Controlling electroosmotic flow in capillary zone electrophoresis

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Abstract

Electroosmotic flow in capillary zone electrophoresis must be controlled for precise and reproducible performance. We found that the addition of any one of several classes of compounds such as amines, amino acids or organic acids to the running buffer stabilizes electroosmotic flow. With the additives in the buffer, the precision of migration times improved to better than 1%. The reproducibilities improved to better than 5%. The system is very stable at various conditions for small molecules and for proteins at very acidic or very basic conditions.

1. Introduction

Capillary electrophoresis (CE) is an analytical separation technique capable of high resolution because of its inherent high efficiency. Unlike in chromatography, the resolution in CE should improve continuously as the migration times decrease (or as the applied voltage is increased). This potential of high efficiencies, high resolutions and short analysis times in CE have attracted the attention of researchers in various fields, and the use of CE is growing exponentially.

To be an acceptable analytical technique, the precision of migration times and peak areas need to be high. If we take chromatographic figures-of-merit as a rough guideline, then the precision of the data should be about 1% and the reproducibility less than 5% [1]. Frequently, CE done in open and untreated capillaries shows much poorer precisions and reproducibilities (viz. [2,3]). One of the reasons for the poorer precision is the difficulty of maintaining constant electroosmotic flow (EOF), which occurs when an external electric field is imposed across a capillary whose inner wall is charged [4]. In such cases, solutes migrate through the capillary as a result of electrophoresis and electroosmosis. The apparent reason for the lack in precision may be due to variations in the capillary silica surface, occurring during and between electrophoretic separations, which cause variation in the EOF.

There are many literature reports on the lack of precision in CE (e.g., [5–11]). There is also a great deal of work attempting to improve the precision of CE, mainly by manipulating EOF; for example, by the use of surface-active additives (e.g. [12–14]), of ions and zwitterions (e.g. [15]), of polymers (e.g. [16]) and of organic modifiers (e.g. [17–20]). Other attempts using pH (e.g. [18]) and chemical derivatization of the capillary wall (e.g. [21–24]) have been reported.

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External electric field has been used also to control EOF (e.g. [25–27]).

In spite of all the efforts mentioned above, there is still a need for a simple and a reliable way to control and maintain a constant EOF. In the present communication we report on the use of several classes of compounds which allow us to control EOF better, and thus to give stable CE systems with excellent precision in migration times without the need for capillary rinsing between injections. The rationale for the choice of the additives will be discussed.

We should stress here that the aim of the present work was to control EOF, and therefore to improve the precision of the data, and not to optimize the efficiencies of the separations. The solutes in this study were not chosen with an application in mind but rather due to convenience and availability. Thus, the results are reported mainly in terms of consistency in migration times and in efficiency and not in terms of efficiency improvement.

2. Material and methods

2.1. Apparatus

Separations were performed on a laboratory-made CE unit. A high-voltage power supply (Glassman, NJ, USA) was used to establish the electrical field across the capillary. The output voltage of the power supply was computer-controlled. Separations were done in polyimide-coated fused-silica capillaries (Polymicro Technologies, CA, USA), 50 and 100 μm I.D. and 375 μm O.D. Separation lengths of the capillaries varied from 40 to 44 cm, and the total length of the capillaries varied from 72 to 74 cm. Detection was done with a Model 200 UVIS absorbance detector (Linear Instruments, CA, USA) at 280 or 200 nm for proteins. About a 1-cm section of the capillary coating was removed by heat and it served as a UV detection window. The signals from the detector were fed to a Model 600 recorder (W + W Electronic, Switzerland) and to a Model D-2000 Hitachi integrator (Merck, Germany).

2.2. Reagents

Buffers

The running buffers were made from either NaH_2PO_4 (Baker, USA), Na_2HPO_4 (Mallinckrodt, USA), KH_2PO_4, CH_3COONa (Merck) or Tris (Serva, Germany). Final pH was adjusted with a 1 M solution of either NaOH, H_3PO_4, CH_3COOH or HCl (Frutarom, Israel). Most of the experiments were done using a 0.02 M NaH_2PO_4 buffer at pH 6.00 adjusted with 1 M NaOH.

