took two forms (Mikkers et al., 1979). The first was called moving boundary or zone electrophoresis. This technique involves the separation of charged compounds within an open tube that has been filled with an electrolyte. A voltage potential is applied across the tube, resulting in differential migration of solutes. This migration, defined as electrophoretic mobility (μ_m), is determined by the relationship between solute charge and average Stoke's radius. At the early stages of development, this technique suffered from two main problems. The first was diffusion. This occurs both from the normal diffusion of a solute within a solvent, as well as from convection heating within the solvent that is due to the applied voltage. The second problem encountered using this technique was detection. Unless the compounds separated had a visible absorption, it was difficult to visualize the separation.

One approach used to overcome diffusional problems involves the use of stabilizers in the separation. Many compounds, including agars, cellulose, paper, silicas, and acrylamide have been used successfully. The use of these stabilizers has revolutionized electrophoresis, permitting the attainment of many of the inherent advantages of electrophoretic separations. Among these are high resolution and the ability to vary separation selectivities. In addition, because large molecules do not appreciably move in the matrix after the voltage has been withdrawn, specific and sensitive staining of the separated compounds can now be easily accomplished.

Conventional gel electrophoresis is today a valuable and commonly used analytical and preparative technique for a wide variety of molecules. They include proteins, nucleic acids, amino acids, peptides, and sugars. However, gel electrophoresis still suffers from a number of drawbacks. Among them are difficulty in obtaining accurate quantitation, long run times, and interactions between the solute and the gel matrix. In addition, the use of gels is still a largely manual technique, labor intensive in its setup, and difficult to automate using instrumentation.

Capillary Electrophoresis Development

A third approach to deal with the diffusional and convective problems associated with electrophoresis is to take advantage of the "wall effect" (Jorgenson and Lukacs, 1981). That is, stability of the electrolyte can be obtained by decreasing the ratio of the cross-sectional area of the separation compartment to its surface area. Convection is minimized primarily because dissipation of voltage induced heat is very efficient under these circumstances. In addition, because of the small size of the compartment, the effects of diffusion are also minimized.

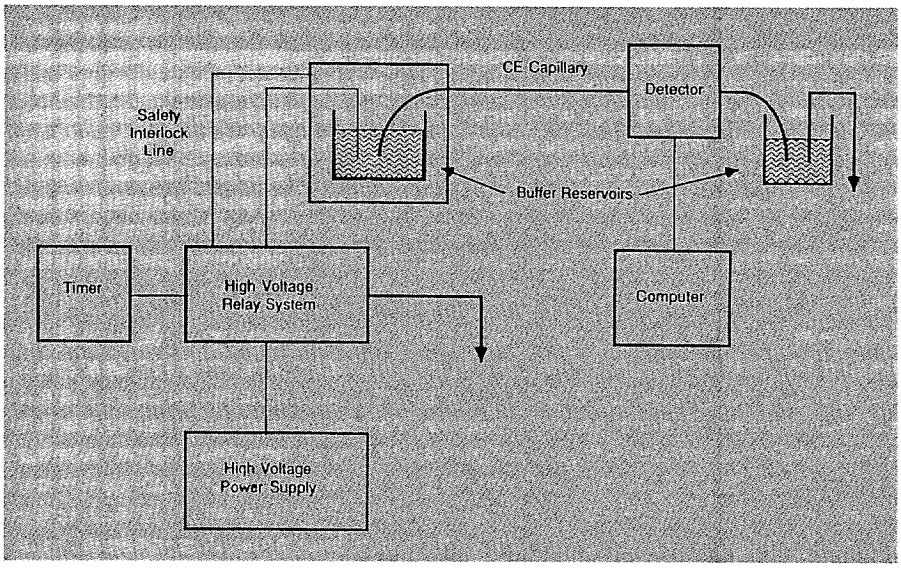
Isotachophoresis was first successfully accomplished (Mikkers et al., 1979) in 1979 using a PTFE tube with an I.D. of 200 microns, using conductivity and UV on-line detection. Using this scheme, high resolution separations of both organic and inorganic ions were performed along with accurate quantitation of all separated species. However, because the output is somewhat unconventional, this technique did not gain widespread acceptance.

