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CAPILLARY ELECTROPHORESIS OF PROTEINS IN BUFFERS CONTAINING HIGH CONCENTRATIONS OF ZWITTERIONIC SALTS

MICHELLE M. BUSHEY and JAMES W. JORGENSON*
Department of Chemistry, University of North Carolina, Chapel Hill, NC 27599-3290 (U.S.A.)

SUMMARY

A method for improving protein separations in capillary zone electrophoresis utilizing high concentrations of zwitterionic buffer additives was examined. Lysozyme and α-chymotrypsinogen A were used as test proteins in untreated fused-silica capillaries in buffers of pH *ca.* 7.0 and 9.0 The zwitterion-containing buffers were compared with buffers containing high ionic salt concentrations and a buffer containing a combination of high ionic salt and high zwitterion concentrations. Over 100 000 theoretical plates were obtained in less than 30 min for both test proteins in a pH 7 buffer containing both trimethylglycine and potassium sulfate. The advantages and disadvantages of this technique compared with those of other methods used to prevent protein adsorption are discussed.

INTRODUCTION

Protein separations are an interesting application of capillary electrophoresis, but satisfactory results are difficult to achieve. Protein separations are especially difficult for two reasons. First, detection of proteins is difficult owing to a lack of good chromophore groups in easily accessible spectral regions. The further development of indirect fluorescence[1] and post-column fluorescence detection systems[2,3], among other detection schemes, may help to alleviate this problem. Second, proteins readily adsorb to fused silica. In a diffusion-limited case, theory predicts of the order of several million theoretical plates for proteins[4], but capacity factors as low as 0.05 can reduce efficiencies 20-fold in protein separations[5]. There have been few examples which demonstrate more than 80 000 theoretical plates for protein separations.

Some of the methods that have been used to counteract protein adsorption include capillary surface treatments[6–10], electrophoresis in buffers with a pH higher than the isoelectric point of the sample proteins[11,12] and electrophoresis in buffers with a very low pH[9,10]. Each of these methods has its own particular advantages and disadvantages. Capillary surface treatments generally provide only moderately improved theoretical plate numbers and suffer from reproducibility problems due to gradual loss of surface coverage. Use of buffers of high pH provides theoretical plate numbers near the theoretical limit but suffers from two disadvantages: instability of fused silica at high pH and the fact that it is not a very universal method as it restricts

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operation to basic pH. Buffers with a low pH also perform well but suffer from problems with high conductivity owing to large hydronium ion concentrations.

It has been shown previously that the use of buffers with high salt concentrations also decreased adsorption of protein to the fused silica capillary in capillary electrophoresis[13]. It was found that K_2SO_4 was superior to NaCl, LiCl, KCl, CsCl, KBr and KNO_3, based on considerations of prevention of protein adsorption and UV absorbance interferences. The study demonstrated a separation of lysozyme with 68 000 plates, trypsinogen with 140 000 plates, horse heart myoglobin with 78 000 plates, β-lactoglobulin B with 95 000 plates and β-lactoglobulin A also with 95 000 plates. The separation was performed in a 0.1 *M* 2-[N-cyclohexylamino]ethanesulphonic acid (CHES) buffer at pH 9.0 that contained 0.25 *M* K_2SO_4 and 1 m*M* EDTA. However, the approach had several drawbacks. Owing to the high ionic strength of the buffer, the applied potential was only 5 kV. This resulted in a long analysis time of just under 80 min. Excessive heating of the buffer also necessitated the use of capillaries of I.D. only 25 μm. This small capillary diameter in turn aggravated problems with detection. As UV detection was used and high salt concentrations interfere with UV detection, each protein concentration was 1% (w/v). Although the high protein concentration caused no overloading problems owing to the high ionic strength of the buffer, a 1% (w/v) protein solution is unrealistically high for routine work.

In an attempt to circumvent some of the problems encountered with buffers of high ionic strength, we decided to try a similar approach using zwitterions instead of ionic salts. Zwitterions will not contribute to the conductivity of the operating buffer, but should be able to associate with the negatively charged capillary surface and with charged protein sites. As the zwitterions do not contribute to the conductivity of the buffer, higher voltages can be used and migration times will be shorter than those which can be obtained with the use of ionic salts. If the zwitterion concentration is sufficiently high, the zwitterion associations with the proteins and capillary surface should both prevent protein adsorption to the fused silica and help to break up protein–protein interactions. In this work we detected the proteins by intrinsic fluorescence so that the concentration of protein in the sample could remain low. Other studies have examined the use of zwitterionic silanes to control electroosmotic flow[14], but we believe this to be the first attempt to utilize zwitterions in the capillary zone electrophoresis (CZE) buffer to minimize adsorption.