Additives

Three classes of additives were studied. The first additive class, which were amines, included 0.01 M solutions of the following compounds: triethylamine (TEA), triethanolamine (Fluka, Switzerland), propylamine, n-pentylamine, piperidine (Sigma, MO, USA), dipropylamine, dipentylamine, tripropylamine, morpholine and histamine (Aldrich, WI, USA). The second class of additives included 0.001 M solutions of the following amino acids: alanine, arginine, asparagine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine and valine (Takara Hohsan, Japan). The aromatic amino acids (phenylalanine, tryptophan and tyrosine) were not used because of solubility and detection problems. The third class of additives, which were acids, included 0.01 M trimethylacetic acid (TMA) (Fluka) and 0.001 M imidazole-4-acetic acid (Sigma).

Solutes

Phenol (Mallinckrodt) or acetone (Frutartom) was used as a neutral marker for measuring EOF. Other solutes included the amino acid tyrosine (Sigma) and the proteins lysozyme, myoglobin and trypsinogen (Sigma). The solutes were dissolved (1 mg/ml) in the running buffer.

2.3. CE procedures

The introduction of each new additive was followed by 3 h of conditioning as described below. In addition, the capillary was conditioned

at 25 kV for 1 h at the beginning of each working day. Injection of the sample into the capillary was made by electromigration at 5 kV for a fixed period of time (1–5 s). Electrophoresis was usually run at 25 kV applied voltage. The current through the capillary did not exceed 50 μA.

2.4. Capillary treatment

Each new capillary was cleaned by flushing, sequentially, with 1 M KOH (Frutarom) for 15 min, then with triply distilled water for 30 min and, finally, with the running buffer containing an additive for few seconds. The capillary was conditioned for 3 h, at 25 kV, to allow equilibration of additive interactions with the capillary wall. Similar procedure was employed whenever a new additive was used. *This treatment eliminates the need for capillary washing between runs*.

3. Results and discussion

It is an experimental fact that most conventional electrophoresis buffers do not yield constant EOF. The exact causes for the variation in EOF are not known with certainty, but they are thought to be related to continuous modification of the wall surface resulting from interactions with buffer components and with solutes. Simple attempts to maintain an homogeneous surface, such as rinsing the capillary with bases, acids, triply distilled water etc. are not helpful in stabilizing EOF. Fig. 1 is a plot of migration time of the EOF marker (phenol) for sequential injections. The figure shows that the migration times decrease continuously with successive injections. This decrease was observed with every buffer studied here. The initial decrease is quite large, but is less pronounced with time; however, even after 8 working hours there is still a decrease in the migration time. The general trend is quite clear and there is very little deviation from that trend.

We control EOF, and therefore migration times, by the use of additives. Three classes of compounds, amines, amino acids and acids were investigated by us as additives. The reasons for
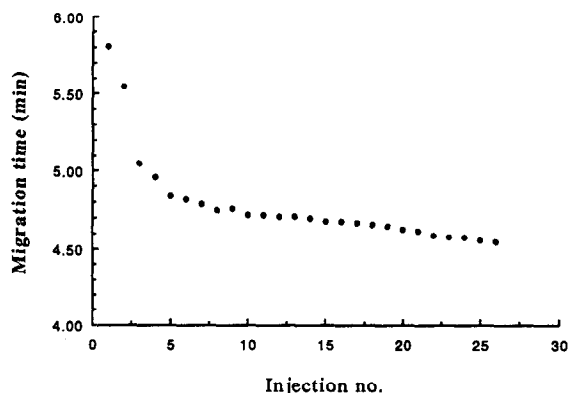

Fig. 1. Migration times of phenol as a function of injection number. No additive in buffer which was 0.02 M NaH$_2$PO$_4$ pH 6. Applied voltage was 25 kV.

choosing these classes of compounds are: from HPLC we know that amines interact with silanol groups, thus masking them from the solutes. Since the amines are positive at the pH used here, they should be electrostatically attracted to the capillary wall. Thus, the amines should shield the wall from impurities in the buffer that otherwise might be adsorbed onto it. The adsorption of impurities will change the nature of the double layer on the wall and therefore change EOF.

