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Dynamic control for ultra-fast separations of organic acids in capillary zone electrophoresis
A new direction to improve resolution

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Abstract

Dynamic control and pH changes in the system have been utilized for the separation of twelve organic acids in less than 3 min using capillary electrophoresis (CE). High-speed separations of organic acids under weak acidic conditions indicate the existence of high electroosmotic flow (EOF) caused by treatment of the capillaries with 0.1 *M* NaOH before each separation. However, strong polyprotic acids can only be detected at higher applied voltages with shorter capillaries, since local EOF decreases significantly when migration time increases. In terms of resolution and speed, the optimal voltage is around 20 kV in 22.5-cm capillaries. The effects of the electric field strength and capillary length on the resolution of organic acids have been investigated to show the existence of dynamic flow. For both methods, dynamic flow is of great importance for the enhancement of resolution for stronger acids. On comparing all of the results, the change in voltage is more efficient for improving the separation resolution in this study. More importantly, this new method can be used in any commercial CE instrument because of its features of high resolution, high speed and simplicity.

Keywords: Electroosmotic flow; Electrophoretic mobility; Dynamic flow; Capillary electrophoresis; Organic acids

1. Introduction

The success of capillary electrophoresis (CE) for the separation of almost all analytes, ranging from small molecules to macromolecules, and from neutral molecules to cations and/or anions, has been well demonstrated in many different fields [1–4]. High separation efficiency and speed are the two most important reasons for its popularity in modern separation science [5]. In free zone CE, separation mainly depends on the differences in migration velocities of analytes. Factors (e.g. pH, species, viscosity, ionic strength of the buffer and electric field strength) controlling the electroosmotic flow (EOF) and electrophoretic mobilities (EPMs) of analytes are important in CE.

Many useful approaches have been employed to control the EOF and EPMs of analytes, to obtain better separation results. The use of a pH gradient is one of the most common modes for the improvement of separation efficiency. For example, the introduction of different ionic strengths of electrolytes into running buffers by mechanical means has been used to generate pH gradients inside capillaries [6,7]. In addition, temperature control is also able to induce pH changes by using buffers with low buffering capacity. This has been performed by passing water at different temperatures through capillaries by mechanical pumping or by voltage programming [8,9]. Alternatively, field amplification, by adding at least

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two different electrolytes into different regions of capillaries, has been demonstrated to generate different local flows. For instance, the improvement in the detection sensitivity of amino acids caused by stacking effects has been shown [10]. Enhancement of resolution for the separation of organic acids by filling capillaries with buffers containing different concentrations of a cationic surfactant in CE has also been reported [11].

Diffusion, the length of the sample injection plug and adsorption of analytes into the capillary wall are three main sources of band broadening [12]. Among them, diffusion is simply proportional to the migration time [13]. It is therefore possible to achieve better resolution when high-speed separations are performed [14]. Examples include separations in microchips and nonaqueous solutions at very high electric field strengths [15–17]. Another important approach for achieving high resolution in high-speed separations is to inject small amounts of sample. However, this generally requires a highly sensitive detection mode, such as laser-induced fluorescence [18].

It is difficult to simultaneously reach optimal resolution and speed in CE because larger values of the sum of EOF and EPMs of analytes are essential in high-speed separations. One example, to show the loss of resolution in high-speed separations, is the separation of acids under alkaline conditions, wherein small differences in the EPMs of anions and very high EOF exist [19]. The separations of organic acids in the presence of a cationic surfactant under acidic conditions is another example [20]. At higher concentrations of cetyltrimethylammonium bromide (CTAB), the reverse direction of EOF is observed. Therefore, resolution is worse, because the sum of the EOF and EPMs of analytes is too large. When the EOF is completely suppressed by coating capillaries with polymers or chemicals, worse resolution may result, caused by the EPMs being too close among completely ionized analytes when high-speed separation is needed [21]. Hence it is not, theoretically, possible to obtain optimal performance in conventional CE without sacrificing one for the other.

