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Optimization of selectivity in capillary zone electrophoresis via dynamic pH gradient and dynamic flow gradient

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ABSTRACT

Two different techniques, dynamic pH gradient and electroosmotic flow gradient, were introduced to control selectivity in capillary zone electrophoresis. These two types of gradients showed dramatic effects on the resolution of organic acids. Dynamic pH gradient from pH 3.0 to 5.2 is readily generated by a high-performance liquid chromatography gradient pump. Electroosmotic flow gradient is produced by changing the reservoirs containing different concentrations of cetylammonium bromide for injection and running. The two gradient techniques are applied to the separation of model anions which are not resolved at constant pH or at constant flow conditions.

INTRODUCTION

Capillary zone electrophoresis (CZE) has played an important role in separation science because of its high efficiency [1–3]. In CZE, the separation performance depends on the electrophoretic mobilities of the analytes and the electroosmotic flow when high voltage is applied. Conventionally, the same electrolyte is used in the capillary tube, the inlet reservoir and the outlet reservoir. In such cases, it is often difficult or impossible to separate a broad range of analytes which have very similar electrophoretic mobilities. Many approaches have been reported to improve the resolution in CZE. Control of the factors governing the electrophoretic mobilities of analytes and the electroosmotic flow are typically used to improve the separation resolution in CZE.

In principle and in practice, changing the pH of the buffer electrolyte seems to be the easiest way to control the electrophoretic mobilities of analytes. For the separation of ionic species such as weak acids or bases, the selection of pH near their dissociation constants (pK_a or pK_b) can generally provide good results. For complex mixtures, however, complete separation may not be possible at any one pH since the components may span a large range of pK values. In such cases, the use of a pH gradient can be advantageous. So far, several approaches for generating pH gradients in CZE have been reported. Boček and co-workers [4,5] used a three-pole, two-buffer system to force the migration of varying ratios of two ions into the capillary during separation. Šustáček *et al.* [6] dynamically modified the pH of the electrolyte at the inlet of the capillary by a steady addition of a modifying electrolyte. A step change in pH can also be used by switching the buffer electrolyte at the column inlet, with $[H^+]$ controlled either directly [7] or by the use of different co-ions [8]. It is also possible to simply introduce a transient pulse of electrolyte at a different pH to enhance separation [9].

There are some subtle considerations relevant to the implementation of a pH gradient. It is necessary for the changing pH zone to actually interact with the analytes. H^+ (and OH^-) is a special case be-

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cause of its very high electrophoretic mobility, allowing it to overtake any positively charged analyte within a reasonable distance into the column, even with a high electroosmotic flow-rate. Still, to guarantee that the final pH of the buffer at the inlet end actually contributes to the selectivity, the pH change must be completed well before the elution of the components that are to be manipulated. Direct control of $[H^+]$ is practical only at low pH, where the concentration is high enough to overcome other ionic equilibria. Even for unbuffered electrolytes [4,5], dissolved CO_2, surface silanol groups, and most importantly the analyte, will have be titrated to alter the mobilities significantly even at neutral pH conditions. To overcome this problem, co-ions have been utilized [8], such as the carbonate–oxalate system. Since the co-ions are at much higher concentrations, the pH is easily altered. Naturally, the co-ions must migrate past the analyte ions to produce any effect. So far, only a step gradient has been generated, although one should be able to alter the ratio of the co-ions to generate a continuous gradient.

To guarantee that each analyte species actually experiences the pH step or gradient, a pH gradient derived from temperature changes has been reported [10]. It is also possible to introduce the pH change from the exit (detector) end of the capillary [11]. Regardless of the signs of the electrophoretic mobilities and the electroosmotic flow coefficient, the net travel of the analytes is then opposite in direction to that of the pH front. One can therefore influence the migration of the early eluting components as well as the late-eluting components. So far, only a step change in an unbuffered system has been demonstrated [11]. To provide accurate and reproducible changes over a wide range of pH, it is best to use a polybasic acid (or base) rather than a monobasic acid (or base) for the buffer electrolyte. The pH of a solution of an acid can be controlled by changing the concentration of a counter ion (*e.g.* Na^+) for a fixed analytical concentration $[A]_0$ of the acid, *i.e.* the fraction ionized. For a monobasic acid the ratio goes from 0 to 1 with the two extremes providing unbuffered conditions. It is also difficult to introduce very low concentrations of any counter ion since trace contaminants can dominate the equilibria. For polybasic (H_nA) acids in between pK values *e.g.* $pK_1 < pH < pK_2$, the ratio goes from 1 to $n-1$ and external control is easy over a wide, well-buffered range. The direction of the pH change is also important. For a change from high to low pH, one can introduce H^+ and decrease the counter ion concentration and expect the front to catch up with the analytes. For a change from low to high pH, one must increase the counter ion concentration and decrease H^+ in the column. The mobility of the counter ion rather than the mobility of H^+ becomes important in establishing the gradient. This further restricts the magnitudes and directions of the electrophoretic mobilities and the electroosmotic flow coefficient if the gradient is generated at the inlet end of the capillary. No such restrictions exist if the pH change is introduced from the outlet end, provided there is a net flow of the counter ion into the capillary.