In 1981 zone electrophoresis in open-tubular glass capillaries was first demonstrated (Jorgenson and Lukacs, 1981). Using an ID of 75 microns and on-line fluoresence detection, 10–30 minute separations of amino acids, dipeptides, and amines with plate counts as high as 400,000 were demonstrated. In the ensuing 8 years a number of variations of the original free zone scheme have developed (for reviews see Compton and Brownlee, 1988; Ewing et al., 1989; Jorgenson et al., 1988). In addition to free zone electrophoresis, a number of other separation mechanisms have been employed using similar hardware arrangements. They include isoelectric focusing (Hjerten et al., 1987), micellar electrokinetic chromatography (MECC) (for review see Snopek et al., 1988), separations based on solute affinity for buffer additives (Gozel et al., 1987; Walbroehl and Jorgenson, 1986), such as inclusion complexes, and traditional gel electrophoresis within the capillary (Cohen and Karger, 1987). Although capillary electrophoresis (CE) has been used primarily as an analytical technique, preparative work has also been successfully accomplished (Rose and Jorgenson, 1988). It is possible to isolate 50 picomoles of a protein or peptide, while still maintaining adequate plate counts and resolution. A wide variety of detection schemes have now also been demonstrated for CE. They include UV/Visible absorbance (Jorgenson, 1984), fluorescence (Green and Jorgenson, 1986), conductivity (Huang et al., 1989), thermooptical (Yu and Dovichi, 1988), electrochemical (Wallingford and Ewing, 1987), and mass spectrometry (Lee et al., 1988; Loo et al., 1989).

Basic Principles

A schematic drawing of a basic CE apparatus is seen in Figure 1. Each end of a glass or fused silica capillary is immersed into a separate reservoir containing an electrolyte and a high voltage electrode. The capillary is also filled with the same electrolyte. Toward one end of the capillary an on-line detector is placed. If the detection mode is optical, the capillary itself is used as the flow cell. Other detection schemes, including conductivity and electrochemistry have also been performed by inserting an electrode within the capillary.

Figure 1. Basic CE Instrumentation.



Sample can be introduced at one end of the capillary by a variety of mechanisms (for review see Jorgenson et al., 1988). They are hydrostatic (gravity driven siphoning), pressure, and electromigration. A hydrostatic injection is accomplished by placing the injection end of the capillary in the sample solution and then raising that end to a level higher than that of the electrolyte at the other end. By carefully controlling both the differential height and the time, accurate and reproducible injections can be made. In the case of a pressure injection, the injection end of the capillary is immersed in the sample solution, and either the sample is pressurized, or alternatively, a vacuum is created at the other end of the capillary. Both hydrostatic and pressure injections introduce a small volume of sample that is representative of the starting mixture. To perform an electromigration injection, the sample end of the capillary is immersed in the sample mixture along with the high voltage electrode. The high voltage is then turned on at a defined voltage for a specific time. Although reproducible injections can be accomplished using this method, an electromigration injection differentially introduces components of a sample mixture on the basis of each component's electrophoretic mobility. Thus, a representative sample will not be analyzed.