Recently, we have developed a very simple method for simultaneously improving the resolution and speed required for the separation of organic acids in CE [22]. The success of this method is due to high EOF and pH changes in the range close to the dissociation constants of acids during separation. In this study, we further investigated changes in the speed and resolution of separation by changing capillary lengths and applied voltages. Although the effects of these two factors on resolution have been emphasized and demonstrated [23–25], different results obtained from this dynamic control mode will be carefully evaluated.

2. Experimental

2.1. Instruments

A commercial electrophoresis instrument from Bio-Rad (BioFocus CE 2000, Hercules, CA, USA) was used. The fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were 35- and 22.5 cm×75 μm I.D. At 5 cm from the outlet end, the polyimide coating was burned off to form the detection window. The absorbance was determined at 225 nm.

2.2. Materials

All chemicals were of reagent grade and were obtained from Aldrich (Milwaukee, WI, USA), except sodium hydroxide, which was from Fisher (Fair Lawn, NJ, USA). The buffer solution used was 1 m*M* NaH_2PO_4, adjusted to pH 4.1 with 0.1 *M* H_3PO_4 . The concentration of each analyte that was injected was $3 \cdot 10^{-4}$ *M*. Mesityl oxide was used to measure EOF coefficients.

2.3. Capillary equilibrium and separation

The capillary was pre-equilibrated with 0.1 *M* NaOH for one day prior to use for electrophoretic separation. Between each run, the capillary was equilibrated with 0.1 *M* NaOH using high pressure (689 476 Pa) for 3 min, then the remaining base inside the capillary was washed out with the running buffer using high pressure (689 476 pa) for 10 s. Analytes were introduced into the capillary by applying a high voltage (5 kV) for 3 s. Separations were performed at different voltages and in capillaries of different lengths, as shown in Section 3.

3. Results and discussion

3.1. Dynamic control

In our previous study, we showed that dynamic control in CE was useful for high-resolution and high-speed separations of organic acids [22]. After treating capillaries with 0.1 *M* NaOH, the EOF generated at an applied voltage was high enough to move organic acids from the anode to the cathode end under weakly acidic conditions in bare fused-silica capillaries. Changes in pH, due to buffer electrolysis, also account for the changes in the EOF and EPMs [26–28]. Although weaker acids were detected within 7 min, very strong polyprotic acids did not migrate out of the capillary within 20 min, in our previous study. We suspected that the dramatic decrease in EOF with time might be one of the most important reasons. If this is true, separations performed at higher electric field strengths in shorter capillaries would seem to be optimal for acids, including weak acids and strong polyprotic acids.

3.2. Effects of the electric field strength

In this dynamic control mode, at least two important transient effects in the separation system must be carefully considered. One is that the decrease in the EOF is time-dependent, which relates to the decrease in the negatively charged density of the capillary surface and the decrease in the pH of the anodic buffer. The other is that differences in pH values among the inlet vial, outlet vial and inside the capillaries, resulting from buffer electrolysis, become more significant with respect to longer migration times [26]. As a consequence of the pH changes (during separation), dissociation (or EPM) of acids will change temporally. In other words, analytes that span the inside of the capillary more often will have a greater chance of undergoing transient changes within the system.

Fig. 1A–D show the separation of twelve analytes in 1 m*M* phosphate buffer solutions (pH 4.1) at 15, 20, 22 and 24 kV in a 22.5-cm capillary. All twelve analytes were separated in less than 3 min at 22 kV. It is important to note that fumaric acid (FA) and 1,2,4-benzenetricarboxylic acid (TBA), which were not detected in our previous study, were well separated in shorter capillaries at 20 and 22 kV. At 15 kV, TBA was not detectable, while it took more than 20 min for FA to be detected. Transient changes in the EOF and pH account for these observations. From pH decreases in anodic buffer vials all less than 0.35 units in 20 min at 24 kV, and less than 0.1 unit in 6 min at 20 kV, we believe that the decrease in EOF should cause more separation problems for stronger acids. Results also show that migration time is not reversibly proportional to the electric field strength, as in isocratic CE. Generally, the variation is due to significant changes in buffer viscosity because of Joule heat generated at a high voltage. However, it is not the case in our study. The relatively low running currents (3.5–8.2 μA from 10–24 kV) observed in the separations at constant temperature (24°C) override the effects of the viscosity changes on the irregular observations. To further verify this question, Fig. 2 shows the values of the EOFs and EPMs of analytes at different applied voltages. The fact that EOF coefficients decrease dramatically as the applied voltages decrease shows that transient decreases in the negatively charged density of the capillary surface exist. In contrast to the EPMs of acids obtained at lower ionic strengths under isocratic conditions, we observed different EPMs of acids at different applied voltages. Furthermore, small variations in the EPM were observed for weaker acids, while they were larger for strong acids. As analytes migrate slowly towards the cathode (stronger acids with larger EPMs towards the anode), they have more of a chance to undergo significant changes within the system.