This study addressed the problem of protein adsorption and investigated the use of buffer additives, namely potassium sulfate and zwitterions, to improve separations of proteins. Lysozyme and α-chymotrypsinogen A were used as test proteins, and buffer solutions containing glycine, glycylglycine, triglycine, sarcosine and betaine were compared with buffers containing potassium sulfate and buffers containing both potassium sulfate and betaine.

EXPERIMENTAL

Materials

Fused-silica capillary tubing with dimensions of 25-μm I.D. and 150-μm O.D. was obtained from Polymicro Technologies. Capillaries were cut to a length of 75 cm with a detection window burned through the polyimide coating 10 cm from the

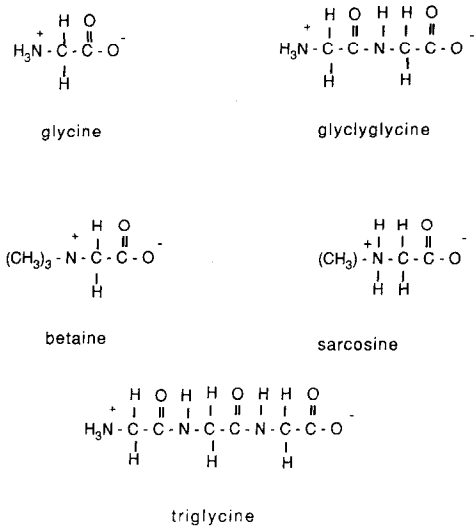

Fig. 1. Structures of the zwitterions used as buffer additives.

grounded end. Glycine, glycylglycine, triglycine, N-methylglycine (sarcosine), CHES, chicken egg-white lysozyme and bovine pancreatic α-chymotrypsinogen A were purchased from Sigma. The two proteins were specifically chosen owing to their relatively high isoelectric points (and their tendency to adsorb strongly) and strong intrinsic fluorescence. Trimethylglycine hydroxide inner salt (betaine) was purchased from Aldrich. Deionized water was further purified with a Barnstead Nanopure system. The structures of the zwitterions used are shown in Fig. 1.

Equipment

Proteins were detected by intrinsic fluorescence with a detector described previously[8]. The excitation wavelength, isolated with a double monochrometer with a 10-nm slit width, was 280 nm and the emission wavelength was isolated with a 340-nm interference filter. Injections were performed with an autoinjector described previously[15].

Electromigration was used to perform injections, and injections were accomplished by applying a potential of 8 kV for 8 s. A 30-kV d.c. reversible power supply was obtained from Spellman High Voltage. A simple cooling system was constructed utilizing a small electric fan to force an air flow over the coiled CZE capillary. As only the buffers containing potassium sulfate demonstrated negative effects from overheating, the cooling system was used only with buffers containing potassium sulfate. All data were collected using an analog-to-digital converter of a multifunction interface board from Scientific Solutions. The multifunction interface board was mounted on an IBM PC/XT microcomputer. Analog signals were filtered with a 100-μF capacitor placed across the analog-to-digital converter input. The data aquisition rate varied between 1 and 3 points per second, depending the particular electrophoretic conditions of individual runs.

