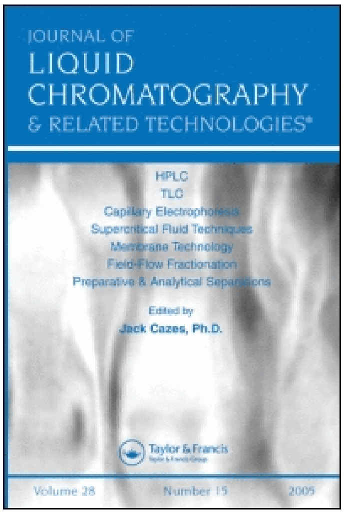

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Influence of Organic Modifiers on the Separation of Carboxylic Acids Using Co-EOF Capillary Electrophoresis

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ABSTRACT

In this paper, a number of different electroosmotic flow (EOF) modifiers, including both organic solvents and ion-pairing reagents, were systematically evaluated for their effect on the separation selectivity of carboxylic acids using the co-EOF mode. Although, all organic solvents modified the EOF to some extent, the best separation selectivity of the tested carboxylic acids was achieved using acetonitrile (15% v/v) as the organic solvent modifier. High concentrations of tetramethylammonium hydroxide (TMAOH) in the background electrolyte (BGE) also improved separation selectivity. This resulted from changes in mobility due to the formation of complexes between TMAOH and the carboxylic acids, and

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the EOF. Excellent separation selectivity of nine carboxylic acids (oxalic, formic, fumaric, malonic, tartaric, citric, succinic, maleic, and acetic) was obtained using 70 mM TMAOH in a 25 mM KH_2PO_4 BGE at pH 6.0. The calibration curves were linear in a concentration range of 0.4–1 mM with detection limits ranging from 4–25 μM. The utility of the proposed method was demonstrated by determining the concentration of carboxylic acids in plant exudates.

Key Words: EOF modifiers; CZE; Carboxylic acids; Plant exudates.

INTRODUCTION

Separation during capillary electrophoresis (CE) is based on the differences between the ionic mobilities of the solutes to be separated. For ionizable solutes, such as carboxylic acids, separation depends mainly on the electrophoretic mobility of the solute and the electroosmotic flow. Manipulation of the electroosmotic flow (EOF) is one of the most important methods for optimizing selectivity. The pH of the background electrolyte (BGE) also contributes significantly to the overall EOF. It is, therefore, common to optimise the EOF via control of the BGE pH to obtain reasonable selectivity.[1–3] Organic modifiers, such as organic solvents and large hydrophobic cations, can also alter the EOF by altering the charge density at the surface of the fused-silica capillary.[4,5] The co-EOF mode, where anions co-migrate with the EOF, is frequently employed for the separation of organic anions by adding cationic surfactants to reverse the direction of the EOF and results in faster analysis times. However, poor resolution of carboxylic acids having similar pKa value is often achieved when using the co-EOF mode.[6]

When added to the BGE in small quantities, organic solvents enhance separation selectivity (resolution) due to their influence on the effective mobility of the ionic solute and the EOF. Sarmini and Kenndler have recently examined the influence of methanol,[7] ethanol,[8] 1-propanol,[9] and acetonitrile[10] on selectivity. They showed, that organic solvents change both the EOF and the effective mobility of the solute by altering the pKa as a result of differences in different solvents. Changes in the EOF resulted directly from the organic solvent altering the zeta potential near the surface of the capillary, as well as from changes in the viscosity and dielectric constant of the BGE. The pKa value of the organic acids increased with increasing organic solvent content of the BGE, which was explained by the concept of “medium effect” or transfer activity coefficient.[4] The transfer activity coefficient quantifies the differences in free energy of a solute ion in two different liquid phases (mediums).

