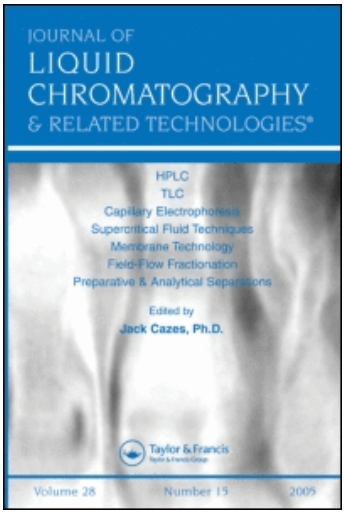

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Use of Capillary Electrophoresis for the Detection of Single-Residue Substitutions in Peptide Mapping

Thomas E. Wheat[a]; Patricia M. Young[a]; Nancy E. Astephen[a]
[a] Divison of Millipore, Waters, Massachusetts

To cite this Article Wheat, Thomas E. , Young, Patricia M. and Astephen, Nancy E.(1991) 'Use of Capillary Electrophoresis for the Detection of Single-Residue Substitutions in Peptide Mapping', Journal of Liquid Chromatography & Related Technologies, 14: 5, 987 — 996
To link to this Article: DOI: 10.1080/01483919108049299
URL: http://dx.doi.org/10.1080/01483919108049299

JOURNAL OF LIQUID CHROMATOGRAPHY, 14(5), 987-996 (1991)

USE OF CAPILLARY ELECTROPHORESIS FOR THE DETECTION OF SINGLE-RESIDUE SUBSTITUTIONS IN PEPTIDE MAPPING

THOMAS E. WHEAT, PATRICIA M. YOUNG, AND NANCY E. ASTEPHEN
Waters, Divison of Millipore
34 Maple Street
Milford, Massachusetts 01757

ABSTRACT

Capillary electrophoresis (CE) can provide an orthogonal separation to the reversed phase HPLC commonly used for peptide mapping, thus ensuring that all possible amino acid substitutions can be detected. The selectivity of a reversed phase separation is based on the relative hydrophobicity of the components while that of CE is derived from charge, mass, diameter and viscosity. Since CE also provides so many options for effecting and modifying a peptide separation, it is useful to define one possible systematic approach to methods development. The application of such an approach to the tryptic peptides of cytochrome *c* yields a method suitable for the identification of differences of a single residue between homologous proteins from different species.

INTRODUCTION

Capillary electrophoresis (CE) should provide a useful and necessary complement to the peptide separations that are routinely effected by high

performance reversed phase liquid chromatography. The latter technique has come to be preferred because of its intrinsically high resolving power. Since its selectivity derives primarily from the relative hydrophobicity of the amino acid side chains, it is well-suited for distinguishing peptides that differ by a single residue. However, since so many alternative peptides can be derived from a given sequence by single substitutions, it is not possible to recognize every possible difference by a change in retention time. In principle, the use of two techniques with different physical and chemical selectivities is more likely to ensure that all substitutions can be identified. Electrophoretic separations should be orthogonal to reversed phase since the selectivity is based on charge, mass, Stoke's radius, and intrinsic viscosity. Excellent peptide separations should be obtained when electrophoretic selectivities are implemented in a capillary format with its intrinsically high efficiency (1-2).

Peptide separations with CE can exploit all the common electrophoretic modes, but it is commonly recognized that the selectivity is sensitive to small changes in pH (3-5). While it has proven possible to carefully adjust resolution with small changes in pH, this approach lacks the ruggedness required for routine use. Unlike reversed phase, CE is not restricted to the pH range of 2 to 8. It is, therefore, possible to manipulate the charge distribution of the peptide sample over a much broader range before attempting the use of specialized buffer additives or other subtle adjustments. Methods development experiments can be planned based on the ionic properties of amino acid side chains. At relatively high pH, above 10, the peptides will be nearly completely deprotonated so the primary source of selectivity among the peptides will be in the differences in the number of acidic residues. In contrast, at low pH, near 2, essentially all peptides will have a net positive charge so resolution will be most affected by the number of basic residues. If more subtle selectivity is required, pH values near the side chain pK's should be examined. For acidic residues, selectivity should be greatest near pH 4 while basic side chains will be most sensitive near pH 10. Tryptic digests of cytochrome *c* provide a practical test of this approach since the protein as isolated from different species provides a panel of homologous peptides differing by a single amino acid residue.