The factors controlling electroosmotic flow, which is governed by the ζ potential on the inner wall of the capillary, include the nature, concentration and pH of the background electrolyte, origin of the capillary and the applied voltage. Adding surfactant [12,13] to the background electrolyte is one simple way to enhance selectivity in CZE since the surfactant can effectively suppress or change the direction of the electroosmotic flow. Organic solvents [14] such as methanol, which can be used to change electroosmotic flow, are also useful to enhance the separation resolution. External electric field [15], which can change the direction and the rate of electroosmotic flow by external voltage, is another approach to improve the separation ability in CZE. Coating the inner walls of the capillary with silated organic compounds [16] and adding salts [17] like NaCl are other options to affect the separation resolution. Field-amplified CZE [18,19], where two different concentrations of buffer electrolytes are used for injection and running, is another powerful method to control the electroosmotic flow.

In this paper, two methods, altering the electrophoretic mobilities of anions by using a dynamic pH gradient and changing the electroosmotic flow-rate by running CZE in two different concentrations of cetylammonium bromide (CTAB), were described to demonstrate their utility in the separation of organic weak acids at acidic conditions.

EXPERIMENTAL

A commercial electrophoresis instrument (Model 3850 Isco; Lincoln, NE, USA) was used for all electrophoretic experiments. High voltages applied in the experiment of dynamic pH gradient and dynamic flow gradient were 10 and 12 kV, respectively. The wavelength was set at 218 nm to detect anions and minimize the background absorption. The fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was 60 cm × 75 μm I.D. At 40 cm from the injection end the polyimide coating was burned off to form the detection window. An integrator (SP Model 4600; Spectra-Physics, San Jose, CA, USA) was used to record all of the data. A high-performance liquid chromatographic (HPLC) gradient pump (two 2150 HPLC pumps and a 2152 HPLC controller; LKB, Gaithersburg, MD, USA) was used to introduce the dynamic pH gradient. The setup is shown in Fig. 1. Coupling is accomplished through a PTFE tube (1.6 mm I.D. × 3.0 mm O.D.) with the buffer at the exit end of the capillary constantly modified by the HPLC pump.

All chemicals were of reagent grade and were obtained from Aldrich (Milwaukee, WI, USA), except that phosphoric acid, sodium phosphate and sodium hydroxide were from Fisher (Fair Lawn, NJ, USA). Buffer solutions of phosphate were prepared from NaH_2PO_4 by adding NaOH or H_3PO_4 to adjust its pH to 5.2, or 4.1 and 3.0, respectively. Acetate buffer solution (pH 6.5) was made by adding NaOH to acetic acid. CTAB was added to buffer solutions to suppress electroosmotic flow. α-Naphthol (a neutral molecule at these pH values) was used to measure the electroosmotic flow. The sample solutions were injected hydrostatistically. The buffer vial at the cathodic end of the capillary was raised to 20 cm high for 4 s to introduce samples into the capillary tube.

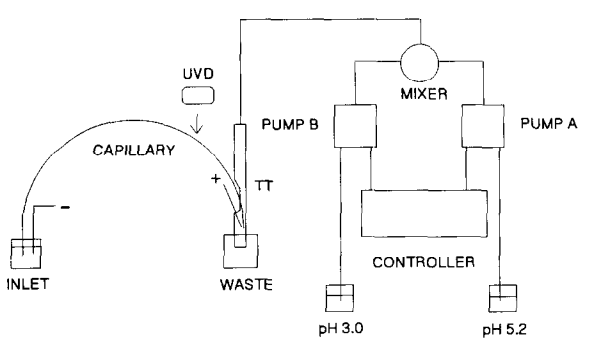

Fig. 1. Schematic of the electrophoresis equipment for generating a dynamic pH gradient. UVD = Ultraviolet detector; TT = PTFE tube, in which a small groove was cut in the middle to insert the electrode and the capillary.