In order to clearly evaluate the existence of dynamic changes in the EOF and pH using this method, the relationships between migration times and electric field strengths for a neutral marker and for nine acids were plotted (Fig. 3). For weaker acids, the ratios are close to those of the neutral marker and are nearly reversibly proportional to the ratios of electric field strengths. The ratios and slopes slightly increase in the order of $T_{10}/T_{24} > T_{10}/T_{22} > T_{10}/T_{20} > T_{10}/T_{15}$. On the other hand, the ratios and slopes increase dramatically for strong polyprotic acids. One thing we should keep in mind is that the EOF and EPMs of weak acids were obtained in the early stages of each separation. Thus, the ratios obtained from weak acids cannot correspond correct-

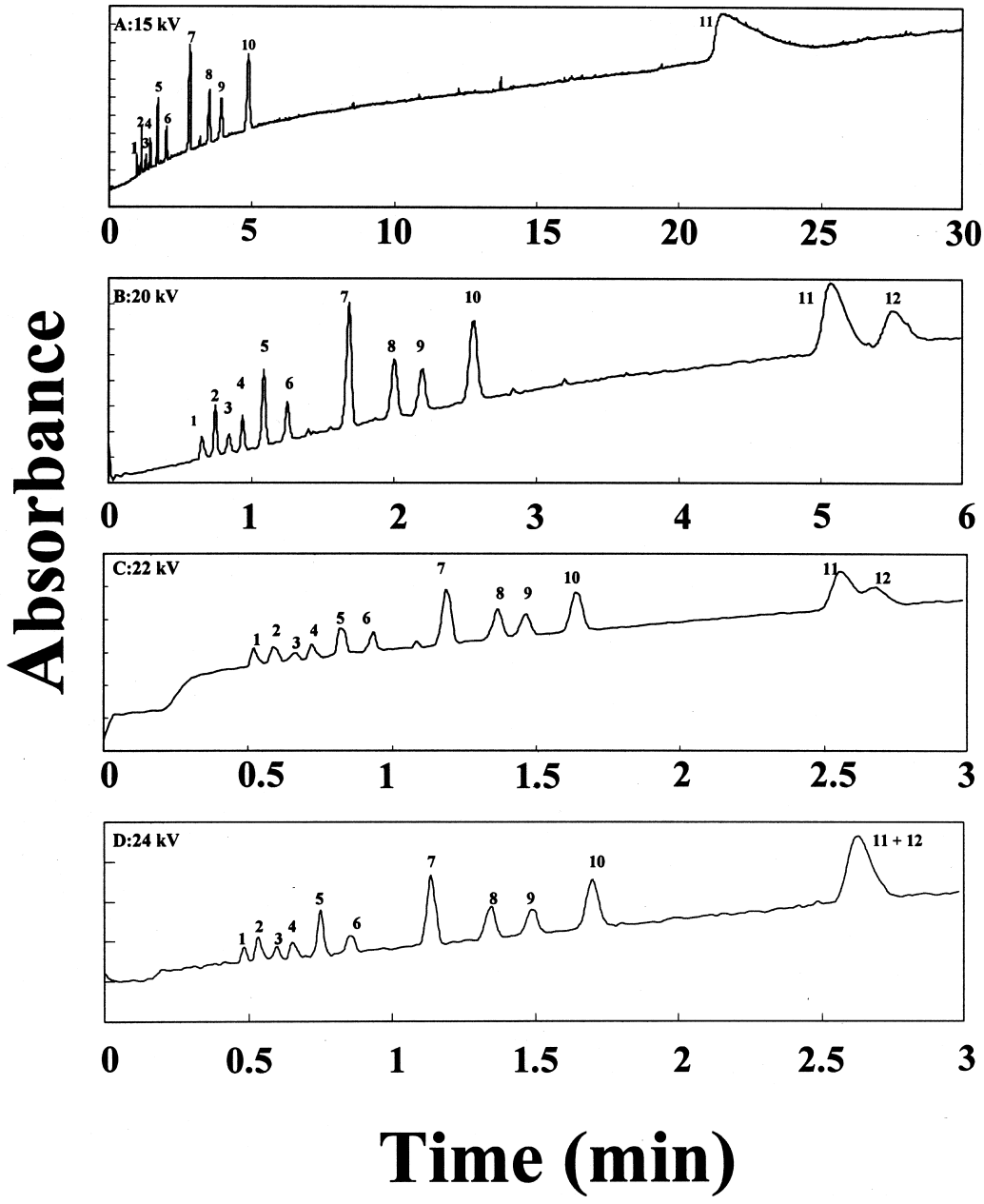

Fig. 1. Separation of twelve analytes in 1 m*M* phosphate buffer, pH 4.1, at different applied voltages. Column, 17.5 cm (effective length)×75 μm I.D.×365 μm O.D. Detection wavelength, 225 nm. Peak identity: 1=Mesityl oxide; 2=*p*-aminobenzoic acid; 3=*p*-hydroxybenzoic acid; 4=*p*-toluic acid; 5=benzoic acid; 6=*o*-toluic acid; 7=*m*-nitrobenzoic acid; 8=*o*-nitrobenzoic acid; 9=phthalic acid; 10=citraconic acid; 11=fumaric acid and 12=1,2,4-benzenetricarboxylic acid.

ly to the significantly dynamic changes in the system. Compared to weak acids, stronger acids span the inside of the capillaries more often. Thus, they face more changes in the system. Overall, these trends clearly show the existence of dynamic changes in the system.