MATERIALS AND METHODS

The preparation of tryptic digests of bovine, chicken, horse, and rabbit cytochrome *c* and the chromatographic separation of these digests has been described previously (6-7). All buffers and other reagents were of the highest available quality.

Capillary electrophoresis was performed with a Waters Quanta 4000 (Waters, Milford,MA). A 75μ X 60cm capillary was selected, and samples were introduced by hydrostatic injection. Separations were monitored at 214nm. Methods development experiments were performed at 20 kV while the comparative peptide mapping was at 28kV. Other conditions are described below.

RESULTS

The separation of the tryptic digest of horse cytochrome *c* at pH 10.5 is shown in Figure 1. The expected sharp, symmetrical peaks are observed. The baseline dip near 5.5 minutes corresponds to the migration of a neutral marker, indicating that some of the peptides still retain a net positive charge. While this separation proved reproducible, the resolution is inadequate. Rather than attempt subtle adjustments of the pH or additions to the electrolyte, the sample was separated at pH 2 (Figure 2). This pattern is completely different from that at pH 10.5 although it has about the same number of peaks. While rapid analyses at these extremes of pH yielding different selectivities may be valuable for some samples, neither gives complete resolution. Where a single analytical method is required, electrolyte pH should be adjusted near the pK's of the amino acid side chains, that is, near 4 for acidic residues and 10 for basic side chains. In the case of tryptic peptides, better selectivity can be expected at the lower pH since most of the peptides have a single basic residue and may vary widely in the number of acidic amino acids.

Tryptic peptides from horse cytochrome *c* were separated at pH 3.5. The pattern is different from those observed at pH 10.5 and pH 2, although fewer peaks are observed and some should be better resolved. When the pH was increased to 4 (Fig 4), the separation improved as peptide migration times increased, and altered selectivity was apparent in relative changes in migration time. Increasing the pH to

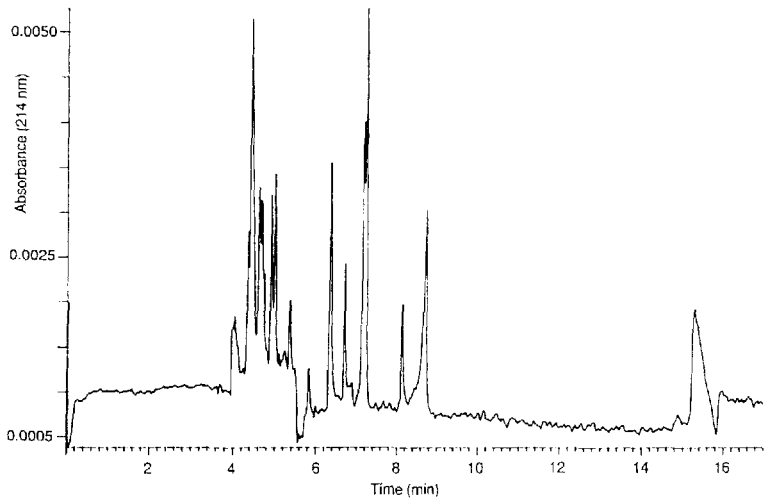

Figure 1. CE Separation of Tryptic Peptides of Horse Heart Cytochrome *c*, pH 10.5. Electrophoresis was performed in 0.05M sodium borate, 0.001M EDTA, pH 10.5 in a 75μ X 60cm capillary Following a 10sec hydrostatic injection, a 20kV potential was applied. The separation was monitored at 214nm.