The capillary was equilibrated for 20 min between each run. Before injecting sample solutions, the capillary was flushed with 0.05 ml of 0.01 *M* NaOH solution, then 0.5 ml of buffer solution to improve reproducibility. A step change in CTAB was created by injecting sample solutions into the capillary containing a low concentration of CTAB, then running with buffer electrolyte containing a high concentration of CTAB at the exit (anodic) end. The capillary was treated as above before each run.

RESULTS AND DISCUSSION

In CZE, the resolution between a pair of adjacent analytes can be calculated from the following equation [20]:

$$R = 0.177\ (m_{\mathrm{eff}1} - m_{\mathrm{eff}2})\ [V/D\ (m_{\mathrm{av}} + m_{\mathrm{eo}})]^{1/2} \qquad (1)$$

where R is the resolution, V is the applied voltage, D is the diffusion coefficient, m_{av} is the average mobility of the two analytes and $m_{\mathrm{eff}1}$, $m_{\mathrm{eff}2}$ and m_{eo} are the effective mobilities of the two analytes and the electroosmotic flow coefficient, respectively. As eqn. 1 shows, the resolution can be enhanced by increasing the difference between the effective electrophoretic mobilities of the analytes and (or) decreasing the sum of electroosmotic flow and the average electrophoretic mobilities of two analytes.

Control of effective electrophoretic mobilities with dynamic pH gradient

Effective electrophoretic mobilities of ions are proportional to the fraction of free ions according to the Tiselius equation (see ref. 21):

$$m_{\mathrm{eff}} = \sum a_i\, m_i \qquad (2)$$

where a_i is the degree of dissociation and m_i is the absolute mobility of the ith ionic form of a molecule. The degree of dissociation again can be calculated from the following equations:

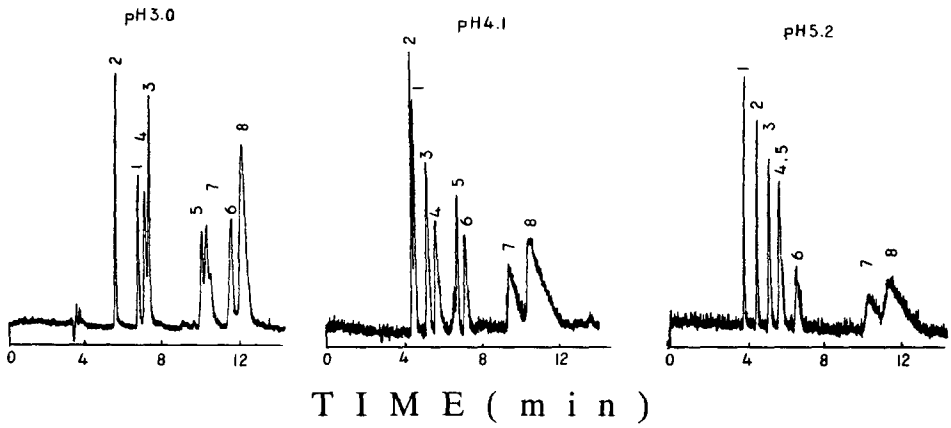

Fig. 2. Effect of change in pH on the migration rates of different anions. Capillary, 60 cm total length (40 cm to detector) × 75 μm I.D.; applied voltage, 10 kV; wavelength, 218 nm; CTAB concentration, 0.35 m*M*; phosphate concentration, 10 m*M*; concentration of other anions, 0.2 m*M*. Peaks: 1 = citraconate; 2 = maleate; 3 = fumarate; 4 = *o*-nitrobenzoate; 5 = *o*-toluate; 6 = benzoate; 7 = *m*-nitrobenzoate; 8 = *m*-toluate.

For divalent anions:

$$a_1 = K_{a1}[H^+]/([H^+]^2 + K_{a1}[H^+] + K_{a1}K_{a2}) \quad (3)$$

$$a_2 = K_{a1}K_{a2}/([H^+]^2 + K_{a1}[H^+] + K_{a1}K_{a2}) \quad (4)$$

For monovalent anions:

$$a = K_a/([H^+] + K_a) \quad (5)$$

where K_{ai} is the ith dissociation constant of the acid.

