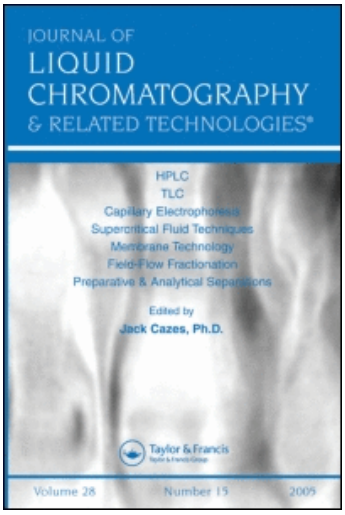

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OPTIMIZATION OF RESOLUTION IN CAPILLARY ZONE ELECTROPHORESIS: EFFECT OF SOLUTE MOBILITY AND BUFFER pH

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ABSTRACT

A reformulated version of the resolution equation in CZE that explicitly relates resolution to migration time is presented. Selectivity and resolution are enhanced with increasing migration time if the increase in time is a result of independent manipulation of electroosmotic flow. If the increase in migration time is a result of simultaneous changes in electroosmotic flow and electrophoretic mobility, selectivity and resolution deteriorate with increasing migration time. Optimum resolution for organic acid isomers is attained at a pH value that is about 0.3 units lower than the average pK of the two acids, even though selectivity continuously increases with decreasing pH. Both selectivity and resolution deteriorate with increasing electroosmotic flow in the same direction as electrophoretic mobility, however, selectivity is more sensitive than resolution to such changes.

INTRODUCTION

Capillary zone electrophoresis (CZE) is gradually gaining acceptance primarily because it offers fast and impressively efficient separations of ionic,

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ionizable and macromolecular species relevant to the area of analytical biotechnology (1,2). When zone electrophoresis is performed in narrow-bore (<100 μm i.d.) columns, heat convection problems are minimized and solute molecular diffusion becomes the predominant cause of peak broadening (3,4). In practice, many experimental parameters such as sample introduction, distortion of the flat flow profile by capillary wall adsorption and/or radial thermal gradients, and solute finite concentration can adversely affect column efficiency and resolution. The subject has attracted the attention of several research groups. For example, Zare and co-workers (5) developed an equation for resolution in CZE that is analogous to the Van Deemter equation in chromatography. In addition to solute diffusion, the equation takes into consideration other zone-broadening mechanisms such as injection plug length and solute-wall interactions. Grushka et al. (6) studied the effect of temperature gradient inside the column as a result of Joule's heating and developed an expression for plate height assuming that the velocity profile within the capillary is parabolic rather than flat. Several other studies have dealt with zone profile in CZE (7,8) and the related technique of micellar electrokinetic capillary chromatography (MECC) (9,10).

In spite of all this activity, the resolution equation that was first developed by Jorgenson and Lukacs (3) where solute longitudinal diffusion is the only zone-broadening mechanism remains the most predominant in CZE literature (1,2,10-14). The fact that efficiencies in excess of 400,000 theoretical plates are routinely obtained in CZE experiments (3) is cited as evidence that other peak-broadening mechanisms can be minimized, if not eliminated, by careful design of the experimental set-up (4).

The resolution equation suggests, as was first pointed out by Jorgenson and Lukacs (3) and widely quoted by others (1,2,4,11-14), that the resolution of analytes is improved if the electroosmotic flow is controlled to slow the migration of the analytes. While this conclusion is obvious by examining the form of the resolution equation, it is, however, based on the assumption that the electroosmotic flow can be independently changed without effecting similar changes in the

electrophoretic mobility of the analytes. Practically, independent control of electroosmotic flow could be achieved by either statically (15) or dynamically (16) coating the capillary wall or by applying an external electric field (17,18). Alternatively, the electroosmotic flow may also be manipulated by changing the applied voltage (19,20), buffer pH (21-23), buffer type and concentration (19-21), and by the addition of organic modifiers (24,25). However, such changes will also invariably affect the electrophoretic mobility of the analytes. In this work a reformulated version of the resolution equation that explicitly relates resolution to migration time is developed and used for the analysis of the effect of simultaneous changes in electroosmotic and electrophoretic flow and pH on selectivity and resolution in CZE.

