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FACTORS AFFECTING FREE ZONE ELECTROPHORESIS AND ISOELECTRIC FOCUSING IN CAPILLARY ELECTROPHORESIS

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SUMMARY

The electrophoretic mobility of charged molecules in a free solution depends on their average charge, mass and shape, and the properties of the solvent media. In addition, differences in solvent media properties usually alter the average charge and shape of the charged molecules or form complexes with the molecules, substantially changing their electrophoretic mobility. In this study, we report the effect of the electrophoretic sample injection, the use of polymer modifiers in the running buffer, and the presence of organic modifiers on the separation of proteins, peptides and DNA molecules in free zone electrophoresis. We also report on the effect of ampholytes and salt concentrations in free zone isoelectric focusing. To reduce convective disturbances in free zone electrophoresis at high field strength, the experiments were run in narrow-bore 25–50-μm I.D. fused-silica capillary tubes. The optical path of the UV detector was designed to increase sensitivity.

INTRODUCTION

The advent of capillary electrophoresis has been one of the highlights of the recent advancements in the separation sciences[1–7]. The HPE™ technique developed by Bio-Rad gives rapid separations of extremely high resolution in a convenient format. In this study, using the HPE 100 system, we have examined the factors relating to the sample loading process, novel separations with polymer additives, the effects of organic solvents and other factors which affect isoelectric focusing procedures.

EXPERIMENTAL

Materials

Substance P fragments SP(1–4), SP(2–11), SP(5–11), thyrotropin-releasing hormone (TRH), and other peptides were purchased from Sigma (St. Louis, MO, U.S.A.). Low-range (88 to 1746 base pairs) DNA size standards were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). DNA 123 base pair ladder (128 to 4182 base pairs) was purchased from Bethesda Research Labs. (Gaithersburg, MD, U.S.A.). Hydroxypropylmethylcellulose (HMC), 4000 cP at 25°C for 2% solution, was obtained from Sigma. Methylcellulose (MC), 4000 cP at 25°C for 2% solution, was purchased from Sigma. Polyethylene glycol (PEG) 35 000 molecular weight was

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supplied by Fluka (Ronkonkoma, NY, U.S.A.). Bio-Lyte® ampholytes, pH range 3–10, for isoelectric focusing were provided by Bio-Rad Labs.

All capillary electrophoresis was performed using the HPE 100 high-performance capillary electrophoresis system from Bio-Rad Labs. The capillaries used were enclosed in cartridges and coated on their internal surfaces with a covalently bonded linear polymer, significantly reducing both adsorption and electroendosmosis[8–10]. The cartridges used were of either 25 or 50 μm I.D., and were of various lengths. In the HPE 100 unit a grating monochromator is used to select wavelengths from a deuterium lamp and light is directed through a window in the cartridge which houses the capillary. The built-in variable-wavelength UV detector has a microfocusing lens system that eliminates the problem of significant light loss common in conventional slit designs. Baseline noise and drift caused by small changes in capillary position are also reduced. Dual power supplies provide up to 12 kV for migration of either anions or cations. Microprocessor control of load times, run times, detector parameters and power supply variations are controlled from a touch panel on the front of the unit. Chemically resistant reservoirs are provided for buffers and electrode contacts. A needle valve directs flow through the capillary for flushing and filling. For these experiments, zone migration times and peak areas were measured using a Hewlett-Packard 3392 integrator.

Methods

Capillaries were purged prior to each experiment by closing the needle valve and flushing the capillary with the running buffer using a microliter syringe. Samples were loaded electrophoretically by applying 8 kV for 8 s. This set of conditions was found to give the best sensitivity without significantly broadening zones. The buffer used for peptide experiments was a 0.1 *M* phosphate buffer containing 0.05% (w/v) HMC.

UV detection of peptides and proteins was performed at 200 nm. DNA was detected at 260 nm. In isoelectric focusing experiments, proteins were detected at 280 nm to avoid strong UV absorption by ampholytes at lower wavelengths.