The choice of amino acids was made to negate the effects that simple amines have on the current density in the capillary. Since amino acids are zwitterions, their contribution to the conductivity of the buffer is smaller than that of amines. Yet, amino acids have an amine group which should interact with the wall surface.

Finally, since amino acids have also an acidic group, we decided to investigate the behavior of acids.

3.1. Additive type: amines

A common practice in HPLC is the addition of amines to the mobile phase to diminish, or even eliminate, the adsorption of basic solutes to the bare silica gel via silanol interactions. The accepted mechanism of the amine action is by competitive interaction with the free silanols on the silica gel. Since the capillary wall in CE also
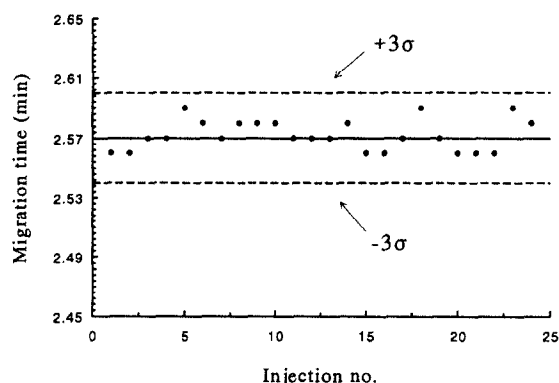
Fig. 2. Migration times of phenol as a function of injection number. 0.001 M TEA additive in buffer which was 0.02 M NaH$_2$PO$_4$ pH 6. Applied voltage was 25 kV.

possesses free silanols, which are responsible for the charge of the surface and, hence, for EOF, it was decided to add an amine to the running buffer to stabilize the wall charge. Fig. 2 shows, in a control chart fashion, the behavior of successive migration times when 0.001 M TEA was added to the buffer. Beside the fact that the migration time is shorter when TEA is present, the figure shows that t_R is constant with a coefficient of variation of about 0.4%. The solid line in Fig. 2 is the average for all the runs. The dashed lines are the $\pm 3\sigma$ lines. The variations in the migration time does not seem to have any trend, i.e. the fluctuations shown are random. The difference in t_R when TEA is present is, most likely, due to the fact that in the TEA study the capillary was not fan-cooled while the

data in Fig. 1 were obtained with temperature control.

Table 1 summarizes the results as a function of applied voltage and of TEA concentration. The data in the table show that the precision in migration time is excellent with relative standard deviations less than 1% in all cases. This precision is sufficient to recommend CE as an analytical tool for routine applications. In addition, the data in the table show that, for a given applied voltage, there is a slight increase in the migration time with an increase in TEA concentration. The increase in migration time might be explained by the increase in ionic strength of the buffer caused by the increasing amine concentration. Similar dependence of EOF on ionic strength was reported by several workers (e.g. [12,17]).

Similar results were obtained with all the amines that were examined here. Table 2 summarizes the results concerning the migration data. The presence of an amine in the buffer results in excellent precision in the migration times. The electroosmotic velocity differs from additive to additive. Table 2 shows that while there is no relationship between the migration times and the additives' pK_a values, there is a good correlation with the ratio of the dielectric constant, ε, to the viscosity, η, as anticipated from the Smoluchowski equation [4]. Undoubtedly, variation in the chemical nature of the amines influences their interaction with the capil-

Table 1

Migration times and standard deviations (in parentheses), as a function of applied voltage and concentration of TEA

Applied voltage (kV)	Migration time (min)			
	$2 \cdot 10^{-4}$ M TEA	$1 \cdot 10^{-3}$ M TEA	0.01 M TEA	0.07 M TEA
5	18.2 (0.05)	19.3 (0.05)	20.6 (0.02)	—
10	8.47 (0.02)	9.20 (0.06)	10.2 (0.03)	—
12.5	6.50 (0.01)	6.43 (0.05)	7.75 (0.03)	7.00 (0.02)
15	5.34 (0.02)	5.70 (0.04)	6.34 (0.02)	6.06 (0.01)
17.5	4.19 (0.01)	4.57 (0.04)	4.77 (0.02)	5.00 (0.01)
20	3.58 (0.02)	3.72 (0.02)	3.90 (0.01)	3.99 (0.01)
25	2.32 (0.02)	2.57 (0.01)	2.74 (0.02)	2.78 (0.02)

Migration times are averages of anywhere between 4 and 20 runs. Solute is phenol; phosphate buffer at pH 6.