EXPERIMENTAL

Apparatus

Data was generated with a Beckman CZE System 2000 (Model P/ACE). The system features a UV detector, an autosampler, a liquid-cooled column cartridge and a PC-based System Gold software package. All experiments were conducted at 25°C and each data point was an average of three determinations. Injections were made using the pressure mode for 2-5 s. Solutes were prepared to be about 1 μg/mL and were monitored at the wavelength of maximum absorption with the highest instrument sensitivity. The buffers were prepared by dissolving the appropriate amounts of reagents in distilled and deionized water and were degassed and filtered through 0.2 μm nylon 66 filters prior to use. A Fisher Accumet pH meter 25 (Fisher Scientific, Fair Lawn, NJ) was used to measure the pH levels. Fused silica capillaries (Polymicro Technologies, Phoenix, AZ) were prepared for use by conditioning with NaOH, H_2O and the appropriate buffer, respectively. All columns were 57 cm (50 cm, injector-detector) x 75 μm, I.D. Electroosmotic flow was monitored with mesityl oxide as the neutral marker.

Reagents

Boric acid, sodium tetraborate, acetic acid and sodium acetate were Fisher Scientific products. Nicotinic and isonicotinic acids were obtained from Aldrich Chemical Co. (Milwaukee, WI). Hydroxypropyl methylcellulose (HPMC) and dextromethorphan were purchased from Sigma Chemical Co. (St. Louis, MO) and levallorphan was kindly supplied by Hoffmann-LaRoche (Nutley, NJ). All chemicals were of the highest purity and were used without further purification.

RESULTS AND DISCUSSION

Effect of migration time on resolution

The resolution (Rs) equation for CZE was first formulated by Giddings (26):

$$Rs = \frac{1}{4} N^{\frac{1}{2}} \frac{\Delta v}{\bar{v}} \qquad 1$$

where N is the number of theoretical plates, $\bar{v}$ is the average migration velocity, and $\Delta v/\bar{v}$ is the relative velocity difference of the two zones being separated. N measures system efficiency and $\Delta v/\bar{v}$ measures selectivity. In this study, selectivity is interchangeably used to denote relative velocity (or migration) difference. Based on eq. 1 and assuming that solute longitudinal diffusion is the only zone-broadening mechanism, Jorgenson and Lukacs arrived at the following expression for resolution (3):

$$Rs = \frac{1}{4}\left(\frac{V}{2D}\right)^{\frac{1}{2}} \frac{\Delta \mu_{ep}}{(\bar{\mu}_{ep}+\mu_{eo})^{\frac{1}{2}}} \qquad 2$$

where $\Delta\mu_{ep}$ is the difference in the electrophoretic mobility of the two zones, $\bar{\mu}_{ep}$ is the average electrophoretic mobility, D is solute molecular diffusion coefficient, V is the applied voltage and μ_{eo} is the electroosmotic mobility. Resolution is seen as a function of applied voltage, differential electrophoretic mobility and

electroosmotic mobility. Terabe et al. (4) modified this expression by introducing a fourth factor, namely, l/L, where L is the total column length and l is the injector-to-detector column length.

Taking into account the fact that average solute migration time ($\bar{t}_m$) for two closely-migrating solutes is related to solute mobility by the expression $\bar{t}_m = (l.L)/(\bar{\mu}_{ep}+\mu_{eo})V$, and that $(\Delta t_m/\bar{t}_m) = \Delta\mu_{ep}/(\bar{\mu}_{ep}+\mu_{eo})$, the resolution equation could be algebraically rearranged to arrive at an expression that explicitly relates the resolution to migration time:

$$Rs = \left(\frac{l.L}{32D}\right)^{\frac{1}{2}} \frac{\Delta t_m}{\bar{t}_m(\bar{t}_m)^{\frac{1}{2}}} \qquad 3$$