RESULTS AND DISCUSSION

Three methods of sample introduction (loading) have been used. We refer to these methods as electroendosmotic loading, electrophoretic loading and hydraulic loading. *Electroendosmotic loading* uses the movement of fluid in the capillary caused by electroendosmosis. In electroendosmotic loading the sample is drawn into the capillary by the electroendosmosis and each ionic species also moves according to its electrophoretic mobility. The amount of an individual sample loaded is thus a combination of the electroendosmosis which moves all species equally, and their individual mobilities which move them unequally[11]. The electroendosmosis also varies from run to run because it is dependent on random adsorption of material to the walls of uncoated capillaries. In *electrophoretic sample loading*, the sample is introduced into the separating capillary by using electrophoretic force only. This is facilitated by coating the capillaries with a polymer to eliminate the electroendosmosis effect. Thus, the amount of sample loaded is directly proportional to its mobility. The HPE 100 unit normally uses electrophoretic sample loading. In the third method of sample introduction, *hydraulic sample loading*, a microliter syringe is used to introduce the

sample into the injection port. This creates a pressure differential between each end of the capillary. This pressure differential (caused by the applied pressure, vacuum or height difference between the ends of the capillaries) moves the sample into the capillary. Hjertén *et al.*[12] pointed out that "in on-tube detection the width of a peak in the electropherogram is not proportional to the width of the zone in the electrophoresis tube: a slowly migrating zone will give a broader peak than a faster zone, even if the two zones have the same width when they pass the UV beam of the detector". Actually the peak width on the chart recorder only indicates the time (seconds or minutes) needed for a zone to pass the detection point. This may be expressed as $w_p = w_z/v$, where w_p is the peak width as seen on the recorder paper, w_z is the zone width in the separating capillary as the zone passes the detection point, and v is the velocity of the zone.

In this experiment, which compared electrophoretic and hydraulic loading, 0.1 *M*, pH 2.5 phosphate buffer was used as both sample buffer and running buffer. A sample solution was prepared with substance P fragments SP(1–4), SP(2–11), SP(5–11), final concentrations 0.5 μg/μl each. In the first part of the experiment, samples were electrophoretically loaded for 8 s. The HPE unit was run under conditions and results as shown in Fig. 1. Then, using the same sample mixture and capillary cartridge, the sample was hydraulically loaded (using applied pressure) into the capillary for different time intervals. Electrophoresis was performed using the same conditions as in Fig. 1. The results are show in Fig. 2.

In electrophoretic loading (Fig. 1), the peaks of SP(1–4), SP(2–11) and SP(5–11) have similar widths. However, when hydraulic loading is used (Fig. 2), the peaks of SP(1–4), SP(2–11) and SP(5–11) have quite different widths. In this case, the peak widths are almost proportional to the retention times. The ratio of peak heights of SP(1–4). SP(2–11) and SP(5–11) in Figs. 1 and 2 are similar. This is because the peak height depends on UV absorption as opposed to the migrating speed of the zone.

To confirm that the broadening of peak widths in Fig. 2 is principally caused by velocity difference as opposed to diffusion, a small peptide, TRH (pGlu-His-Pro-NH_2), was run under the conditions shown in Fig. 1. In this separation, however, the

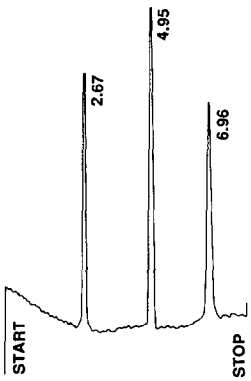

Fig. 1. Substance P fragments SP (1–4), SP(2–11) and SP(5–11) loaded electrophoretically for 8 s at 8 kV. Electrophoresis at 8 kV in 20 cm × 25 μm coated capillary. UV detection at 200 nm. Concentrations 0.5 μg/μl each. Retention times in minutes as indicated.