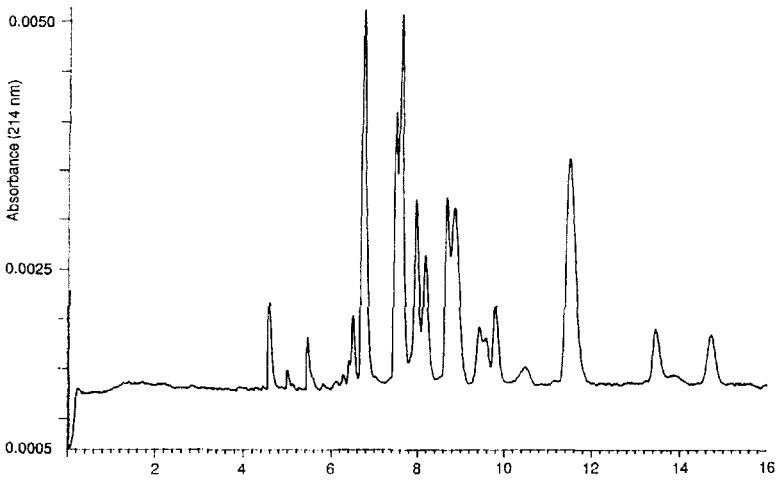

Figure 2. CE Separation of Tryptic Peptides of Horse Heart Cytochrome *c*, pH 2.0. Electrophoresis was performed in 100mN phosphoric acid, pH 2.0, in a 75μ X 60cm capillary Following a 10sec hydrostatic injection, a 20kV potential was applied. The separation was monitored at 214nm.

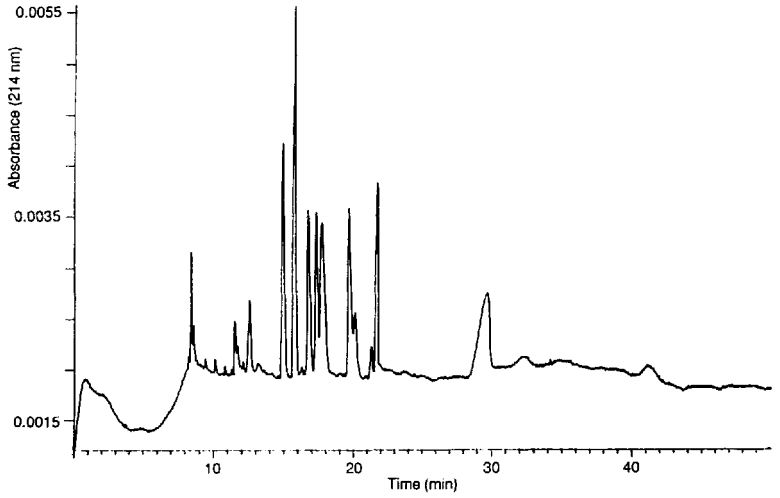

Figure 3. CE Separation of Tryptic Peptides of Horse Heart Cytochrome *c*, pH 3.5. Electrophoresis was performed in 0.025M sodium citrate, pH 3.5, in a 75μ X 60cm capillary Following a 10sec hydrostatic injection, a 20kV potential was applied. The separation was monitored at 214nm.

4.5 (Fig 5) provided additional resolution while extending migration times, even in some cases well beyond previous run times. As a compromise between resolution and run time, pH 4.0 was selected for further evaluation by comparative peptide mapping of cytochromes *c* from various species. The running conditions were increased to 28 kV, the highest value compatible with the linear portion of the Ohm's Law plot for this capillary and buffer.

Separations were performed for tryptic digests of cytochrome *c* from beef (Fig 6), chicken (Fig 7), rabbit (Fig 8), and horse (Fig 9). As expected, these maps are grossly similar, but there are differences that should reflect single amino acid substitutions among these species. The changes were defined by identifying the peaks in each electropherogram. This was accomplished by injecting each peptide, as purified by reversed phase, in the elctrophoretic separation, both singly and spiked into the complete digest. The peptides in the electropherogram are numbered in the order in which they elute in a reversed phase separation in the presence of TFA (7). The orthogonal selectivity associated with electrophoresis is reflected