The amount of negative charge on the inner wall of the capillary and the analyte ions should be a function of pH of the buffer electrolyte as indicated by eqns. 3–5. For a bare silica column at these pH, electroosmotic flow is generally towards the cathode (injection end). For separation of these anions by migration towards the anode, we need to reverse the electroosmotic flow by adding a cationic surfactant, CTAB. At 0.35 m*M* CTAB, the amount of the positive charge on the inner wall of the capillary caused by the adsorption of CTAB increases as pH decreases since the degree of dissociation of the SiOH groups decreases as pH decreases. That is, electroosmotic flow toward the anodic direction increases when pH decreases. Our results show that $m_{eo} = -3.15 \cdot 10^{-4}$, $-1.8 \cdot 10^{-4}$ and $-1.31 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} at pH 3.0, 4.1 and 5.2, respectively. The experiments here thus take advantage of the combined results of the changes in electrophoretic mobilities and in electroosmotic flow. However, since m_{eo} is negative (same direction as m_{eff}), the numerator in eqn. 1 dominates in determining the resolution. We note that m_{eo} is smaller than m_{eff} for Na^+ ($= 5.0 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}) so that the pH change can be introduced from the outlet end.

Fig. 2 shows the separation of the model anions at pH 3.0, 4.1 and 5.2. The calculated electrophoretic mobilities are listed in Table I. The separations of *o*-nitrobenzoate from fumarate, and *o*-toluate from *m*-nitrobenzoate are impossible at pH 3.0. At pH 4.1, there is overlap between the peaks of citrac-

TABLE I

OBSERVED ELECTROPHORETIC MOBILITIES (m_{ep}) OF ANIONS

Electroosmotic flow coefficient (m_{eo}; × 10^{-4} cm^2 V^{-1} s^{-1}) is negative as electroosmotic flow is toward the anode. m_{eo} = −3.15 at pH 3.0, −1.80 at pH 4.1 and −1.31 at pH 5.2. [Anions] = 0.2 m*M*.

Anions	$-m_{ep}$ (× 10^{-4} cm^2 V^{-1} s^{-1})		
	pH 3.0	pH 4.1	pH 5.2
Citraconate	3.1	7.0	8.2
Maleate	4.1	7.1	7.4
Fumarate	2.7	5.7	6.0
o-Nitrobenzoate	2.8	5.0	5.3
o-Toluate	0.8	4.1	5.3
Benzoate	0.3	3.6	4.4
m-Nitrobenzoate	0.7	2.3	2.4
m-Toluate	0.1	1.9	2.3

onate and maleate. Serious tailing on the late eluting peaks and the separation of *o*-toluate from *o*-nitrobenzoate are problems at pH 5.2. It is worth noting that the electrophoretic mobilities of *m*-isomers are lower than those of *o*-isomers. A likely reason is steric effects in the *o*-isomers which decrease the interaction between the anions and CTAB. The tailing problem is more serious for later eluting peaks at higher pH. Two factors that contribute to this are the stronger interaction between anions and CTAB due to increased dissociations of anions at higher pH and the larger difference of mobilities between the buffer ions and the anions at higher pH [22,23]. Based on literature values [24,25] of dissociation constants and mobilities we have calculated the effective mobilities of this set of anions at these pH. The trends are comparable to those in Table I but the absolute magnitudes are different. In all cases except for fumarate, the calculated values are larger than the experimental values. We note that the literature values are for systems at infinite dilution and for 20 or 25°C. Differences are expected in the presence of the buffer ions and CTAB. Further, since our column temperature is higher than ambient during electrophoresis, the viscosity of water decreases and the effective mobilities should be higher as observed.

As Fig. 2 shows, it is impossible to separate all of the model anions with an isocratic buffer electrolyte. The problem can be overcome by introducing a dynamic pH gradient from pH 3.0 to 5.2 as Fig. 3 shows. The resolution was enhanced, the separation took less than 11 min, and the tailing problem was also reduced by this method. Even though the gradient starts immediately after injection, the components do not meet the moving front until they migrate further down the column. However, every component is guaranteed to meet the moving front in this mode of operation.