The term $1/(\bar{u}_{ep}+\mu_{eo})$ or $1/(\bar{\mu}_{ep}[1+(\mu_{eo}/\bar{\mu}_{ep})])$ appears in the expressions for $\bar{t}_m$ and $\Delta t_m/\bar{t}_m$. Consequently, selectivity and resolution are directly affected by the magnitude and sign of the ratio $(\mu_{eo}/\bar{\mu}_{ep})$. In this study, the effect of migration time on selectivity and resolution under two qualitatively different experimental CZE situations is investigated. In the first situation, electroosmotic flow is independently changed and in the second, changes in electroosmotic flow are accompanied by simultaneous changes in electrophoretic mobility to keep the ratio $\mu_{eo}/\bar{\mu}_{ep}$ approximately constant. In the first experiment two solutes, dextromethorphan and levallorphan, and mesityl oxide as a neutral marker were electropherogrammed at pH 9.2 with 10 mM borate buffer containing varying concentrations of HPMC, a known electroosmotic flow suppressor (4). Under these conditions, the solutes are positively charged and travel towards the cathodic detector's end in the same direction as the electroosmotic flow. Examination of the data presented in Table I reveals that while $\bar{\mu}_{ep}$ is practically unchanged, $(\mu_{eo}/\bar{\mu}_{ep})$ varied from 4.54 with 0.0% HPMC to 2.37 with 0.20% HPMC. Figure 1 shows plots of selectivity $(\Delta t_m/\bar{t}_m)$ and resolution (Rs), calculated according to eq. 3 as a function of migration time. It shows that both parameters are enhanced with increasing migration time, indicating that the natural diffusional zone-broadening effect associated with longer migration times is more than offset by the enhancement of selectivity. Figure 2

Table I. Solute migration times at different concentrations of HPMC in 10 mM borate buffer at pH 9.2

% HPMC	Dextromethorphan		Levallorphan		Mesityl Oxide		$x = \mu_{eo}/\mu_{ep}$
	t_m[a]	μ_{ep}[b]	t_m[a]	μ_{ep}[b]	t_m[a]	μ_{eo}[b]	
0.0	2.89	11.86	3.19	6.77	3.70	42.34	4.54
0.05	3.12	11.14	3.41	6.87	4.01	39.07	4.34
0.10	3.60	11.61	4.13	6.03	4.91	31.91	3.62
0.15	4.13	11.73	4.85	6.10	5.98	26.20	2.94
0.20	4.67	11.85	5.56	6.48	7.22	21.70	2.37

[a]t_m in minutes ±1.0%; [b]$\mu x 10^5 cm^2/Vs$. Applied voltage = 20 kV; Column length: total = 47 cm, Inj-Det = 40 cm; Instrument: Beckman Model P/ACE System 2000.

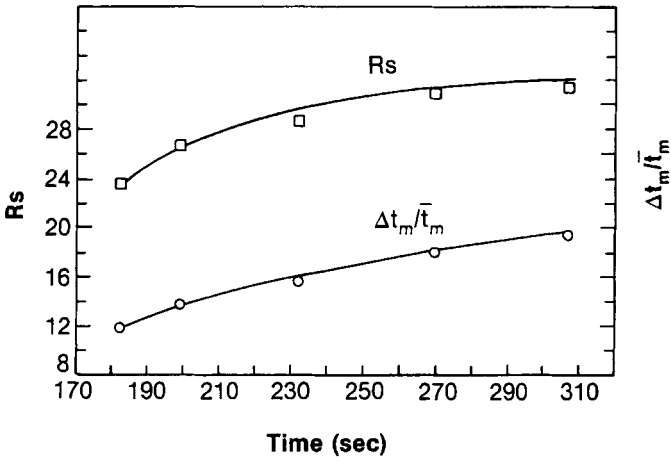

FIGURE 1 Dependence of selectivity and resolution on migration time under conditions of constant solute electrophoretic mobility. Applied Voltage: 20 kV; Buffer: 10 mM borate with different percentages of HPMC; pH: 9.2; Solutes: 1 -dextromethorphan, 2 - levallorphan.

gives the electropherograms obtained at different HPMC concentrations and clearly demonstrates that selectivity is enhanced with the addition of HPMC. Theoretical resolution as calculated by eq. 3 is also enhanced, however, it appears that the peaks are broadened with the addition of HPMC. This is perhaps due to chromatographic zone-broadening mechanisms such as solute partitioning in the HPMC column surface film.