Table 2
Migration times of phenol, and their standard deviations as a function of the amine used to control EOF

Amine	pK_a	ε/η	Migration time (min)	Standard deviation
Triethanolamine[a]	7.76	47.4	2.69	0.01
Propylamine	10.74	15.04	3.35	0.007
Dipropylamine	11.00	7.19	3.91	0.006
Tripropylamine	10.66		4.72	0.005
Histamine	9.83		4.74	0.008
Pentylamine	10.75	5.5	4.87	0.008
Trietylamine	10.72	7.49	5.1	0.02
Morpholine	8.3	4.41	5.32	0.01
Dipentylamine	≈11		5.69	0.016
Piperidine	11.12	4.74	6.12	0.01

Applied voltage was 25 kV; amine concentration was 0.01 M except histamine whose concentration was 0.001 M; buffer was 0.02 M NaH$_2$PO$_4$ at pH 6.0. Migration times are averages of anywhere between 4 and 20 runs. Values of dielectric constants ε, and viscosities, η, are mainly at 25°C [28].
[a] Indicates values obtained without fan-cooling of capillary.

lary wall, thus giving different EOF and migration times for each additive. In addition, some of the data were collected on various capillaries which might also cause the observed scatter in the migration data.

Day-to-day reproducibility was also examined and representative results are given in Table 3. Typical values for day-to-day reproducibilities are 5% or less as shown in the table. Similar

Table 3
Day-to-day reproducibility in migration times of phenol with TEA in buffer

Day	Migration time (min)	Standard deviation
1	5.56	0.008
2	5.89	0.006
3	6.17	0.006
4	5.80	0.02
Average	5.86	0.25[a]

Applied voltage was 25 kV; TEA concentration was 0.01 M; 0.02 M NaH$_2$PO$_4$ buffer at pH 6.0.
[a] Standard deviation of the average migration time of the daily migration time averages.

values were obtained for all amines examined irrespective of whether they are primary, secondary or tertiary amines.

3.2. Additive type: amino acids

As mentioned above, a possible disadvantage in the use of amines as buffer additives is the relatively high current which can result from their presence. Consequently, it was decided to examine amino acids which, being zwitterions, should be poorer conductors. Moreover, amino acids present an amine-type additive with additional functional groups which might influence the selectivities toward charged and uncharged solutes. Amino acids, as well as other zwitterions, were used previously in CE but mainly to eliminate surface adsorption of proteins (e.g. [15,29]). We examine here sixteen different amino acids as additives to control and stabilize EOF. Most of the common amino acids were studied with the exception of the aromatic ones (phenylalanine, tyrosine and tryptophan).

Table 4 shows typical results with four amino acid additives with different functional groups. The migration time values are the average of anywhere from 8 to 32 runs. As a further example, Fig. 3 shows the stability of the EOF, as measured by the migration times of phenol, when serine was the additive. The relative standard deviation of the data in Fig. 3 is 0.16%.