Once a sample has been introduced, the sample end of the capillary is returned to the electrolyte, and the high voltage is turned on. Typical running voltages can vary from 5 to 30 kV, and typical run times from 1–45 minutes. As the run progresses, the individual components of the sample migrate toward the detector. The migration time in zone electrophoresis for a particular solute is given by: $t = L^2/\mu V$, where t is the migration time, L is the tube length, μ is the solute's total mobility, and V is the applied voltage (Mikkers et al., 1979; Jorgenson and Lukacs, 1981). The migration time has two components. The first and most obvious is the electrophoretic mobility (μ_m). Thus, if a solute is negatively charged, it will migrate toward the anode, while positively charged molecules will migrate toward the cathode. In addition to the electrophoretic mobility, a second powerful force drives the movement of solutes. The high voltage induces bulk fluid flow within the capillary that is due to electroendoosmosis. This component of the solute's overall mobility is μ_{osm}. This occurs because the wall of the fused silica capillary is negatively charged, and soluble cations in the electrolyte (largely hydrated H^+) will loosely associate with the capillary wall. These hydrated cations are pulled toward the cathode inducing a bulk fluid flow in this direction. Thus, the overall mobility of any solute will be the sum of the two forces, electrophoretic mobility (μ_m) and electroendoosmosis (μ_{osm}). A typical vector diagram is seen in Figure 2. For most large molecules, the size of the osmotic vector is larger than the electrophoretic vector. Therefore, regardless of whether the solute molecule is a cation or an anion, all molecules will migrate toward the cathode. However, cations will migrate faster than the osmotic flow, while anions will migrate slower than the osmotic flow. The osmotic flow is generally determined with the injection of some neutral compound. Because of this property, both anions and cations may be analyzed in a single run.

Capillary electrophoresis is a very powerful separation tool. This is due to the large number of theoretical plates possible in the separation. The separation efficiency, expressed in terms of the total number of theoretical plates N, is $N = \mu V/2D$, where μ is the solute mobility, V the voltage used, and D is the solute's diffusion coefficient (Mikkers et al., 1979; Jorgenson and Lukacs, 1981). This expression leads to an interesting prediction, being that the total number of theoretical plates is directly proportional to the voltage used. Thus, the use of very high voltages can result in a very large number of theoretical plates. Further, because N is inversely proportional to the solute diffusion coefficient, this equation predicts that higher efficiencies can be obtained for larger, rather than smaller molecules. Under ideal conditions, greater than 1,000,000 plates have been demonstrated. Under more normal operating conditions 100,000–200,000 plates can be routinely achieved.

In principle, CE can be used to separate a wide variety of molecules. For molecules containing good chromophores, direct UV/Visible or fluorescence detection serves quite well. However, the

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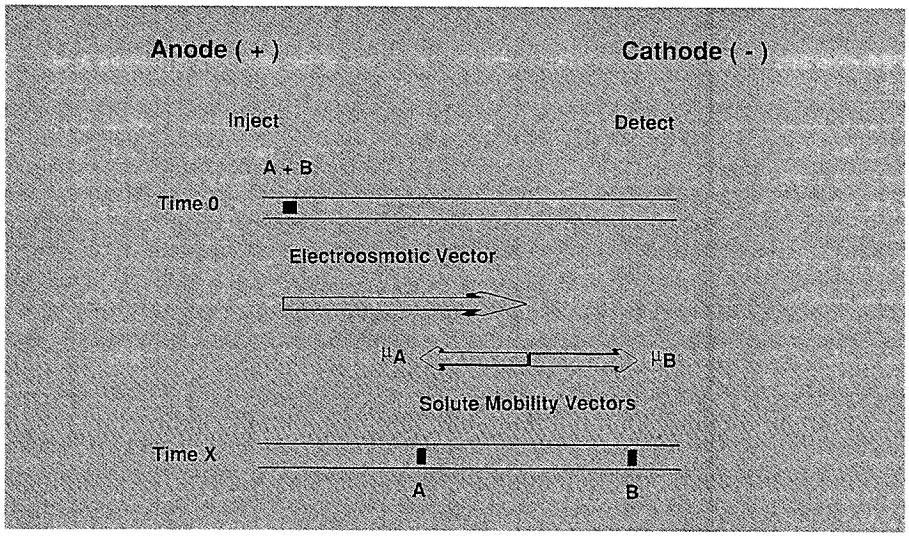
Figure 2. Mobility of a solute in a fused silica capillary.



wide diversity of non-absorbing compounds has driven the development of a variety of alternative detection schemes. Both conductivity and electrochemical detectors have been demonstrated for CE. In addition laser driven optical systems offer greatly increased sensitivity. Direct techniques like absorbance and fluorescence have already been demonstrated using a laser as the light source (Nickerson and Jorgenson, 1988; Cheng and Dovichi, 1988). In addition, indirect laser induced fluorescence appears to offer a universal and sensitive detection mode (Kehr and Yeung, 1988). Detection is one area where significant development will likely occur within the next few years.