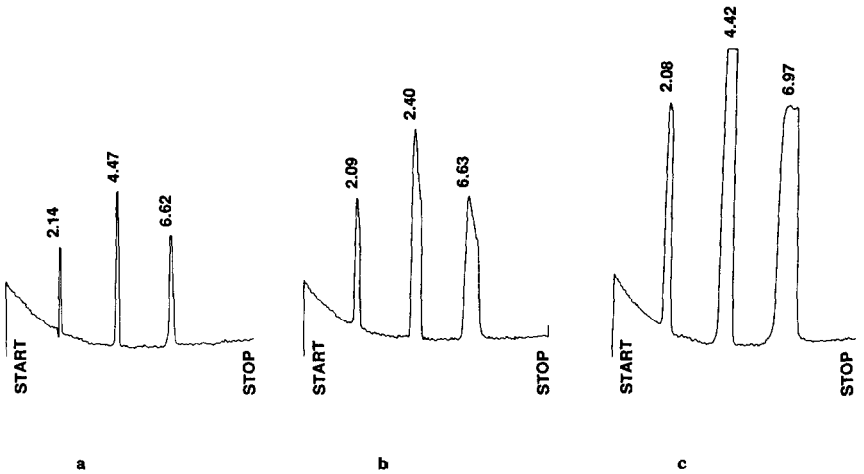

Fig. 2. Substance P fragments SP(1–4), SP(2–11) and SP(5–11) loaded hydraulically to create different length starting zones. Electrophoresis at 8 kV in 20 cm × 25 μm coated capillary. UV detection at 200 nm. Concentrations 0.5 μg/μl each. Starting zone widths: (a) 0.6 cm; (b) 2.0 cm; (c) 3.0 cm. Retention times in minutes as indicated.

voltage was interrupted for varying periods during the run. The peak shapes for various stopping periods are shown in Fig. 3. The results indicate that the peaks have not spread significantly despite pauses of up to 30 min during the run (an entire run

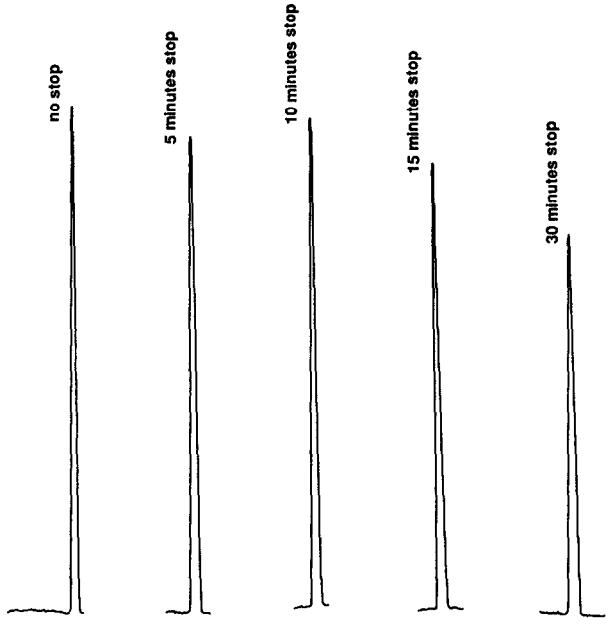

Fig. 3. Sample diffusion in capillary with various stopping periods. Sample: TRH (pGlu-His-Pro-NH_2), 50 ng/μl. Electrophoresis at 8 kV in 20 cm × 25 μm coated capillary. UV detection at 200 nm. "Stop" was performed at 1 min after starting for various intervals. Normal electrophoresis was then completed.

without stopping took only 5 min). Normally, when a zone broadens as a result of diffusion, the peak height also decreases. This effect is not apparent in Fig. 2, indicating that the observed peak broadening is caused by velocity differences in the migrating ions.

Most high-performance capillary electrophoresis (HPCE) separations are performed without any support or sizing medium, (polyacrylamide, agarose, etc.). Separation takes place as a result of the different mass–charge ratios of the sample components. Sample components with similar mass–charge ratios (*e.g.*, various size polynucleotides, or the monomer, dimer and trimer of albumin) may not be separated by free zone electrophoresis. To separate these samples, some molecular sieving is essential. Although gel-filled capillaries have been used in capillary electrophoresis, this technique is inconvenient and does not yield reproducible results[13,14]. DNA fragments and the monomer, dimer and trimer of albumin may be separated by using linear polymers, such as methylcellulose or polyethylene glycol as additives in the HPCE buffer. These additives generate a molecular sieving effect which facilitates the separation. Conditions and results are shown in Figs. 4–6. Solutions of linear polymer such as methylcellulose or polyethylene glycol pass easily through narrow-bore capillaries. This method is more convenient and yields more reproducible results than those obtained when a gel-filled capillary is used.

In most cases, the fractions collected from reversed-phase high-performance liquid chromatographic (HPLC) separations have sample concentrations well above the sensitivity limit of the HPE 100 unit (approximately 5 ng/μl for proteins and peptides). The fractions may contain a large portion of the organic solvent acetonitrile. As acetonitrile has no charge, it should have no effect on the sample separation in the HPCE process[8]. Fig. 7 shows results obtained with acetonitrile present in the sample.