Similar results were obtained for all 16 amino acids studied irrespective of whether the additive was polar, hydrophobic, acidic or basic. Table 4 demonstrates that EOF is independent of the

Table 4
Effect of amino acids in buffer on precision of migration time

Amino acid additive	Migration time (min)	Standard deviation
Lys	4.29	0.009
Arg	4.37	0.007
Asp	4.53	0.017
Ser	4.40	0.007

Solute was phenol; applied voltage was 25 kV; buffer as in Table 3; amino acid concentration was 0.001 M.
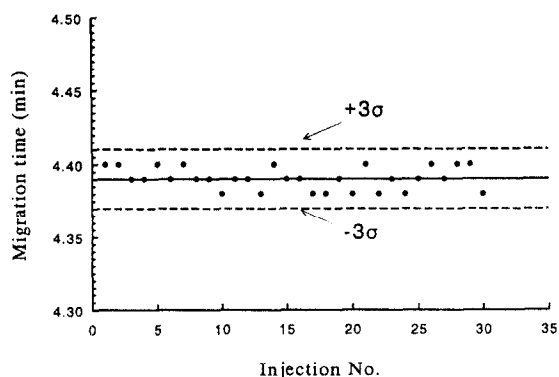
Fig. 3. Migration times of phenol as a function of injection number. 0.001 M Serine additive in buffer which was 0.02 M NaH_2PO_4 pH 6. Applied voltage was 25 kV.
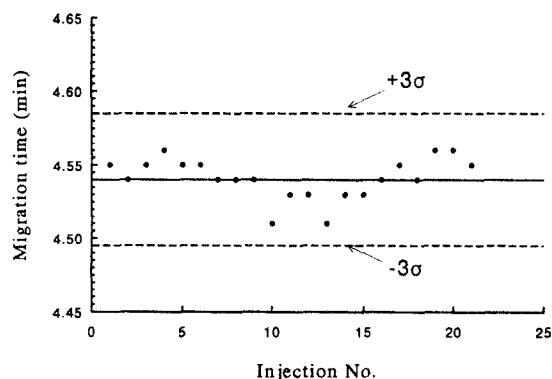

Fig. 4. Migration times of phenol as a function of injection number. 0.01 M TMA additive in buffer which was 0.02 M NaH_2PO_4 pH 6. Applied voltage was 25 kV.

nature of the amino acid additive. When tyrosine was used as a test solute, its migration times were also independent of the nature of the additive. Thus, at least for the solutes that we have examined, no selective interactions were observed with the amino acids.

Table 5 shows typical reproducibilities for some amino acid additives. The number of days over which the data were amassed is shown in the table. The results are alike those observed in chromatography. Similar results were obtained with all amino acids studied irrespective of the solutes used or the nature of amino acids.

3.3. Additive type: organic acids

Since amino acids have an acidic group as well as an amine group, organic acids were investigated as additives to control EOF. Fig. 4 shows typical results when TMA was used as an addi-

tive. Imidazole-4-acetic acid affected EOF in a similar fashion. Much to our surprise, excellent precision was also obtained with this class of additive.

3.4. Effect of buffer pH

The stabilizing effect of the amines or the acid additives occurs over a wide range of buffer pH. Fig. 5 shows the electroosmotic velocity, monitored by acetone as the solute, as a function of pH when TEA was present in the running buffer. The data were collected at pH 5 and higher, since at lower pH TEA is no longer effective as EOF stabilizer. Fig. 6 shows similar

Table 5
Effect of amino acids in buffer on the reproducibility of solute velocity

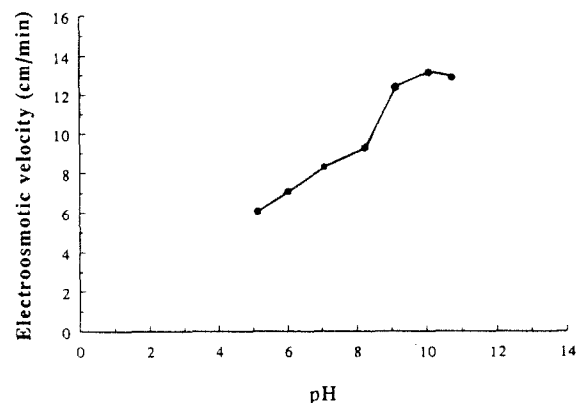| Amino acid	No. of days	Velocity (cm/min)	Standard deviation
Gly	4	9.5	0.095
Val	4	9.5	0.35
Arg	3	9.5	0.25
Cys	3	9.3	0.51

Experimental conditions as in Table 4.