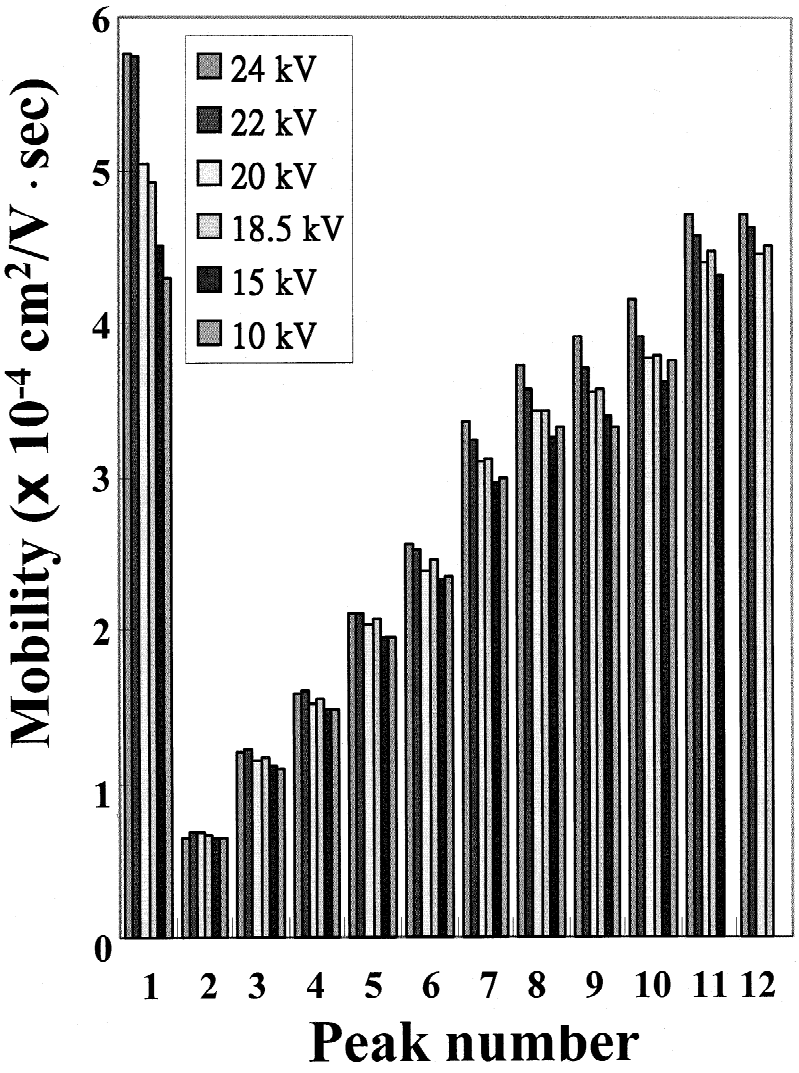

Fig. 2. EOF coefficients and EPMs of twelve analytes, obtained at different applied voltages. Conditions as in Fig. 1.

3.3. Effects of capillary length

To further show the existence of dynamic flow inside capillaries, we performed separations of organic acids in capillaries of two different lengths, 22.5 and 35 cm. To minimize any possible effect of Joule heat on the changes in migration velocity, the same electric field strength, 525 V/cm (low current observed), was chosen for the separations in the two capillaries. Fig. 4 shows the EOF coefficients and EPMs of analytes obtained from the two capillaries. It is interesting to note that lower EOF coefficients were obtained from the longer