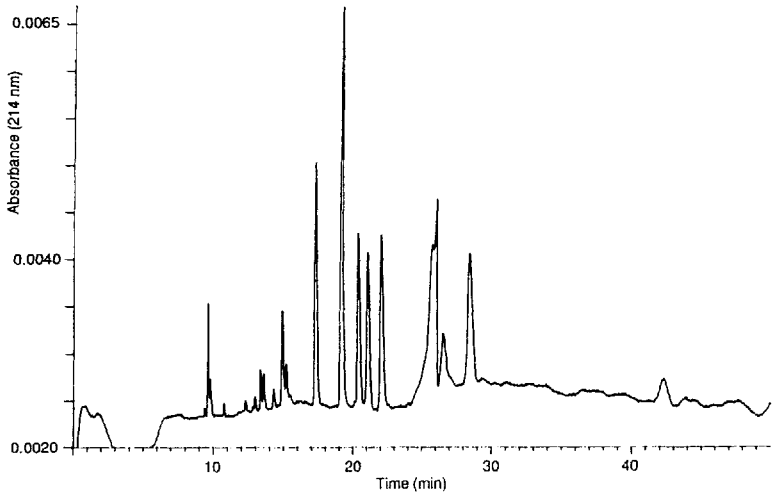

Figure 4. CE Separation of Tryptic Peptides of Horse Heart Cytochrome *c*, pH 4.0. Electrophoresis was performed in 0.025M sodium citrate, pH 4.0, in a 75μ X 60cm capillary Following a 10sec hydrostatic injection, a 20kV potential was applied. The separation was monitored at 214nm.

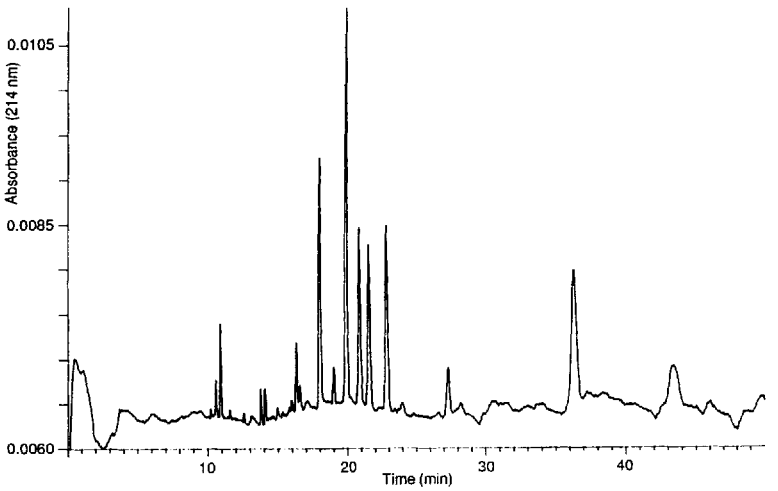

Figure 5. CE Separation of Tryptic Peptides of Horse Heart Cytochrome *c*, pH 4.5. Electrophoresis was performed in 0.025M sodium citrate, pH 4.5, in a 75μ X 60cm capillary Following a 10sec hydrostatic injection, a 20kV potential was applied. The separation was monitored at 214nm.

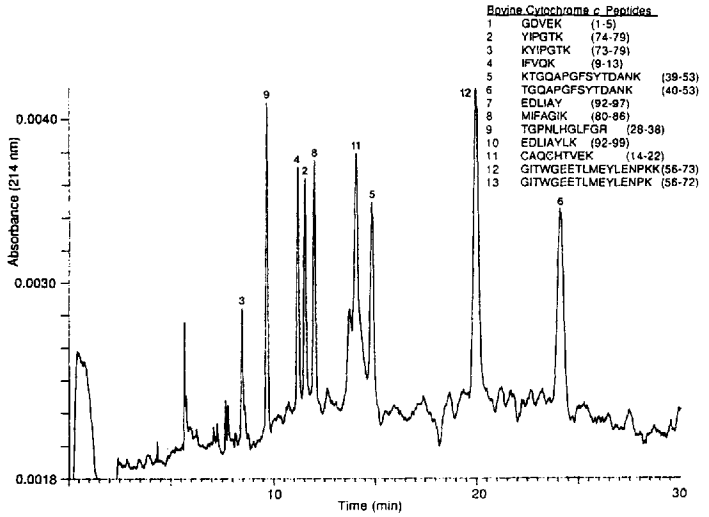

Figure 6. CE Separation of Tryptic Peptides of Bovine Heart Cytochrome *c*, pH 4.0. Electrophoresis was performed in 0.025M sodium citrate, pH 4.0, in a 75μ X 60cm capillary Following a 10sec hydrostatic injection, a 28kV potential was applied. The separation was monitored at 214nm.