TABLE I

ELECTROOSMOTIC FLOW AND PROTEIN RESULTS IN EACH BUFFER SYSTEM

Buffer	*pH*	*E (kV)*	*I (μA)*	*Endoosmotic flow, $\mu_{eo} \times 10^4$ ($cm^2/V \cdot s$)*	*No. of theoretical plates*	
					Lysozyme	*α-Chymotrypsinogen A*
0.04 *M* Phosphate	6.9	30	15	3.91	No useful data	
0.04 *M* Phosphate–2 *M* glycine	6.4	30	11	3.62	No useful data	
0.04 *M* Phosphate–1 *M* glycylglycine[a]	6.1	30	11	2.93	No useful data	1500
0.04 *M* Phosphate–0.5 *M* triglycine[a]	6.2	30	11	2.50	No useful data	500
0.04 *M* Phosphate–2 *M* sarcosine	6.7	30	9.5	2.08	220	10 000
0.04 *M* Phosphate–2 *M* betaine	7.5	30	8.5	3.12	3700	18 800
0.04 *M* Phosphate–0.1 *M* K_2SO_4[b]	7.0	20	55	2.20	28 700	75 000
0.04 *M* Phosphate–0.25 *M* K_2SO_4[b]	7.0	10	47	1.37	142 000	146 000
0.1 *M* CHES–0.25 *M* K_2SO_4[b]	9.0	10	40	2.25	119 000	195 000
0.1 *M* CHES–2 *M* betaine	9.0	30	45	4.11	No useful data	600
0.04 *M* Phosphate–2 *M* betaine–0.1 *M* K_2SO_4[b]	7.6	20	34	1.88	128 000	189 000

[a] Concentration limited by solubility.

[b] Capillary cooled in fan air flow.

Methods

Fused silica was treated with a series of KOH and HCl rinses as follows: 10 min with 1 *M* KOH, 10 min with 0.1 *M* KOH, 10 min with water, 10 min with 0.1 *M* HCl and 20 min with the electrophoresis buffer. In addition, capillaries were subjected to a 5-minute 0.1 *M* KOH rinse, 5-min 0.1 *M* HCl rinse and 5-min water rinse before new buffers were introduced. No other surface treatment was used, and capillaries were not silylated. A few millimeters of polyimide coating were burned off the injection end of the capillary to prevent sample from becoming trapped between the fused silica and polyimide coating during injections.

All phosphate-containing buffers were made from the same 0.1 *M* sodium phosphate stock solution (pH 6.9). Appropriate amounts of the additive investigated were added to aliquots of the stock solution and diluted with deionized water to make a 0.04 *M* sodium phosphate buffer. In order to avoid unnecessary variability in ionic strength, the pH was not readjusted following additions of zwitterions. However, the pH 9 CHES buffers were adjusted with NaOH after the addition of buffer additives and dilution to the desired concentration.

Table I lists the buffers used, the additive concentrations, pH, current and applied voltage for each buffer system investigated. All protein solutions were 0.1% (w/v) in each protein and the proteins were dissolved in the electrophoresis buffer. Indole was used as a neutral marker of electroosmotic flow for each buffer system. All migration times were based on the first statistical moment and all theoretical plates were also determined by statistical moments.

RESULTS AND DISCUSSION

In choosing the zwitterions, the first consideration was to chose compounds that were zwitterionic over a wide pH range, the second was that these compounds should have a high solubility in the buffer system tested and the third was that as the zwitterions increase in size, ideally they should not increase in hydrophobicity. If the compounds chosen are hydrophillic there is less likelihood that they will promote protein denaturation.

In addition to information on parameters, Table I also lists electroosmotic flow and protein information for each buffer system tested. When no protein information is listed, the proteins had either undergone irreversible adsorption to the capillary surface in that particular buffer, or the peak shape was so poor that no useful information could be extracted. High concentrations of potassium sulfate increase the conductivity of the buffer so much that lower applied potentials had to be used compared with buffers not containing potassium sulfate. In addition, each of the buffers which contained potassium sulfate had to be cooled, as mentioned under Experimental, in order to permit application of the potential levels listed in Table I. Both glycylglycine and triglycine are not as soluble as the other zwitterions used and so could not be dissolved at a 2 *M* concentration. These two compounds have the added disadvantage of strong UV absorption at short wavelengths, which precludes the use of this type of detection system.

Comparing the phosphate-only buffer with the phosphate–zwitterion buffers, current and electroosmotic flow decreases are observed for each zwitterion tested. The observed decrease in electroosmotic flow is a combination of lower pH, higher