In the second experiment the migration times of nicotinic and isonicotinic acids were measured at pH 4.5 using acetate buffer at different concentrations and applied voltages. The results are presented in Table II. The effect of applied voltage and buffer concentration on μ_{eo} and μ_{ep} have been shown to be qualitatively similar in nature (19). Both mobilities increase with increasing applied voltage and decreasing buffer concentration in such a way as to result in insignificant variations in their ratio. As a consequence, decreasing μ_{eo} does not necessarily result in

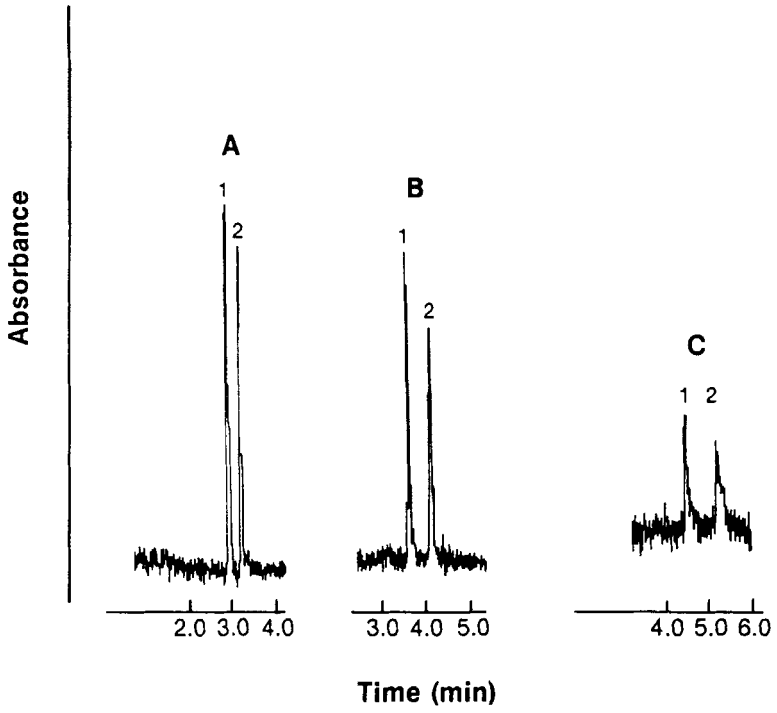

FIGURE 2 Effect of electroosmotic flow on resolution at constant solute electrophoretic mobility. Applied Voltage: 20 kV; Buffer: 10 mM borate; pH: 9.2; A: 0.0% HPMC; B: 0.1% HPMC; C: 0.2% HPMC. Solutes: Same as Figure 1.

enhancement of selectivity. Figure 3 shows plots of $(\Delta t_m/\bar{t}_m)$ and Rs as a function of migration time. $\Delta t_m/\bar{t}_m$ increased slightly with increasing migration time (decreasing applied voltage and/or increasing buffer concentration) but more or less remained within a narrow range. Alternatively, resolution deteriorates with increasing migration time in contrast with the results presented in Figure 1. This is attributed to the fact that as the migration time increases, diffusional zone-broadening is increased with no countering improvement in differential migration difference. While the original resolution equation (eq. 2) predicts that resolution improves with increasing voltage (decreasing migration time) it does not directly

Table II. Solute migration times at different applied voltages and acetate buffer concentrations

Migration time[a] (min)			
Applied Voltage (kV)	Buffer Concentration (mM)	Nicotinic Acid	Isonicotinic Acid
30	50	4.38	4.46
25	50	6.17	6.29
20	50	8.30	8.45
15	50	12.03	12.26
10	50	19.92	20.31
30	35	4.28	4.36
25	35	5.70	5.80
20	35	7.48	7.62
15	35	10.57	10.77
10	35	16.89	17.21
30	25	4.20	4.28
25	25	5.17	5.27
20	25	6.89	7.02
15	25	9.97	10.16
10	25	16.13	16.44
30	10	4.01	4.08
25	10	5.01	5.10
20	10	6.47	6.59
15	10	8.88	9.05
10	10	13.73	13.99

Migration times ±1.0%; Column length: total = 57 cm; Inj-Det = 50 cm; Instrument: Beckman Model P/ACE System 2000; pH = 4.5.