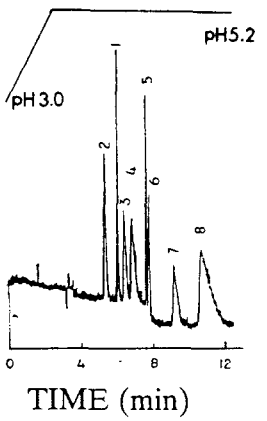

Fig. 3. Influence of a pH gradient on the separation of organic anions. Conditions as in Fig. 2 except a pH gradient is introduced through the outlet end of the capillary as indicated by the top plot in this figure.

TABLE II

ELECTROOSMOTIC FLOW COEFFICIENT (m_{eo}) AT DIFFERENT CONCENTRATIONS OF CTAB

m_{eo} is negative for electroosmotic flow toward the anode.

[CTAB] (μM)	m_{eo} ($\times 10^{-4}$ cm^2 V^{-1} s^{-1})
4.0	5.7
6.0	2.9
10.0	0.4
14.0	0.03
40.0	−0.8

Control of the electroosmotic flow by step change in CTAB

To reduce the problem of tailing caused by the difference of mobilities between the buffer ion and the model anions, acetate buffer was used instead of phosphate buffer. The use of pH 6.5 was based on the following two reasons. First, to minimize the effect of variations in pH on electrophoretic mobilities the pH of the buffer solution is chosen to be much higher than most of the pK_a. Second, the effect of the surfactant on the electroosmotic flow is larger at higher pH since the dissociation of SiOH on the inner wall of capillary increases as pH increases.

Table II shows that CTAB not only can suppress electroosmotic flow, it can also change its direction at higher concentrations of CTAB, consistent with published reports [12,13]. Table III shows that the electrophoretic mobilities of the model anions are almost constant when the concentration of CTAB changes. Slight variations on electrophoretic mobilities of the anions may be due to the formation of an ion-pair between the anions and CTAB at higher concentration of CTAB. This is evident for the larger anions, naphtholate and coumarate. So, the

TABLE III

ELECTROPHORETIC MOBILITIES (m_{ep}) OF ANIONS AT DIFFERENT CONCENTRATIONS OF CTAB

[Anion] = 0.2 mM, except [pyruvate] = 0.5 mM and [α-naphtholate] = 50 μM.

Anion	$-m_{ep}$ ($\times 10^{-4}$ cm^2 V^{-1} s^{-1})			
	6 μM CTAB	10 μM CTAB	14 μM CTAB	40 μM CTAB
Citraconate	7.6	7.4	7.4	7.4
Maleate	7.0	6.8	6.7	6.9
Phthalate	7.0	6.8	6.7	6.8
Pyruvate	6.2	6.1	5.8	5.9
o-Nitrobenzoate	5.3	5.1	5.0	4.9
Benzoate	5.1	4.9	4.8	4.8
α-Naphtholate	4.8	4.3	4.2	3.6
Coumarate	4.7	4.3	4.0	4.1

dominant term in eqn. 1 is the denominator when the CTAB concentration is varied. Fig. 4 shows all anions except phthalate and maleate can be separated in less than 18 min at 6 μM CTAB buffer electrolyte. As the CTAB concentration increased to 10 μM, α-naptholate and coumarate cannot be separated and there is little separation between the peaks of phthalate and maleate. The resolution between *o*-nitrobenzoate and benzoate decreases dramatically as the CTAB concentration changed to 40 μM where electroosmotic flow is in the same direction as the electrophoretic mobilities of anions. The increase in $m_{ep} + m_{eo}$ results in the poor resolution of these two anions because the difference between their electrophoretic mobilities is very small. However, the resolution between coumarate and α-naphtholate increases and the elution order changes. This may have resulted from the complexation of CTAB with α-naphtholate and with coumarate. So, Fig. 4 shows it is impossible to separate all the model anions under isocratic condition.