capillary. This again shows that the local EOF decreases with time. The smaller EPMs of the weak acids and the larger EPMs of the strong acids obtained from the longer capillary also indicate the existence of dynamic changes. From the similar rates of decrease of the pH in anodic buffer (results not shown) using the two capillaries, the effects of differences in the electrolysis rates on the separation can be ruled out. This is consistent with the theoretical prediction because the same electric field strengths were applied for the separations.

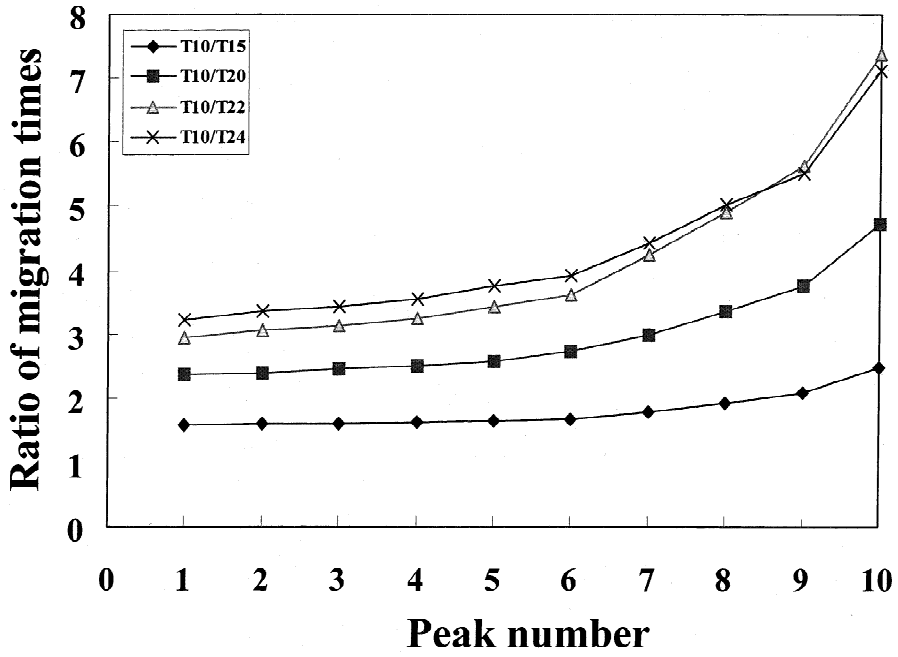

Fig. 3. Trends of the ratios of migration times for each analyte at 10 kV to those at 15, 20, 22 and 24 kV, respectively. Conditions as in Fig. 1.