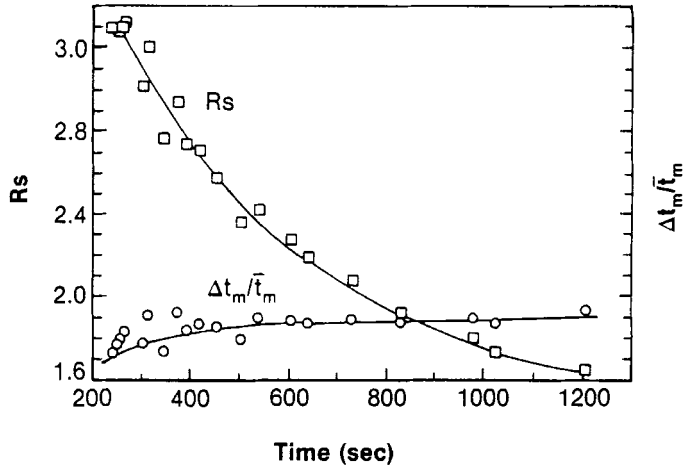

FIGURE 3 Dependence of selectivity and resolution on migration time under conditions of approximately constant $\mu_{eo}/\bar{\mu}_{ep}$ ratio. Solutes: Nicotinic and isonicotinic acids; Other conditions: Same as Table II.

address the effect of other experimental parameters that directly affect migration time such as changes in buffer type, concentration, addition of organic modifier and salts. The reformulated equation presented in this study offers a direct relationship between resolution and migration time irrespective of the experimental cause for changes in migration time.

Effect of pH on the separation of organic acid isomers

Smith and Khaledi (14) analyzed the migration behavior of a set of substituted phenols and devised a theoretical model that describes mobility in CZE as a function of fundamental constants, namely the acid dissociation constant and the mobility of the fully-dissociated ions. This model was used for the pH optimization of resolution. Terabe et al. (4) devised a theoretical model for the determination of the optimum conditions for the separation of isomeric acids having only slightly different dissociation constants. The method was used for the

optimization of the CZE separation of oxygen-isotopic benzoic acid isomers. The following presents our theoretical treatment of the dependence of selectivity, which was not directly addressed by the above cited literature, and resolution on pH and electroosmotic flow following the approach adopted by Terabe et al. (4).

The mobility of a weak acid (HA) in CZE can be described by the following relationship (4,14,27):

$$\mu = \alpha\mu_o + \mu_{eo} \qquad 4$$

where $\alpha = K/(K+[H^+])$, μ_o is the electrophoretic mobility of the anionic form of the acid and K is the acid dissociation constant.

The relative velocity difference of two acids having close dissociation constants K_1 and $K_2 = rK_1$, could be described by the following expression:

$$\frac{\Delta V}{\bar{V}} = \frac{\mu_1 - \mu_2}{\bar{\mu}_{ep} + \mu_{eo}} = \frac{\alpha_1\mu_{o1} - \alpha_2\mu_{o2}}{\bar{\alpha}\bar{\mu}_o + \mu_{eo}} \qquad 5$$

Assuming that $u_{o1} \simeq u_{o2}$ for two isomeric acids, eq. 5 could be rearranged as follows:

$$\frac{\Delta V}{\bar{V}} = \frac{\Delta\alpha}{[\bar{\alpha} + \frac{\mu_{eo}}{\bar{\mu}_o}]} \qquad 6$$

Accordingly, the relative velocity difference (selectivity) of two isomeric acids could be pH optimized by obtaining the maximum of the right-hand side of eq. 6 as a function of pH. Similarly, the resolution could be pH optimized at constant voltage by optimizing the mobility ratio in eq. 2. The expression is first rearranged as follows:

$$\frac{\Delta\mu_{ep}}{[\bar{\mu}_{ep} + \mu_{eo}]^{\frac{1}{2}}} = (\bar{\mu}_o)^{1/2} \frac{\Delta\alpha}{[\alpha + \frac{\mu_{eo}}{\bar{\mu}_o}]^{\frac{1}{2}}} \qquad 7$$

Figure 4A gives plots of the function $\Delta\alpha/\bar{\alpha}$ which represent the relative velocity difference of two acids, with $K_1 = 1 \times 10^{-5}$ and different values of r, as a function of pH under the condition of zero electroosmotic flow. The figure shows that