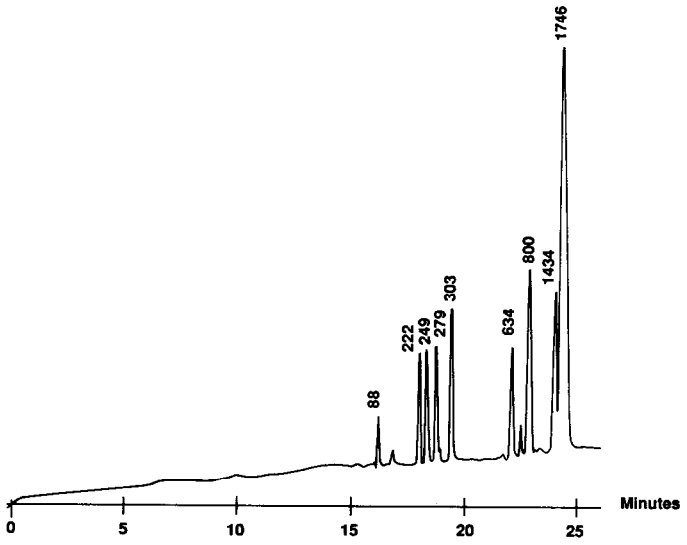

Fig. 4. Molecular sieving of DNA size standard, 88 to 1746 base pairs (as indicated at individual peaks), concentration: 0.2 μg/μl. Separation buffer: 0.089 M TBE, pH 8.0, 7 *M* urea, 0.5% HMC. Electrophoresis at 8 kV in 50 cm × 50 μm coated capillary. UV detection at 260 nm.

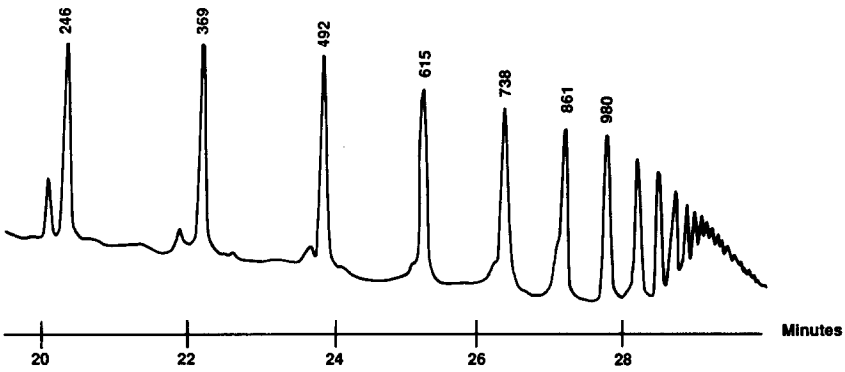

Fig. 5. Molecular sieving of 123 base pair ladder, 123 to 4182 DNA base pairs, concentration: 0.5 μg/μl. Separation buffer: 0.089 *M* TBE, pH 8.0, 0.5% MC. Electrophoresis at 8 kV in 50 cm × 50 μm coated capillary. UV detection at 260 nm.

With 0% to 60% acetonitrile in the sample, the separation patterns are not significantly different over a set of nine peptides. Because HPCE analysis is unaffected by the presence of acetonitrile in the sample, it can be an excellent tool for a further analytical separation following reversed-phase HPLC. Additionally, such separations do not require evaporation of the organic solvent or the concentration of the sample.

Because the buffer-contacting surfaces of the HPE 100 unit are resistant to most

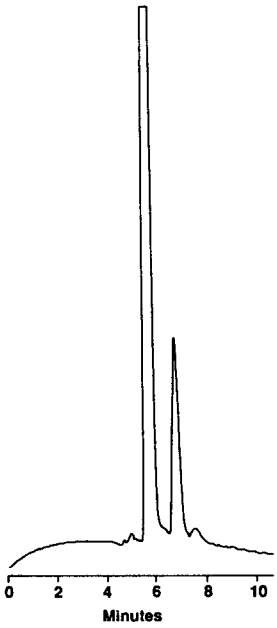

Fig. 6. Separation of bovine serum albumin monomer, dimer and trimer, concentration: 1 μg/μl. Electrophoresis at 8 kV in 20 cm × 25 μm coated capillary. UV detection at 200 nm. Separation buffer: 0.1 *M*, pH 2.5 phosphate with 5% PEG.