Fig. 5 shows the ratio of migration times for each analyte in the two different capillaries. The ratios remained almost constant (1.6) for weaker acids, then gradually increased, to 2.6, for citraconic acid (CA). The ratio of migration times for each analyte obtained from these two capillaries should be 1.56, if dynamic flow was nonexistent. In other words, transient changes exist in the system. Comparison of the results from Figs. 3 and 5 indicates that strong acids undergo more changes in the system. The trends also clearly show why it is difficult to separate stronger acids in longer capillaries and at lower electric field strengths.

3.4. Resolution

The resolution of two species is proportional to the differences of the EPMs of the two species, while it is reversibly proportional to the sum of the average EPM of the two species and the EOF [23]. In this dynamic control mode, better resolution is due primarily to the fact that (1) differences in EPMs among acids are more significant because separations were performed under more weakly acidic conditions and (2) the sum of two driving forces, EOF and EPM, is smaller because the two driving forces are in opposite directions.

In this report, resolution was calculated as being twice the difference in migration times of two species over the sum of the peak widths corresponding to the two species. As shown in Fig. 6, higher separation resolution was obtained at a lower applied voltage. It is different from the theoretical prediction that resolution is proportional to the square root of the applied voltage under isocratic conditions. Again, small local EOF and pH changes account for higher resolution between any two strong acids at lower applied voltages. However, separations at lower applied voltages suffer from very long migration times and broader peak widths. In terms of resolution and speed, it is better to perform separations at around 20 kV.

Fig. 7 shows the effects of capillary length on the resolution. The square root of the ratio of two capillary lengths is 1.25, so the resolution ratio obtained from 35- and 22.5-cm capillaries should be about 1.25, under isocratic conditions. However, the ratios of the resolution using two different capillary

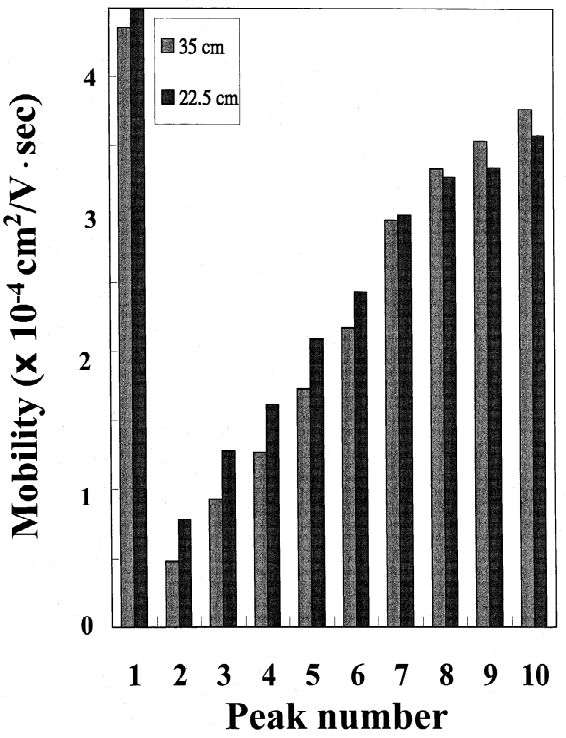

Fig. 4. EOF coefficients and EPMs of ten analytes obtained at 525 V/cm in 22.5 and 35 cm capillaries. Conditions as in Fig. 1.

lengths were 1.4, 2.1 and 3.6 for *p*-aminobenzoic acid (p-ABA) and *p*-hydroxybenzoic acid (p-HBA), *m*-nitrobenzoic acid (m-NBA) and *o*-nitrobenzoic acid (o-NBA), and o-NBA and phthalic acid (PHA), respectively. Therefore, in addition to the effects of capillary length on resolution, other factors could be involved. The existence of dynamic flow, due to changes in the EOF and pH, should be one of the most important factors. For very long separation times, peaks become too broad to reduce the resolution, as found between PHA and CA. Therefore, it is essential to perform high-speed separations to obtain higher resolution in this dynamic mode.