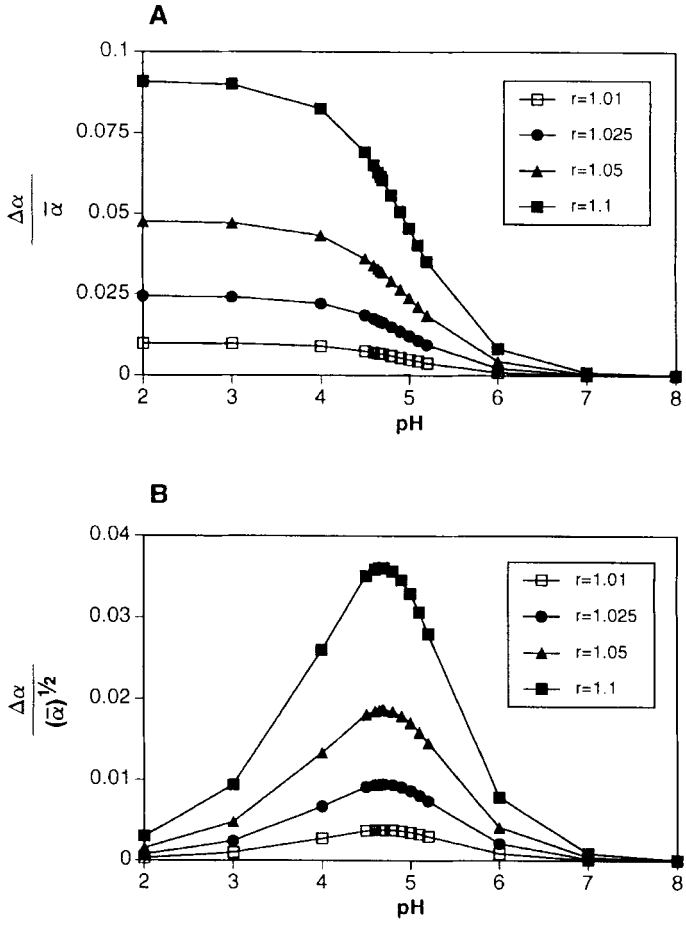

FIGURE 4 Dependence of A: $\Delta\alpha/\bar{\alpha}$ and B: $\Delta\alpha/(\bar{\alpha})^{1/2}$ on pH at different r values. $K_1 = 1.0 \times 10^{-5}$; $r = K_2/K_1$.

selectivity continuously improves with decreasing pH (decreasing ionization) and that maximum separation is achieved at low pH where α is infinitely small. Maximum selectivity, however, does not translate to maximum resolution because improved selectivity at low pH is achieved at the expense of having an extremely long migration time. This is clearly illustrated in Figure 4B which shows plots of

Table III. Optimum pH for the separation of acid isomers as a function of the ratio of their dissociation constants

r (K_2/K_1)	Average pK	Optimum pH	Δ^a
1.001	4.9997	4.6991	0.301
1.005	4.9989	4.6981	0.301
1.010	4.9978	4.6971	0.301
1.020	4.9957	4.6961	0.300
1.050	4.9894	4.6921	0.297
1.100	4.9793	4.6851	0.294
1.200	4.9604	4.6721	0.288
1.300	4.9430	4.6601	0.283

[a]Δ = Average pK - Optimum pH; $K_1 = 1 \times 10^{-5}$.

$\Delta\alpha/(\bar{\alpha})^{1/2}$ as a function of pH at different values of r. As the pH is decreased the resolution improves to a maximum at a pH value slightly below the average of the dissociation constants of the acids but resolution subsequently decreases with further decreases in pH. This is attributed to the fact that the slight improvement in relative velocity difference with decreasing pH is offset by the diffusional zone broadening associated with increasing migration times. Terabe et al. (4), theoretically analyzed a similar situation and observed that optimum resolution is always obtained at a pH value that is smaller than the average pK of the two acids by log 2 (or exactly 0.301 units) regardless of the ratio r (K_2/K_1). While this is accurate for small values of r ($r<1.01$), our calculations show that it is only approximate for $r>1.01$. The results reported in Table III indicate that the differences between the average pK of the two acids and optimum pH decrease slightly with increasing r. The reason for the discrepancy is that Terabe et al. assume that the two acid constants (K_1 and K_2) are approximately equal, while we