Fig. 5. Electroosmotic velocity as a function of pH. Solute was acetone. Applied voltage 25 kV; 0.02 M phosphate buffer, 0.01 M TEA in buffer.
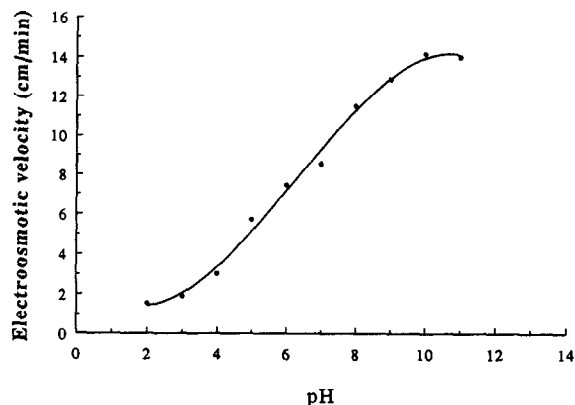
Fig. 6. Electroosmotic velocity as a function of pH. Solute was acetone. Applied voltage 25 kV; 0.02 *M* phosphate buffer, 0.01 *M* TMA in buffer.

results for TMA as additive. Acetone was chosen as solute since it is uncharged throughout the pH range studied. In both figures, the filled circles, drawn to represent the experimental points, are wider than ± 1 standard deviation of the measurements. Therefore, both types of additives can be used to stabilize EOF over a wide range of pH. To achieve the wide pH ranges shown, the phosphate buffer was prepared from either NaH_2PO_4 or Na_2HPO_4 depending on the actual pH.

Several additional observations should be made with regard to Figs. 5 and 6: (a) In the pH range studied the TMA changes from being almost completely unionized to being completely ionized (negatively charged). TMA's effectiveness in controlling EOF seems to be independent of its ionization state. TEA changes from being completely ionized (positively charged) at the low pH to being only about 50% charged. Here too, the effectiveness of TEA as EOF controller seems to be pH independent. (b) Whether the additive was an acid or a base, the electroosmotic velocity of the acetone probe was roughly the same, both in magnitude and direction, at a given pH, for both additives. The magnitude of the EOF for each additive seems to be a function of its charge, viscosity and dielectric constant. (c) The increase in EOF with an increase in pH is a well understood phenomenon (e.g. [12,23]). The sigmoid shape of the curve in Fig. 6 is typical of

acid–base titration curves. The behavior of the EOF most likely mirrors the concentration of the ionized silanol groups on the capillary surface. Thus, by measuring the EOF as a function of the pH, the titration of the capillary surface silanols can be monitored. However, and more importantly, Figs. 5 and 6 indicate that the additives used here do not control the EOF by strongly adsorbing to the capillary surface. If additive adsorption was the stabilizing mechanism, then the pH dependence of the EOF would have been much smaller [23]. It is our opinion that the additives used in this study work more like supporting electrolytes in polarography: by introducing a larger number of charge carriers a narrower wall double layer is obtained with a better defined wall ζ potential, resulting in a more stable EOF.

The discussion until now centered on small and neutral solutes. To examine the applicability of the additives to stabilize the migration times of large solutes, we used lysozyme, trypsinogen and myoglobin as test molecules. The running buffer was either Na_2HPO_4 (pH 11) or NaH_2PO_4 (pH 2). These pH values were chosen since they minimize the adsorption of the proteins to the capillary wall. Again, we found that with additives in the buffer the migration time precision improved. For example, at pH 11 the precision improved from 7% to less than 1% when using either TEA, TMA or isoleucine as the additive.

3.5. Effects of additives on peak shape

The additives discussed above affect favorably not only the migration data but also peak shape and width. For example, in the absence of additives typical relative standard deviation in plate height (*H*) measurements is between 20 and 40%. The presence of an additive improves the precision in efficiency drastically as Table 6 shows. With TEA additive the relative standard deviation is at worst about 15% and mostly less than 10%. Similar results were observed with all additives studied here.