viscosity and possible changes in the double layer due to high zwitterion concentrations. Comparing the phosphate-only buffer with the phosphate–K_2SO_4 buffers, current increases and electroosmotic flow decreases are observed for K_2SO_4-containing buffers. The high ionic strength of the K_2SO_4 buffers decreases the double layer thickness and zeta potential of the capillary wall[16]. A decrease in zeta potential is in turn proportional to a decrease in electroosmotic flow. An increase in viscosity is the likely explanation of a further decrease in both current and electroosmotic flow on comparing the phosphate–0.1 *M* K_2SO_4 buffer with the phosphate–0.1 *M* K_2SO_4–2 *M* betaine buffer, as the betaine-containing buffer was visibly more viscous than the buffer without betaine. Increasing the pH from near 7 to 9 produces an increase in the electroosmotic flow, as expected, because more of the surface silanols are ionized at pH 9. This effect is seen on comparing the phosphate–0.25 *M* K_2SO_4 buffer with the CHES–0.25 *M* K_2SO_4 and the phospate–2 *M* betaine buffer with the CHES–2 *M* betaine buffer.

As expected, the sodium phosphate-only buffer does not prevent irreversible adsorption of either of these two proteins. Likewise, the buffer containing glycine provides no useful data. Buffers containing glycylglycine and triglycine begin to show some positive results. Lysozyme is still irreversibly adsorbed in these two systems, but α-chymotrypsinogen A is able to migrate through the capillary, although the resulting

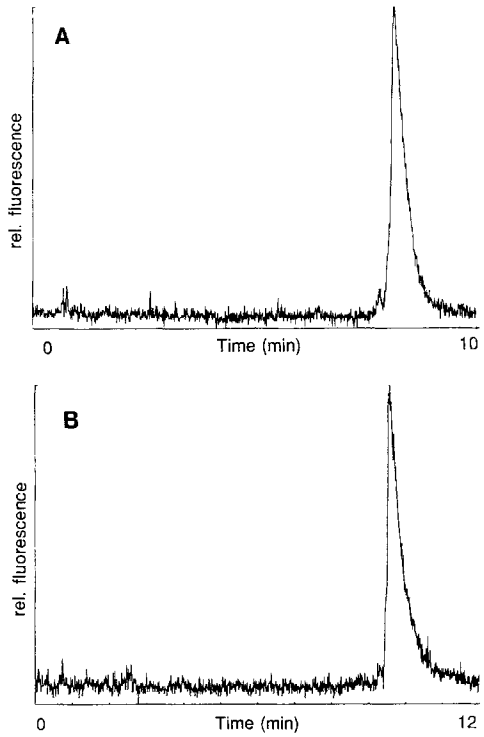

Fig. 2. Electropherograms of α-chymotrypsinogen A. Buffers: (A) 0.04 *M* sodium phosphate–1 *M* glycylglycine, pH 6.1, 11 μA; (B) 0.04 *M* sodium phosphate–0.5 *M* triglycine, pH 6.2, 11 μA. Applied potential, 30 kV in both runs; capillary, 25-μm I.D., 150-μm O.D.; injections, 8 kV for 8 s each.

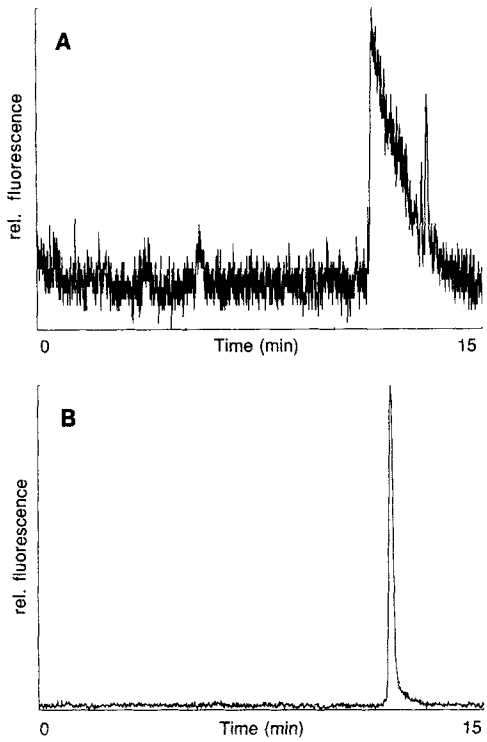

Fig. 3. Electropherograms of (A) lysozyme and (B) α-chymotrypsinogen A in 0.04 *M* sodium phosphate–2 *M* sarcosine, pH 6.7, 9.5 μA. Other conditions as in Fig. 2.

peak shapes are poor. Fig. 2 shows the electropherograms of α-chymotrypsinogen A in these two buffer systems.