3.5. Capillary length vs. electric field strength

In Table 1, the effects of the capillary length and electric field strength on resolution are compared. Although the improvement in resolution obtained by changing the applied voltage from 24 to 10 kV is only slightly better than that obtained by changing the capillary length from 22.5 to 35 cm, the separation performed by controlling the applied voltage is much faster and less costly. For stronger acids, slight changes in pH (around pH 4.1) do not significantly

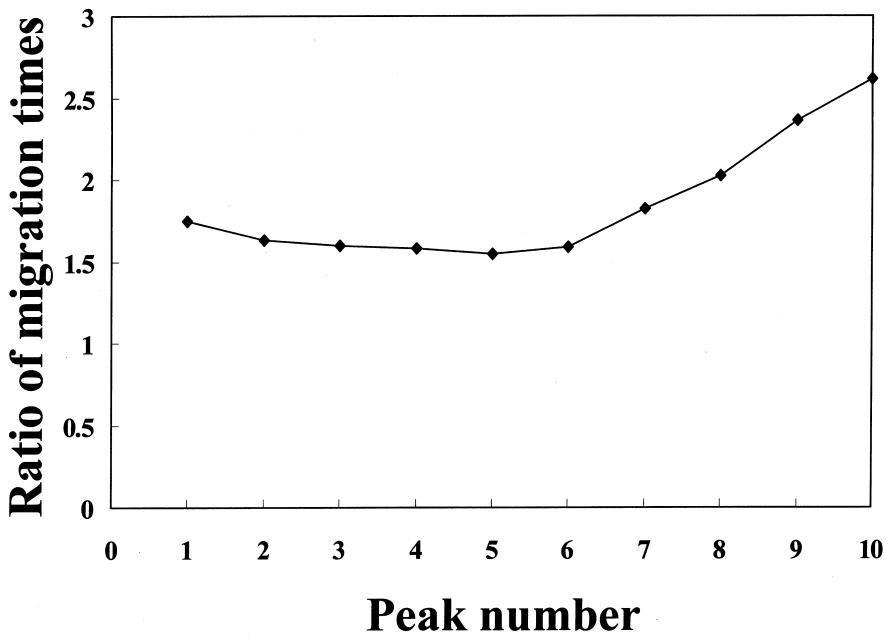

Fig. 5. Trend of the ratio of migration times for each analyte at 525 V/cm in 35 cm to 22.5 cm capillaries. Conditions as in Fig. 1.