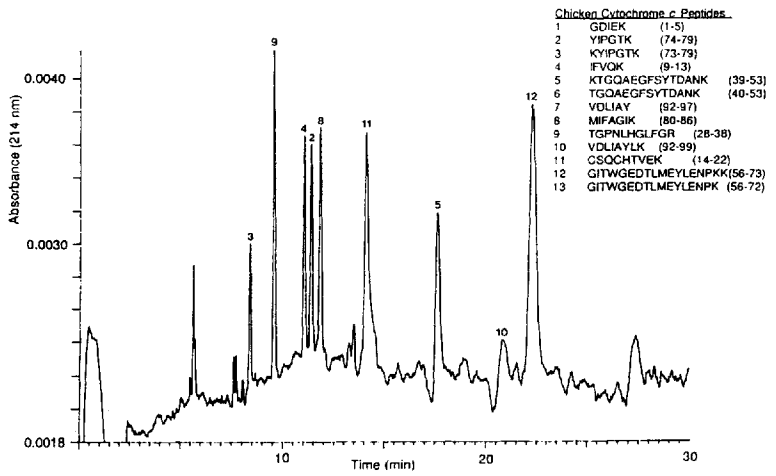

Figure 7. CE Separation of Tryptic Peptides of Chicken Heart Cytochrome *c*, pH 4.0. Electrophoresis was performed in 0.025M sodium citrate, pH 4.0, in a 75μ X 60cm capillary Following a 10sec hydrostatic injection, a 28kV potential was applied. The separation was monitored at 214nm.

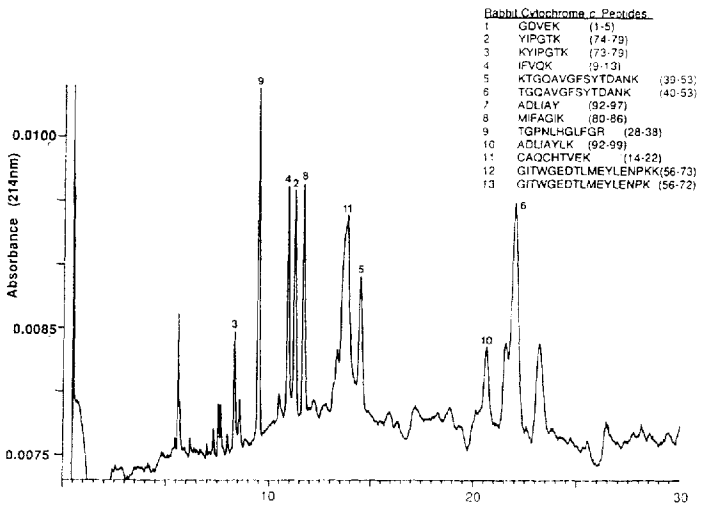

Figure 8. CE Separation of Tryptic Peptides of Rabbit Heart Cytochrome *c*, pH 4.0. Electrophoresis was performed in 0.025M sodium citrate, pH 4.0, in a 75μ X 60cm capillary Following a 10sec hydrostatic injection, a 28kV potential was applied. The separation was monitored at 214nm.

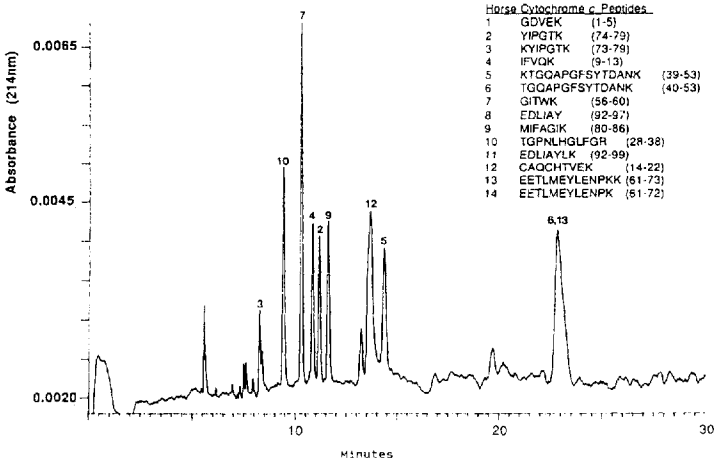

Figure 9. CE Separation of Tryptic Peptides of Horse Heart Cytochrome *c*, pH 4.0. Electrophoresis was performed in 0.025M sodium citrate, pH 4.0, in a 75μ X 60cm capillary Following a 10sec hydrostatic injection, a 28kV potential was applied. The separation was monitored at 214nm.

immediately in the observation that there are no peaks that are adjacent to one another in both separations.