Results for the sarcosine system are shown in Fig. 3. Lysozyme is able to migrate through the capillary in this system but the peak shape is still extremely poor. The results are much better for α-chymotrypsinogen A in the sarcosine buffer, but the theoretical plate numbers are low at only 10 000. The phosphate–betaine system seems to perform better than the phosphate–sarcosine system in preventing adsorption of these two proteins, although the observed increase in the number of theoretical plates might be explained by the pH difference between the two buffers. Even so, these two proteins still show signs of tailing in the betaine buffer and the theoretical plate numbers are still low. Although betaine should remain a zwitterion at pH 9.0, the CHES–betaine buffer performs much more poorly than the phosphate–betaine buffer, as evidenced by the fact that lysozyme adsorbs irreversibly and the number of theoretical plates obtained for α-chymotrypsinogen A is low. Both of these electropherograms are shown in Fig. 4.

The best results for prevention of protein adsorption are observed in buffers containing high concentrations of potassium sulfate. The buffers containing 0.25 *M* potassium sulfate seem to perform best, at both pH 7.0 and 9.0. These two electropherograms are shown in Fig. 5. Although the numbers of theoretical plates obtained for

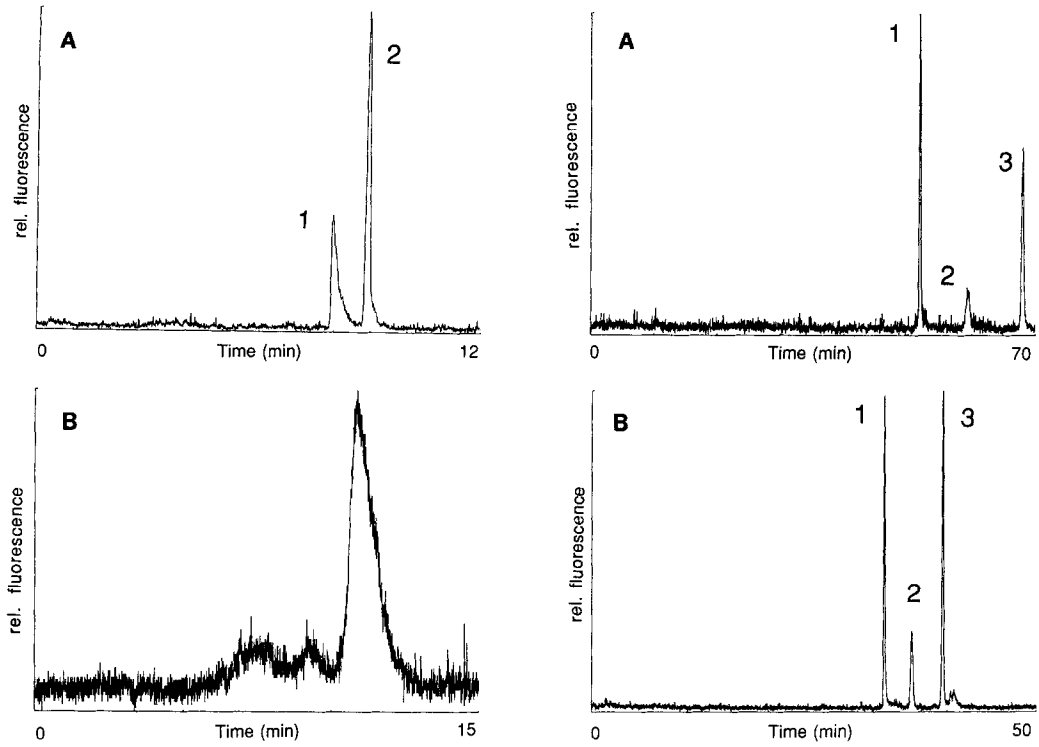

Fig. 4. Electropherograms of (A) lysozyme (peak 1) and α-chymotrypsinogen A (peak 2) in 0.04 *M* sodium phosphate–2 *M* betaine, pH 7.5, 8.5 μA and (B) α-chymotrypsinogen A in 0.1 *M* CHES–2 *M* betaine, pH 9.0, 45 μA. Other conditions as in Fig. 2.