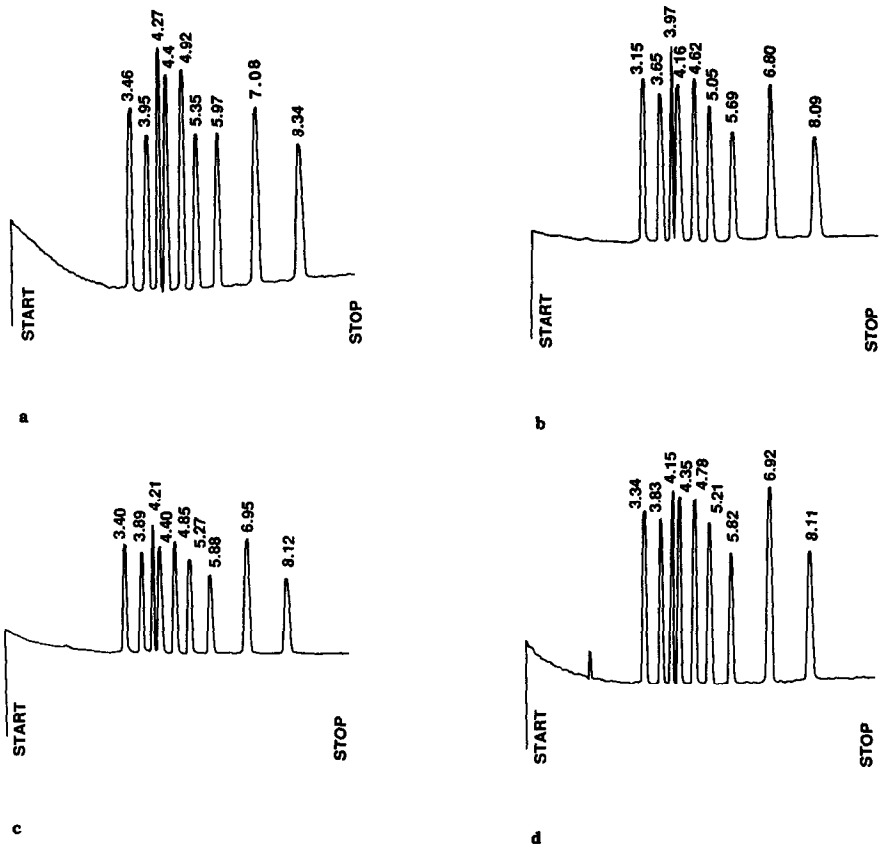

Fig. 7. Effects of acetonitrile in peptide samples, samples injected electrophoretically. Electrophoresis at 8 kV in 20 cm × 25 μm coated capillary. UV detection at 200 nm. Peptides in order of appearance are: bradykinin, angiotensin II, α-melanocyte-stimulating hormone, TRH, luteinizing hormone-releasing hormone, [2–5]leucine enkephalin, bombesin, methionine enkephalin and oxytocin, 50 ng/μl each. Percentage acetonitrile in sample: (a) 0%; (b) 20%; (c) 40%; (d) 60%. Retention times in minutes.

organic solvents except acetone, it is possible to perform electrophoresis with organic solvents present in the separation buffer. Fig. 8 shows a separation performed with 50% acetonitrile present in the separation buffer, using the same sample and conditions as in Fig. 7. Although the peaks retain good resolution, the separation pattern is changed. This indicates that although the presence of organic solvent in the buffer does not destroy the HPCE separation, it may have an influence on the separation mechanism. This characteristic may prove useful in the separation of some difficult hydrophobic samples.