In the separation of the bovine digest (Fig 6), peaks 4, 2, and 8 represent peptides with one basic side chain and no acidic residues. These peptides are five, six, and seven residues and migrate inorder of increasing size. Peak 9 is an eleven residue peptide that migrates more rapidly because it includes two basic residues while peak 3 elutes even earlier as a six residue peptide with two basic side chains. The expected dominant effects of charge and mass on electrophoretic migration are, therefore, observed. In addition, it becomes apparent that some peptides are missing from the map. Those are the peptides with the largest net negative charge so it is likely that the separation was not run long enough to observe them.

When the electropherograms of the digests derived from different species are compared, the effect of a single difference between homologous peptides can be observed. For example, residue 44 is proline in bovine cytochrome and glutamate in the chicken protein. This substitution is reflected in decreased mobility of peak 5 (residues 39-53) in map of chicken cytochrome *c* (Fig 7). The cytochromes also yield a second peptide (40-53) that is shorter by one lysine residue and that migrates much more slowly. In the case of chicken cytochrome *c*, this peptide does not elute within the specified run time.

Peak 7 in the map of the horse protein (Fig 9) is not observed in the other maps because it is derived from the introduction of a new cleavage site. A lysine replaces the glycine found at position 60 in the other proteins. It is interesting to note that this five residue peptide with one basic side chain is well-resolved from a second five residue peptide (Peak 4; residues 74-79)that should have the same charge properties.

DISCUSSION

These results confirm that CE provides a complementary separation technique to the reversed phase HPLC commonly used for peptide mapping. Useful differences in selectivity are observed with subtle changes in the pH of the electrolyte. Such changes can be systematically examined with a protocol based on the expected charge

distribution of the peptides in the mixture. In this way, a method can be developed that can detect single amino acid differences within a panel of homologous peptides. The relative migrations of such peptides can be related to their structure, and as expected, the dominant selectivity is derived from the charge and mass of the peptides. However, other factors, such as, three-dimensional effects, can contribute additional selectivity. Thus, capillary electrophoresis thus provides a useful tool extending the repertoire of the protein chemist by providing more information about a mixture of peptides and by ensuring that all possible peptides with in a mixture are separated.

REFERENCES

1. Jorgenson, J.W., and Lukacs, K.D., Zone Electrophoresis in Open-Tubular Glass Capillaries, Anal. Chem., 53, 1298, 1981.

2. Jorgenson, J.W., and Lukacs, K.D., Capillary Zone Electrophoresis, Science, 222, 266, 1983.

3. Grossman, P.D., Wilson, K.J., Petrie, G., and Lauer, H.H., Effect of Buffer pH and Peptide Composition on the Selectivity of Peptide Separations by Capillary Zone Electrophoresis, Anal.Biochem., 173, 265, 1988.

4. Young, P.M. and Merion,M., Capillary Electrophoresis Analysis of Species Variations in the Tryptic Maps of Cytochrome C, in Current Research in Protein Chemistry: Techniques, Struycture, Function,Villefranca J.J., ed., Academic Press, Boston, 1990, p.217.

5. Strickland, M., and Strickland, N., Free-solution Capillary Electrophoresis Using Phosphate Buffer and Acidic pH, Amer.Lab., 22(17),60, 1990.

6. Young, P.M., and Wheat, T.E., Optimization of High-Performance Liquid Chromatographic Peptide Separations with Alternative Mobile and Stationary Phases, J.Chrom., 512, 273,1990.

7. Young, P.M., and Wheat, T.E., Alternative Mobile Phases for Enhanced Chromatographic Selectivity and Increased Sensitivity in Peptide Separations, Peptide Res., In press.