Peptide Mapping Using Capillary Electrophoresis

One of the most commonly used techniques for protein analysis is the development of a peptide map. The first step in this process is the specific digestion of a protein through chemical or enzymatic means. Once the digestion is complete, the resulting peptide mixture is separated, yielding a complex set of peaks. This separation, or fingerprint, is characteristic for every protein. Point mutations in the protein can often be detected using this type of analysis. Most commonly, the peptide separation is accomplished using an HPLC reverse phase separation. The separation is rooted in hydrophobic interactions between the packing material and the amino acid side chains. Both sequence and conformation contribute to the selectivity. While many single residue substitutions affect retention, not all such substitutions can be recognized with certainty. Therefore, alternative selectivities must be used in order to accurately recognize all substitutions that can take place. CE can supply this kind of selectivity, easily separating peptides on the basis of charge. Therefore, specifically in the area of peptide mapping, and more generally, in the area of peptide separations, CE is a valuable tool when used in conjunction with HPLC for peptide analysis.

In this study we have used cytochrome c from horse and chicken mitochondria as model compounds. Although the sequence from these two proteins is very similar, several point mutations exist. HPLC and CE are used for the development of peptide maps. The maps given by the two techniques are quite different. However, small differences between the two species can be distinguished using either separation. In addition, HPLC was used in order to purify individual peptides. These peptides were subjected to amino acid analysis as well as CE analysis. Examples will be shown in which the additional selectivity of CE can easily separate peptides co-purified in the HPLC separation.

Materials and Methods

Protein Digestion

Cytochrome c (Sigma) from chicken and horse mitochondria (1mg/500μl) were suspended in 0.1 M ammonium bicarbonate buffer at pH 8.0. Trypsin (0.1 mg/ml) was dissolved in 500 μl of buffer and added to the cytochrome c solutions. The digests were incubated for 24 hours at 37°C. Trypsin was then inactivated by heating at 100°C for 5 minutes, and the digests frozen in 100 μl aliquots at −20°C.

HPLC Separation

Aliquots of the digest were diluted 1:10 with eluent A prior to chromatography. Peptides were resolved using reverse phase HPLC at 35°C with a 2 mm×15 cm Waters Delta Pak™ C18, 5 μm, 300 Å column on a Waters Peptide Analyzer. The separation conditions were as follows: Eluent A: Water/ 0.1% trifluoroacetic acid (TFA); B: acetonitrile/ 0.1% TFA. The following gradient was employed:

Time (min)	Flow (ml/min)	%A	%B	Curve
0	0.25	95	5	*
3	0.25	90	10	6
13	0.25	87	13	6
67	0.25	60	40	6
77	0.25	40	60	6
87	0.25	40	60	6

Peptides were collected manually for further analysis

Capillary Electrophoresis Separation

All CE separations were performed using a breadboard system. Injections were made hydrostatically and all detection was at 214 nm. The separation conditions used were as follows:

pH 7.0 Analysis: Buffer: 50 mM sodium phosphate at pH 7.0 containing 10% acetonitrile; Voltage: 14000V; Current: 60 μA; Capillary: 75 μm×60 cm fused silica (Polymicro Technologies, Phoenix AZ). All separations were conducted at constant voltage.

pH 2.0 Analysis: Buffer: 100 mN Phosphoric acid; Voltage: 12000V; Current: 84 μA; Capillary: 75 μm× 60 cm fused silica. All separations were conducted at constant voltage.