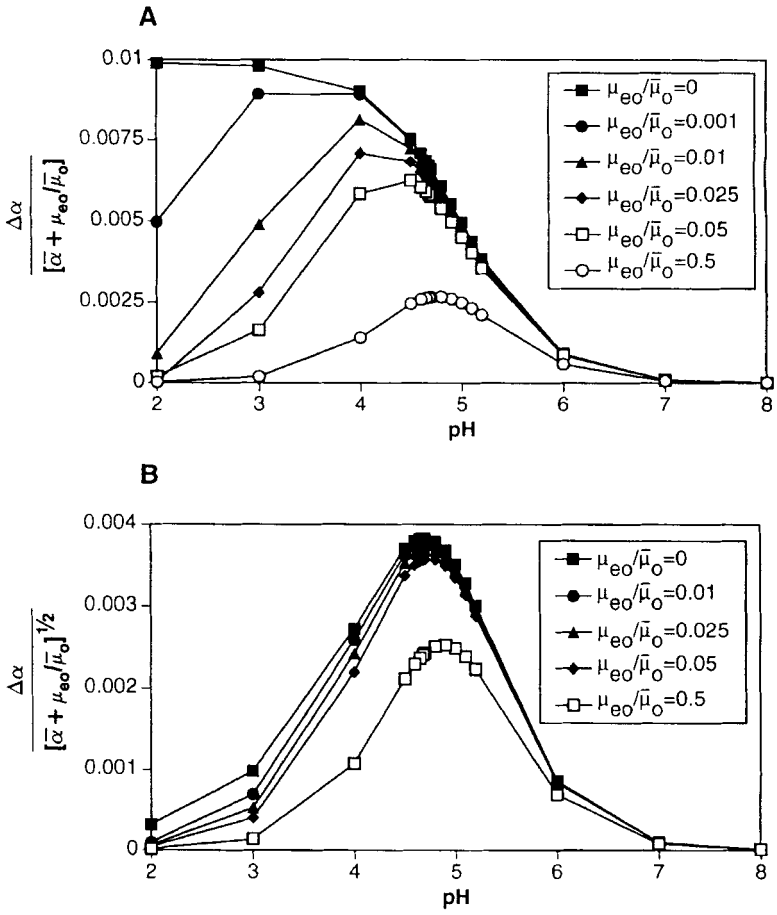

FIGURE 5 Dependence of A: $\Delta\alpha/[\bar{\alpha}+(\mu_{eo}/\bar{\mu}_o)]$ and B: $\Delta\alpha/[\bar{\alpha}+(\mu_{eo}/\bar{\mu}_o)]^{1/2}$ on pH at r = 1.01 and different values of $\mu_{eo}/\bar{\mu}_o$. $K_1 = 1.0 \times 10^{-5}$; $r = K_2/K_1$; $\bar{\mu}_o$ = average electrophoretic mobility of the fully dissociated acid ions.

make no assumption on their relative values. It is to be noted that values of r >1.1 are typical of organic acid isomers such as, for example, the meta- and para-substituted pairs of benzoic acids (28).

Finally, the effect of electroosmotic flow on relative selectivity and resolution of acid isomers is illustrated in Figures 5A and 5B, respectively. It is observed that

both selectivity and resolution deteriorate with increasing electroosmotic flow in the same direction as the electrophoretic mobility. Figure 5, however, shows that selectivity is more sensitive than resolution to any variation in electroosmotic flow. For example, $\Delta v/\bar{v}$ at pH = 4.7 and $(\mu_{eo}/\bar{\mu}_o) = 0.5$ is reduced by as much as 60% of its maximum value achieved in the absence of electroosmotic flow, while Rs is only reduced by 37% for a similar change in electroosmotic flow conditions. Moreover, it is observed that in the presence of electroosmotic flow, plots of selectivity vs. pH and resolution vs. pH appear qualitatively similar with a maximum for each at about the same pH value.

Smith and Khaledi (14) reported a theoretical model for the pH optimization of the resolution of organic acids and showed a plot of resolution vs. pH that peaks at a given pH value. Their figure was obtained by optimizing the mobility ratio of the resolution equation assuming a constant number of theoretical plates N. In light of the discussion presented in this work, it is believed that Smith and Khaledi have in effect optimized selectivity rather than resolution. The fact that they obtained a plot with a maximum at a given pH value rather than continuously increasing function with decreasing pH reflects the presence of electroosmotic flow in their system, which makes plots of selectivity vs. pH and resolution vs. pH appear qualitatively similar.

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