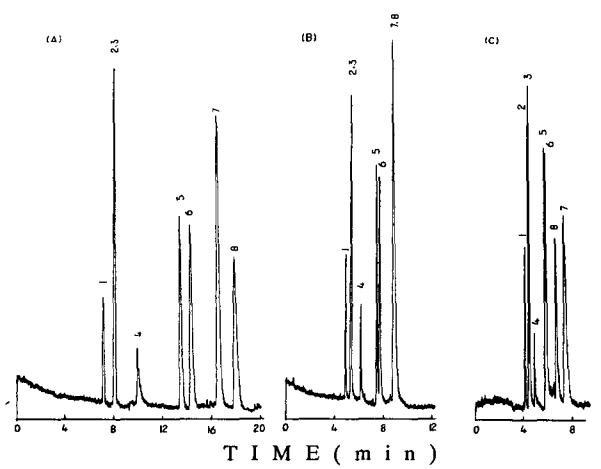

Fig. 4. Effect of changes in concentration of CTAB on the migration rates of different organic anions. CTAB concentration: (A) 6 μM, (B) 10 μM, (C) 40 μM; capillary, 60 cm total length (40 cm to detector) × 75 μm I.D.; applied voltage, 12 kV; wavelength, 218 nm; acetate concentration, 5 mM; concentration of other anions, 0.2 mM except pyruvate concentration, 0.5 mM and α-naphtholate concentration, 50 μM. Peaks: 1 = citraconate; 2 = maleate; 3 = phthalate; 4 = pyruvate; 5 = *o*-nitrobenzoate; 6 = benzoate; 7 = α-naphtholate; 8 = coumarate.

Helmer and Chien [18] mentioned that the relationship between the bulk velocity, m_b, and the local electroosmotic velocity, m_l, in the two buffer regions can be expressed by the following equation:

$$m_b = x\, m_{l1} + (1 - x)\, m_{l2} \quad (6)$$

where x is the fraction of length filled with buffer 1 and m_{l1} and m_{l2} are the local electroosmotic flow-rates when buffer 1 and 2 are used individually, respectively. This means that if we create a step change of two buffers containing different concentrations of CTAB it is possible to monotonically change the electroosmotic flow. Fig. 5 shows a computer simulation of the electroosmotic flow rate resulting from a step change in CTAB concentration, in agreement with the above discussion. The change is indeed monotonic, but not linear. The rate of change can be controlled by the magnitude of the buffer step. It is interesting to note that the electroosmotic flow can never be reversed in this mode. It is also irrelevant which direction the analytes are moving relative to the flow, since there is only one flow-rate to consider [18]. All analytes experience the same flow gradient regardless of where the front is. Fig. 6 shows the effect on the enhancement of the separation of model anions by a step change in the concentration of CTAB. Comparing the results of Fig. 6A and B, it is obvious that the bulk electroosmotic flow gradient can be controlled by stepping to different concentrations of CTAB. Improvements in resolution and reduction in separation time are simultaneously achieved by the method of step change in CTAB concentration.