More important is the effect of the additive on the peak shape in the case of large molecules. As

Table 6
Plate heights and standard deviations (in parentheses), as a function of applied voltage and concentration of TEA

Applied voltage (kV)	H (μm)			
	$2 \cdot 10^{-4}$ M TEA	$1 \cdot 10^{-3}$ M TEA	0.01 M TEA	0.07 M TEA
5	6.1 (0.7)	6.1 (0.7)	6.5 (0.1)	–
10	3.9 (0.1)	3.4 (0.1)	3.8 (0.3)	–
12.5	3.0 (0.2)	3.1 (0.1)	3.3 (0.1)	3.7 (0.3)
15	3.1 (0.2)	2.8 (0.2)	2.9 (0.2)	3.6 (0.4)
17.5	2.8 (0.2)	2.6 (0.1)	3.1 (0.03)	3.0 (0.01)
20	3.0 (0.3)	2.6 (0.2)	3.1 (0.2)	3.0 (0.1)
25	2.4 (0.4)	2.4 (0.4)	3.2 (0.1)	2.8 (0.3)

Other experimental conditions as in Table 1.

an example, Fig. 7 shows the peak which results from an injection of lysozyme at pH 11. Without the additive, or a pre-run wash, the resulting lysozyme peak is broad and badly tailing. The plate count was a very poor 5000. After a buffer and triply distilled water wash the peak shape improved and the plate number increased to

Fig. 7. The effect of adding TEA to the buffer on the efficiency and the peak shape of lysozyme. 0.02 M Na$_2$HPO$_4$ buffer pH 11, 25 kV.

30 000. With TEA in the running buffer the lysozyme peak becomes much narrower with a plate count of 250 000. All of the above values of H for lysozyme are much lower than the theoretically predicted value. The peak still tails but the overall performance is far superior with the additive in the buffer. Similar results were obtained with myoglobin and trypsinogen. Their peak shapes improved in the presence of any of the additives examined here. Note, again, that the presence of the additive eliminates the need for washing the capillary between injections.
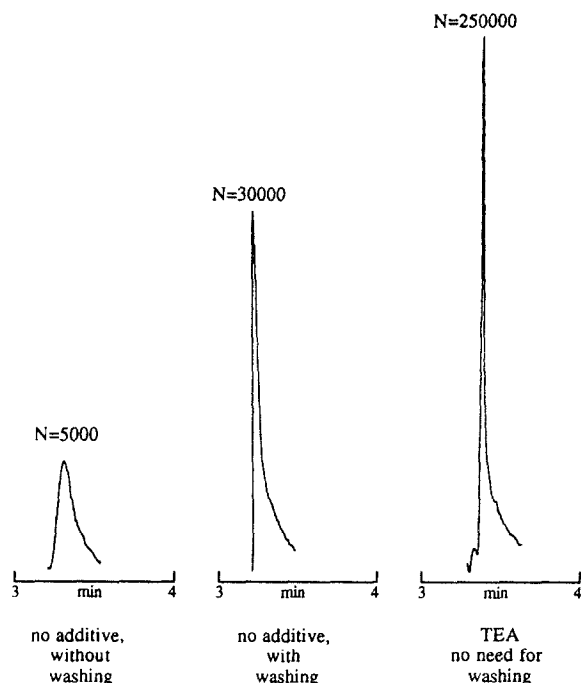
4. Conclusions

Amines, amino acids and acids control EOF and, therefore, stabilize the migration times of solutes in CE. In the presence of these additives, the precision and reproducibility both in migration times and in plate heights improves drastically. With additives, CE can compete, as far as figures-of-merit are concerned, with more established separation techniques, such as HPLC. The additives described here work well with small as well as with large solute molecules. The results discussed above coupled with the fact that such a chemically wide range of additives work in a similar fashion indicate that their modus operandi is related probably more to the presence of charged species than to chemical interactions.

The goal of the present work was to improve

precision. While it is important to optimize CE separation, it is also vital to improve the precision of the method. Our results clearly demonstrate that the use of additives does provide the necessary precision which is essential for practical use.

Acknowledgement

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