Data was collected on a Waters 840 Data Station at 5 points per second. 1mV=0.001 absorbance units.

Amino Acid Analysis

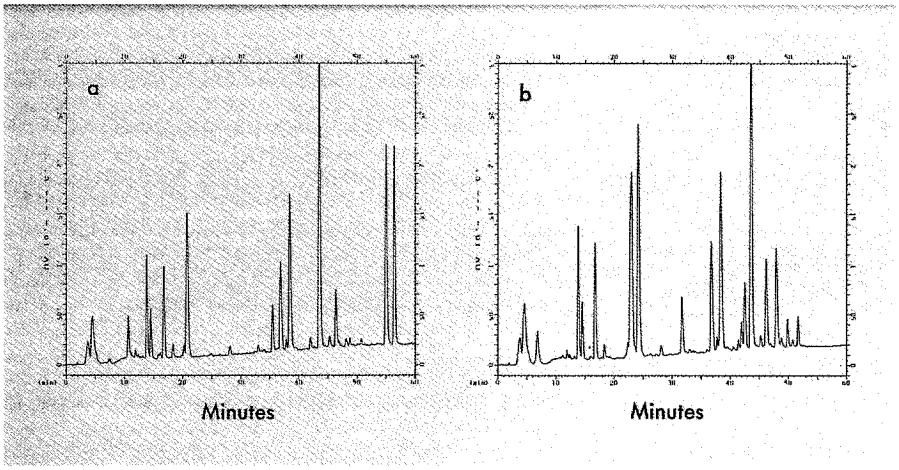
Peptide fractions were analyzed using the Pico·Tag™ amino acid analysis methodology (Bidlingmeyer et al., 1984).

Results

HPLC Separation and Analysis

Cytochrome c from horse and chicken mitochondria were chosen as model proteins due to their well characterized primary structure. Tryptic peptides of each protein were analyzed by high resolution reverse-phase HPLC with photodiode array detection. The results are seen in Figure 3. The mapping procedure was facilitated by the presence of native chromophores, a covalently bound heme group, and single Trp residue, that were easily detected by their characteristic spectra. In addition, amino acid analysis was used to help identify peaks. There are 11 single residue substitutions between horse and chicken cytochrome c. Five of these differences are not readily detected by reverse-phase HPLC since they appear to reside on small, hydrophilic peptides that are not well retained. Of particular interest is the apparent co-elution of peptides 39–53 and 40–53, differing by a single Lys residue, while peptides 56–72 and 56–73, containing an additional Lys residue are clearly resolved.

Figure 3. HPLC separation of cytochrome c. a: Chicken cytochrome c. b: Horse cytochrome c.



Capillary Electrophoresis Separation and Analysis

The cytochrome c digests were analyzed by CE using two different buffer systems. The first system chosen was a neutral pH phosphate buffer. Maps of both chicken and horse cytochrome c can be seen in Figures 4a and 4b. As in the HPLC analysis, the maps appear similar in many respects. However, several differences can also be noted in the separations. Additional information can also be obtained in this analysis. The use of a neutral pH allows the determination of the overall charge of the peptide. As a reference, the neutral marker formamide was subjected to an identical analysis. This can be seen in Figure 4c. All peptides that migrate more quickly than formamide (faster than 12.5 minutes) have an overall positive charge, while those that migrate more slowly have a negative charge. This quality of information is very difficult to obtain in an HPLC analysis.

The second buffer system used for the CE analysis of chicken and horse cytochrome c is phosphoric acid at pH 2. Under these conditions, all peptides will carry a positive charge. Therefore, separation will be based primarily on the charge/stoke's radius relationship of each peptide. The CE analysis is seen in Figure 5. Again, although the maps are quite similar, differences are quite easy to see. In order to identify the individual peaks in this separation, HPLC purified fractions were also separated in this buffer system. The results are seen in Figure 6. The peak identifications seen in Figure 5 are derived from these analyses. In most cases, a single peak in the HPLC separation yielded one peak in the CE analysis. However, for both species, the HPLC peak that was thought to contain residues 39–53 and 40–53 based on amino acid

Figure 4. CE separation of Cytochrome c. All separations using 50 mM sodium phosphate at pH 7.0. a: Chicken cytochrome c. b: Horse cytochrome c. c: Formamide.