Isoelectric focusing in polymer-coated tube capillary electrophoresis is a two-step process[12,15,16]. Isoelectric focusing in uncoated tubes is not possible because electroendosmosis prevents the formation of stable focused zones. Proteins are mixed with Bio-Lyte ampholytes and hydraulically loaded to fill the capillary. Focusing is performed between electrodes filled with 0.01 *M* phosphoric acid and 0.02 *M* sodium hydroxide. Mobilization of focused zones is accomplished by adding salt to one of the

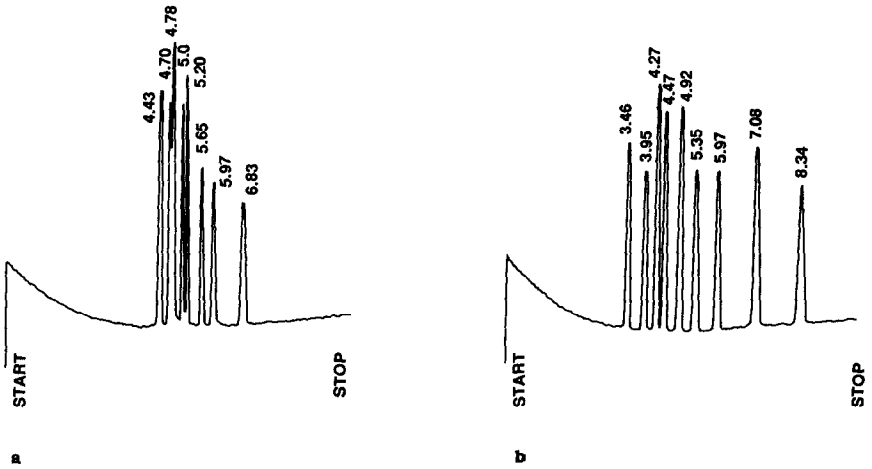

Fig. 8. Effect of 50% acetonitrile in running (electrophoresis) buffer. Electrophoresis at 8 kV in 20 cm × 25 μm coated capillary. UV detection at 200 nm. Running buffer: (a) 0.1 *M*, pH 2.5 phosphate final with 50% acetonitrile; (b) 0.1 *M*, pH 2.5 phosphate without acetonitrile.

electrolytes. A typical separation is shown in Fig. 9. The correct concentrations of Bio-Lyte ampholyte and added salt are essential for good results. According to our study, 1% to 2% ampholyte yields an acceptable result, so it might be appropriate to have a stock HPCE ampholyte, pH 3–10 solution, 2% concentration, for mixing with samples up to 50% in volume, prior to use. Any non OH^- anion in the catholyte or any non H^+ cation in the anolyte will cause the isoelectric focusing zone to move. The mobility and the concentration of this extra ion determines how fast the zone moves. Although as little as 20 m*M* NaCl added into the catholyte will cause zone movement, adding 80 m*M* NaCl to the catholyte yields better reproducibility. Reagents should not be exposed to air. Sodium hydroxide absorbs carbon dioxide rapidly, preventing the formation of stable focused zones in isoelectric focusing.

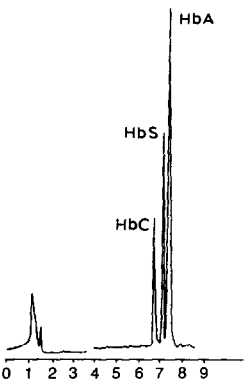

Fig. 9. Isoelectric focusing of hemoglobin in Bio-Lyte ampholytes, pH range 3–10, between the catholyte, 10 m*M* phosphoric acid and the anolyte, 20 m*M* NaOH. Focusing at 8 kV in 12 cm × 25 μm coated capillary. Mobilized by replacing anolyte with 80 m*M* NaCl + 20 m*M* NaOH. UV detection at 280 nm. Time scale in minutes.

CONCLUSIONS

For an HPCE apparatus equipped with an on-line detector, electrophoretic sample loading provides superior quantitative results and sharper resolution than hydraulic sample loading. Electrophoretic loading, made possible by coated capillaries, is therefore the method of choice for an analytical capillary electrophoresis instrument. Solutions of large linear polymers such as HMC, MC and PEG may be used to provide a molecular sieving effect which facilitates the separation of DNA fragments and the monomer, dimer and trimer forms of proteins. The presence of acetonitrile (up to 60%) in a peptide sample has no significant influence on HPCE separations. Therefore fractions collected following reversed-phase HPLC may be separated directly using the HPCE technique. A successful HPCE separation may still be obtained with up to 50% acetonitrile present in the HPCE running buffer. Therefore, the buffer solution may be modified as needed with organic solvents for special purposes such as solubilizing hydrophobic samples. Isoelectric focusing is possible because of the coated capillaries which eliminate electroendosmosis and allow stable focused zones. However, care must be taken to avoid carbonate formation in the anolyte, causing zone drift.

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