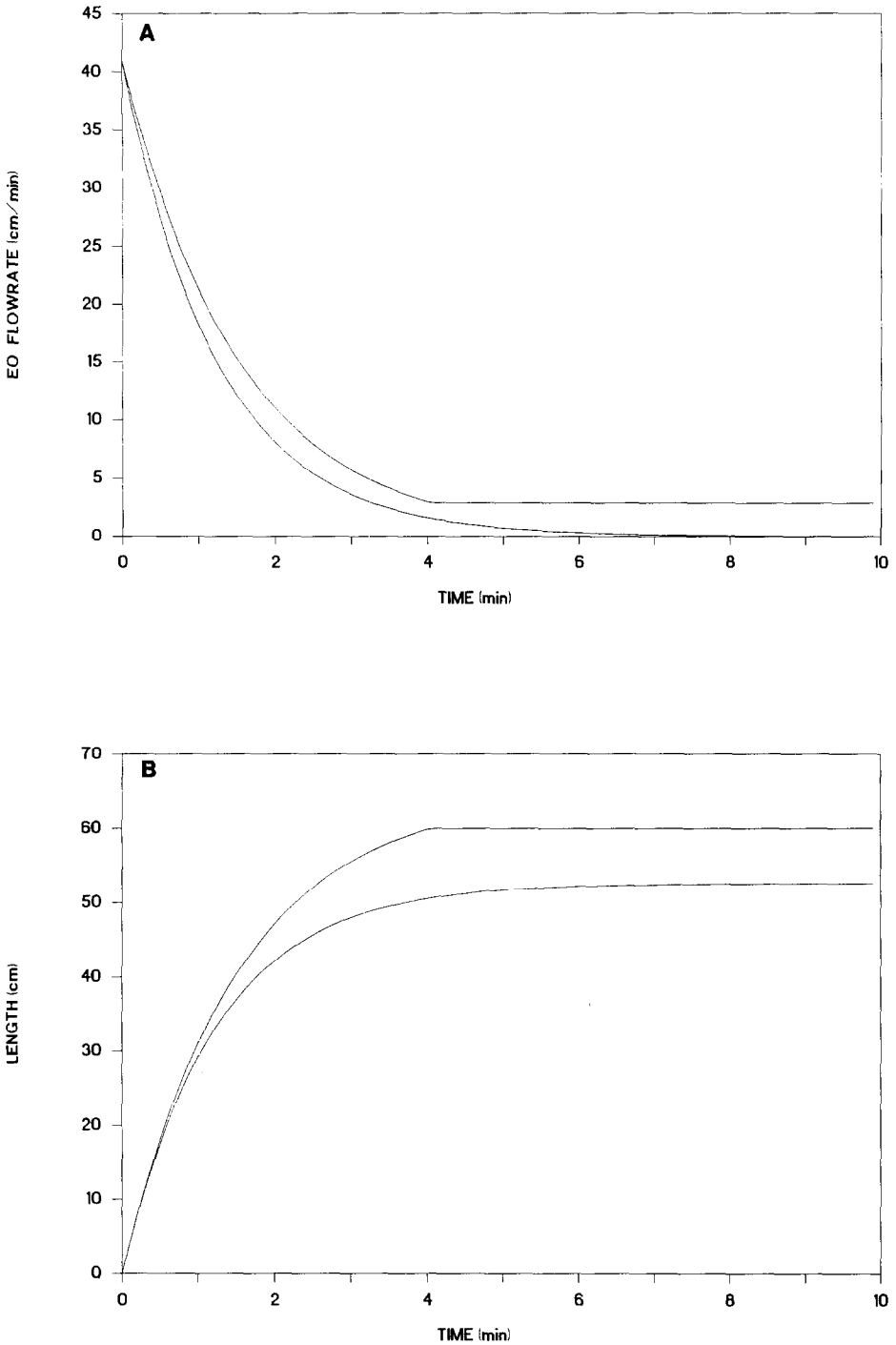

Fig. 5. Computer simulation of electroosmotic flow gradient for a step change in CTAB concentration. The data in Table II were used. Initial CTAB concentration is 4 μM; final CTAB concentration is 10 μM for top trace and 40 μM for bottom trace. (A) Flow-rate as a function of time; (B) location of moving boundary from the exit end of a 60-cm capillary as a function of time. Applied voltage, 12 kV; column length, 60 cm (40 cm to detector).

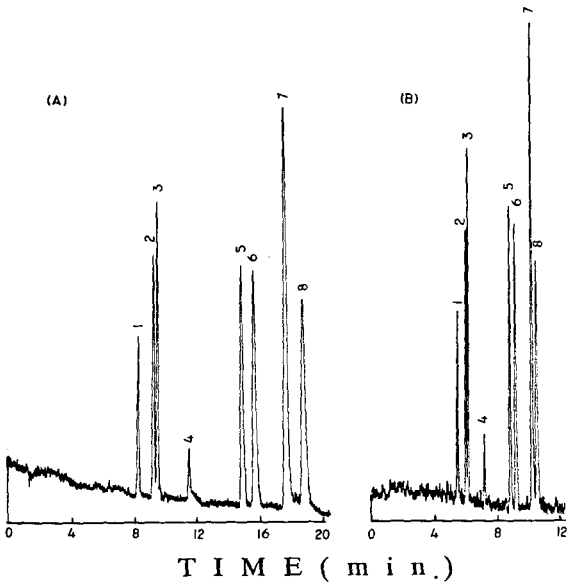

Fig. 6. Influence of a step change in CTAB concentration on the separation of organic anions. Before injection, the concentration of CTAB is 4 μM while the running concentrations are 10 μM (A) and 40 μM (B). Conditions as in Fig. 4.

The control of electroosmotic flow rate and electrophoretic mobilities of analytes are important to improve the resolution and reduce the separation time in CZE. Many approaches have already been used to control the electroosmotic mobilities of analytes in order to enhance the separation resolution. In this work, we demonstrated the effect of a dynamic gradient in pH on the effective electrophoretic mobilities of several model anions by introducing the change at the outlet end of the capillary. Altering the electroosmotic flow is another known method for increasing the resolution. In this paper, a simple gradient approach, which is based on the combination of the effect of a surfactant on electroosmotic flow and the idea of field-amplified electroosmosis, is outlined. Eight organic acids are successfully separated by this method in less than 11 min.

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