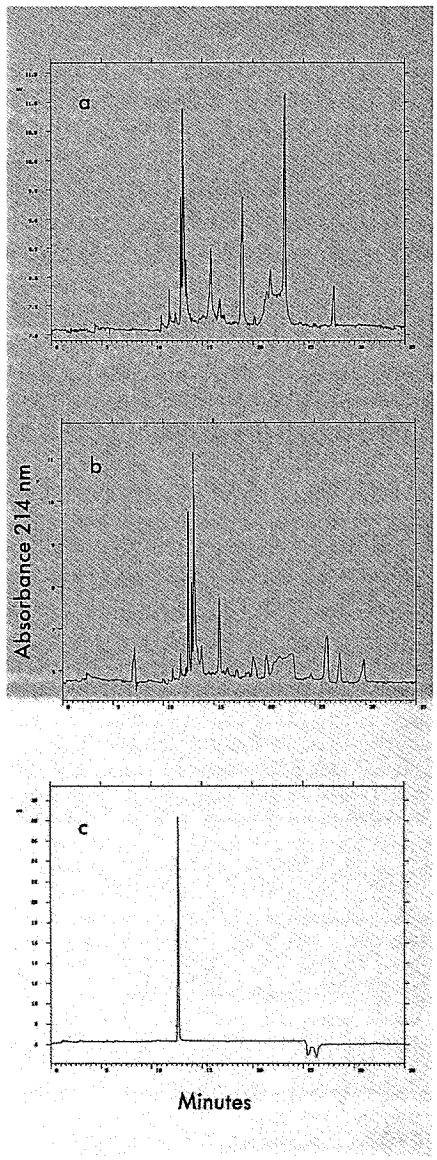
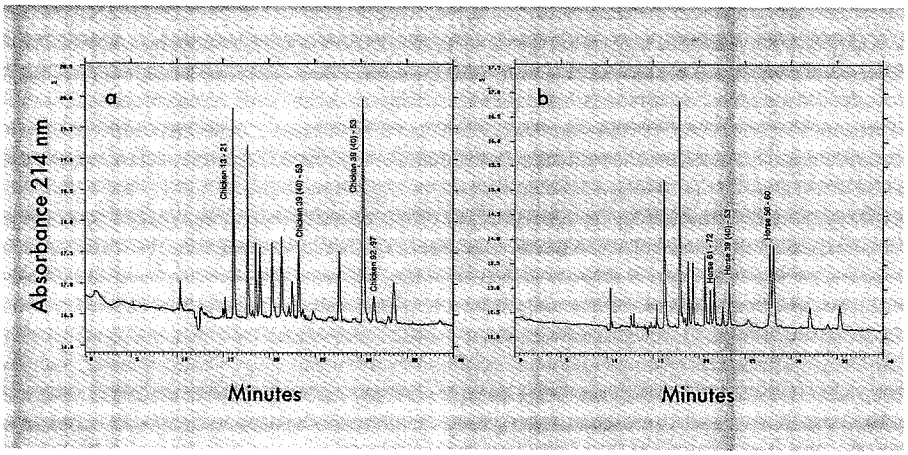
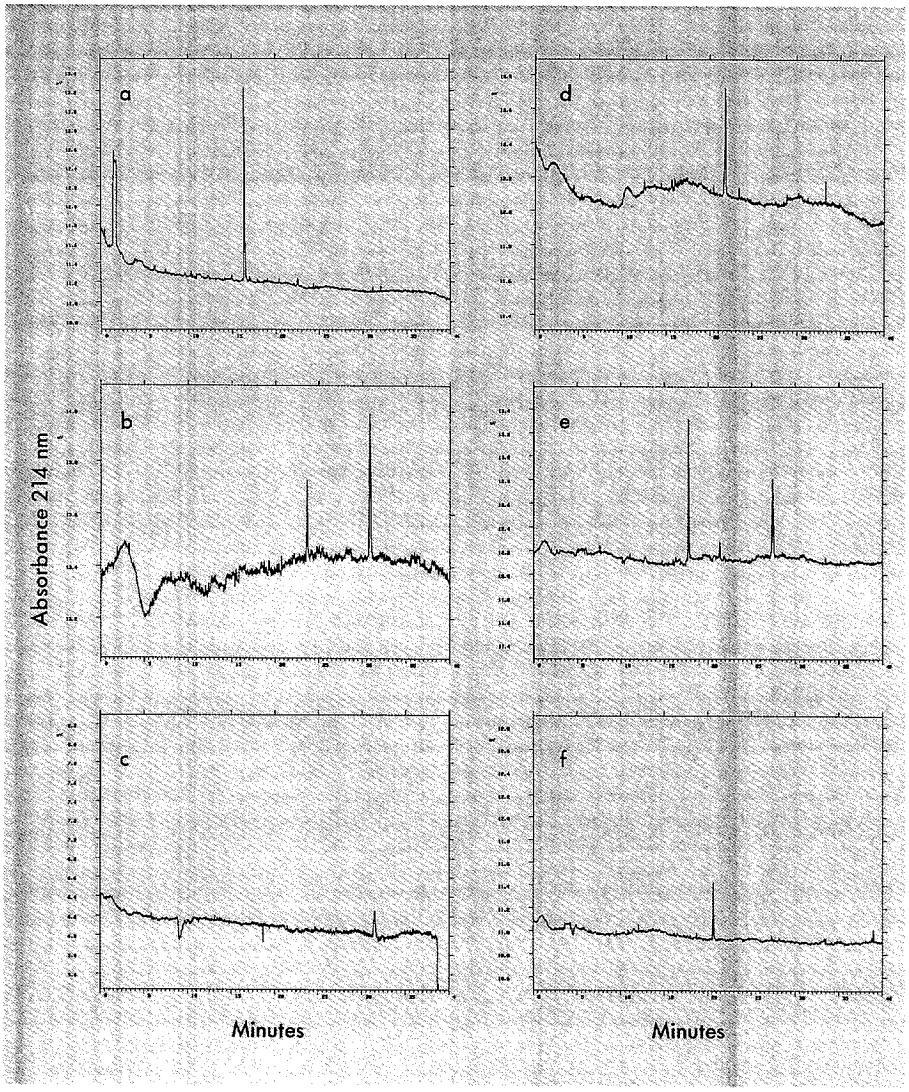


Figure 5. CE separation of Cytochrome c. All separations using 100 mN phosphoric acid. a: Chicken cytochrome c. b: Horse cytochrome c.



analysis, indeed yielded two CE peaks. For both species residue 39 is Lys. The additional positive charge conferred by this residue should result in a faster migration time. Thus, the first peak in the CE analysis is 39–53, while the second peak is 40–53.

Figure 6. CE separation of HPLC fractions. a: Chicken 13–21. b: Chicken 39–53 and 40–53. c: Chicken 92–97. d: Horse 56–60. e: Horse 39–53 and 40–53. f: Horse 61–72.



Discussion

The development of capillary electrophoresis as an analytical tool further expands the repertoire of techniques available to the chemist and biochemist. The very high separation efficiencies coupled with very small sample consumption make this technique ideal for those situations where purity determinations of a precious sample are required. In addition, the selectivities offered by CE are complementary to those most commonly used in HPLC. Thus, the two techniques, when used in tandem, provide a powerful combination for the analysis of complex mixtures of molecules, as well as the determination of sample purity.