Fig. 5. Electropherograms of (A) lysozyme (peak 1), indole (peak 2) and α-chymotrypsinogen A (peak 3) in 0.04 *M* sodium phosphate–0.25 *M* K_2SO_4, pH 7.0, 47 μA, 10 kV and (B) lysozyme (peak 1), indole (peak 2) and α-chymotrypsinogen A (peak 3) in 0.1 *M* CHES–0.25 *M* K_2SO_4, pH 9.0, 40 μA, 10 kV, both with an air-cooled capillary. Other conditions as in Fig. 2.

both of the proteins are high at both pH values, the analysis time is exceptionally long in both buffers. This is a result of both the lower electroosmotic flow and the low applied voltage. Lowering the concentration of potassium sulfate from 0.25 to 0.1 *M* dramatically decreases the analysis time but also provides less satisfactory results, as the theoretical plate numbers are lower and the peaks exhibit more signs of tailing. This electropherogram is shown in Fig. 6.

Interesting results are given by the buffer containing both betaine and potassium sulfate. Although the buffer contains only 0.1 *M* potassium sulfate, it provides theoretical plate numbers similar to those obtained with the buffer that contains 0.25 *M* potassium sulfate. The combination buffer also performs better than the buffer that contains 2 *M* betaine alone. An advantage of the combination buffer over the potassium sulfate-only buffers is that with the lower salt concentration and the added viscosity provided by the 2 *M* betaine, higher voltages can be applied and analysis time is halved with no decrease in efficiency. Electropherograms for buffers 0.1 *M* potassium sulfate, both with and without betaine, are shown in Fig. 6.

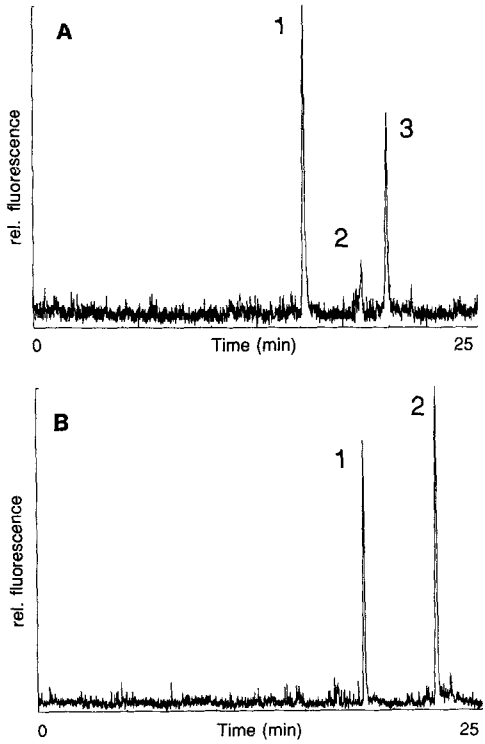

Fig. 6. Electropherograms of (A) lysozyme (peak 1), indole (peak 2) and α-chymotrypsinogen A (peak 3) in 0.04 *M* sodium phosphate–0.1 *M* K_2SO_4, pH 7.0, 55 μA, 20 kV and (B) lysozyme (peak 1) and α-chymotrypsinogen A (peak 2) in 0.04 *M* sodium phosphate–2 *M* betaine–0.1 *M* K_2SO_4, pH 7.6, 34 μA, 20 kV, both with an air-cooled capillary. Other conditions as in Fig. 2.

Addition of zwitterions or ionic salts to buffers could be one way to help tailor electroosmotic flow velocities to desired values. The use of zwitterions for this purpose has the added advantage that the increase in viscosity may help to decrease band broadening due to suppression of convection.

Although suppression of protein adsorption by use of buffers with high pH values provides larger theoretical plate numbers than those obtained with the method outlined here, very basic proteins such as lysozyme can seldom, if ever, be run in those sytems. Capillary surface treatment methods, as mentioned earlier, suffer from reproducibility problems as the treated surface is unstable. Buffers with low pH work reasonably well in minimizing protein adsorption, but the method suffers from the same disadvantage as buffers with high ionic salt concentrations, namely, high conductivity. The use of buffers that combine high zwitterion concentrations with ionic salts provides reasonable theoretical plate numbers for proteins such as lysozyme and α-chymotrypsinogen A, which normally suffer irreversible adsorption in CZE. These buffers not only provide good separation efficiencies but also permit the use of higher voltages, with reasonably fast analysis times.

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