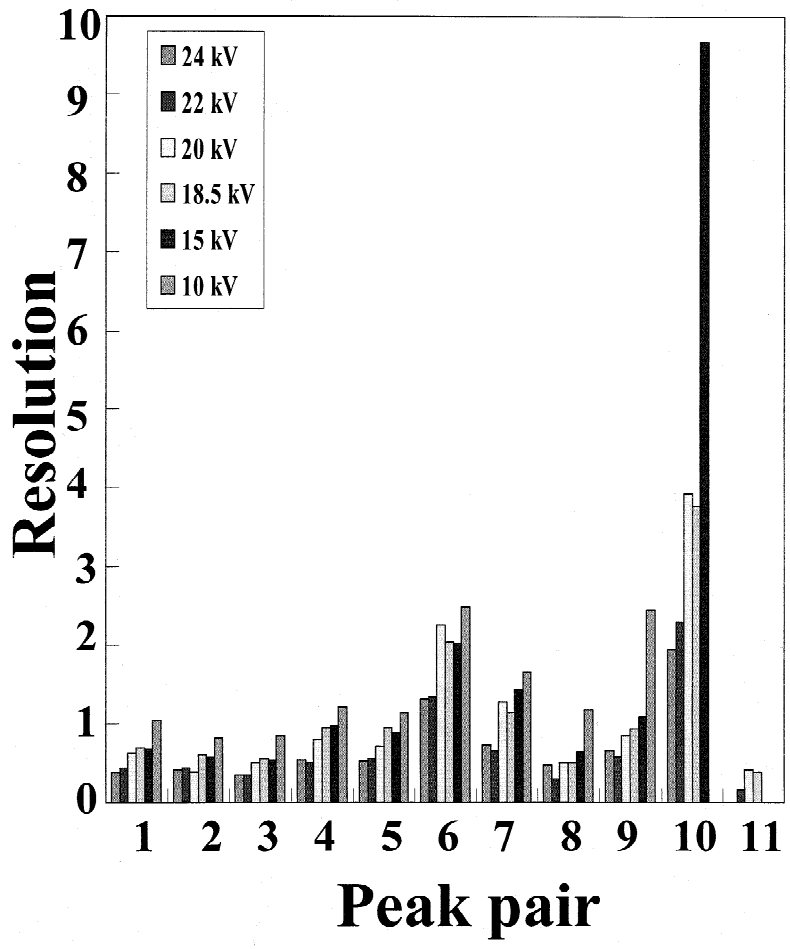

Fig. 6. Effects of applied voltage on resolution between two neighboring species for twelve analytes. Peak pair 1 means peaks 1 and 2, peak pair 2 means peaks 2 and 3, and so on. Conditions as in Fig. 1.

affect their dissociation. Thus, controlling the migration velocity is a more effective way of improving resolution for stronger acids than for weaker acids.

4. Conclusion

Dynamic control in fused-silica capillaries under weak acidic conditions is a very promising technique for the separation of organic acids. By taking advantage of high EOF and pH changes inside capillaries, high-resolution separations of twelve organic acids were achieved in less than 3 min. Transient effects are larger for stronger acids, which further supports the view that the changes in pH and decreases in

Table 1
Comparison of the effects of electric field strength and capillary length on resolution

Peak pair[a]	Resolution ratio	
	R_{10}/R^{b}_{24}	$R_{35}/R^{c}_{22.5}$
p-ABA and *p*-HBA	2.0	1.4
p-TA and BA	2.2	1.4
m-NBA and *o*-NBA	2.3	2.1
o-NBA and PHA	2.5	3.6
PHA and CA	3.7	1.8
CA and FA	5.0[d]	–

[a] BA (benzoic acid) and p-TA (*p*-toluic acid); [b] 10 to 24 kV; [c] 35 to 22.5 cm and [d] the ratio was taken from 15 to 24 kV.

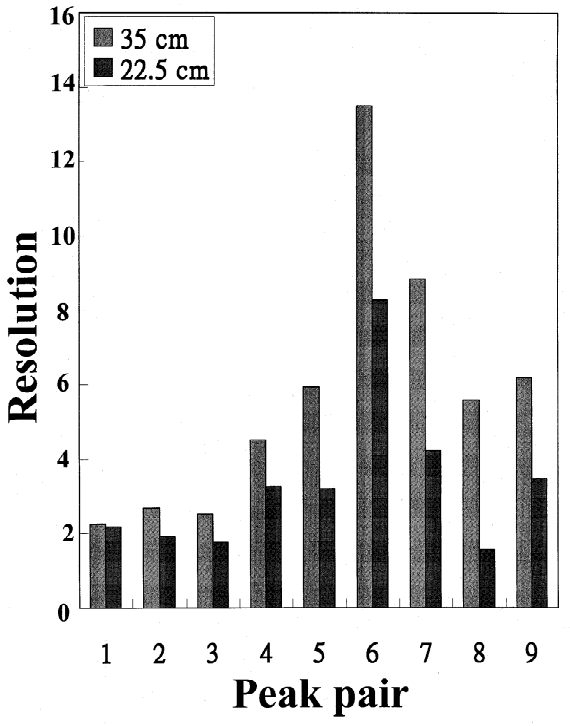

Fig. 7. Effects of capillary length on resolution between two neighboring species for ten analytes. Conditions as in Fig. 6.

EOF are important in this dynamic control mode. In addition, the effects of the changes in capillary length and electric field strength on resolution agree with the existence of dynamic flow and pH changes inside capillaries. The results also show that changing the applied voltage is more efficient than changing the capillary length as a means of improving the resolution. This is especially true for stronger acids.

Because of several features, including high resolution, simplicity and high speed, of this new method, several interesting studies, such as voltage programming and the separation of peptides and acidic drugs from biological samples, are presently under intensive investigation in our group. Real-time observations of the transient phenomena inside capillaries and more advanced theoretical considerations are also being undertaken. All of these studies contribute to the further development of high-resolution and ultra-fast separations in CE.

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