The separation selectivity of organic anions can also be manipulated by the addition of cationic surfactants and ion-pairing reagents. The most commonly used cationic surfactants in co-EOF include tetradecyltrimethylammonium bromide (TTAB), cetyltrimethylammonium bromide (CTAB), and hexadimethrine bromide (HDB).[11–15] However, poor selectivity was frequently obtained due to the fast separation. In order to improve selectivity, conventional reserved-phase liquid chromatography (RPLC) ion-pairing alkylammonium salts were proposed to be used in CE by Terabe and Iki;[16,17] these were successfully used to separate peptides and phenolic acids,[18] pyridylamino derivatives,[19] metal complexes,[20] and aminosalicylic acids and related impurities.[21] In principle, the selectivity can be altered by the addition of the ion-pairing reagent to the running buffer because of the formation of complexes between the ionic solute and the ion-pairing reagent, and changes in the EOF due to the presence of the surfactant.[22]

Therefore, it should be possible to control separation selectivity of similar carboxylic acids by the addition of organic modifiers to the BGE. Our previous reports show that the fast separation of carboxylic acids was obtained using co-EOF mode using the cationic surfactants.[6,12–14] However, poor resolutions between solutes with a similar pKa value were achieved. The aim of the work was, systematically, to examine the effect of organic solvents and ion-pairing reagents affecting the EOF and, consequently, the electrophoretic mobility of carboxylic acids having similar pKa's, in order to improve the selectivity. Such a method would be of great utility in both soil and plant science, as these carboxylic acids play an important role in the ecosystem.

EXPERIMENTAL

All carboxylic acids obtained from Sigma Aldrich (Sydney, Australia) were of analytical grade and were used without further purification, while tetramethlyammoniumhydroxide (TMAOH) was obtained from Aldrich (Milwaukee, WI) as an aqueous 25% (w/w) solution, and TTAB was obtained from Sigma Aldrich (Sydney, Australia). Standards of all organic acids tested were prepared daily by dilution with Milli-Q^{TM}, from 10 mM stocks. Background electrolytes were prepared by dissolution of an appropriate amount of KH_2PO_4 in Milli-Q water, which contained appropriate amounts of surfactants and organic solvents. All electrolytes were filtered through disposable Millipore 0.45 μm membrane filters and degassed in an ultrasonic bath prior to use. Electrolyte pH was adjusted with 0.1 M NaOH or 0.1 M H_3PO_4.

Root exudate hydroponic solutions were also filtered through a 0.45 μm membrane filter before injection into the CE system. These solutions were obtained from a concurrent experimental study. Fully mature plants (*Thlaspi goesingense*) were transferred to a 10 mL flask containing nutrient solution under asceptic conditions, and allowed to exude for 24 hrs. The full exudate hydroponic solution contained 0.7 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.4 mM KCl, 0.5 mM KH_2PO_4, 0.7 mM $Mg(NO_3)_2$, 0.3 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 0.3 mM KNO_3, 0.5 mM MES, 10 μM H_3BO_3, 0.2 μM $Na_2MoO_4 \cdot 4H_2O$, 20 μM $MnCl_2 \cdot 4H_2O$, 5 μM $CuSO_4 \cdot 5H_2O$, 12.5 μM $FeCl_3 \cdot 6H_2O$, 12.5 μM EDDHA, 5 μM $ZnSO_4 \cdot 7H_2O$, 5 μM $Cd(NO_3)_2 \cdot 6H_2O$, O, and 2 μM $NiCl_2 \cdot 6H_2O$. The final pH of this solution was adjusted to 6.1 by addition of NaOH. After 24 hrs, the samples were stored at 4°C until analysed by CE.

All electropherograms were obtained using a Quanta 4000 (Waters, Milford, WI). The system was controlled by Millennium (Waters, Milford, WI) software. Separation was carried out using fused-silica capillaries with 50 μm I.D × 80 cm total length (72.5 cm effective length), or 50 μm I.D. × 40 cm total length (32.5 cm effective length). The UV detector was set at 185 nm.

New capillaries were pretreated with the following cycles: 0.1 M NaOH for 20 min, 0.01 M NaOH for 20 min, deionized water for 10 min, and, finally, the phosphate BGE for 20 min. The capillary was rinsed with BGE for 2 min between each run. Samples were injected in the hydrostatic mode at 10 cm for 30 s. The capillary was held at 25°C and the applied constant voltage was −20 kV. Benzyl alcohol, 0.05% (v/v), was used as a neutral marker for the determination of electroosmotic flow, and electrophoretic mobilities were calculated from the equation described previously.[23] Identification of each solute was verified by spiking with known standards.

RESULTS AND DISCUSSION

Effect of Organic Solvents

In co-EOF mode, poor resolution is often obtained between carboxylic acids having similar pKa value, because the carboxylic acids migrate in the same direction as the EOF. The influence of organic solvents on the EOF has been quantified by Jansson and Roeraade using Eq (1):[24]

$$\mu_{eo} = -\left(\frac{\varepsilon_0 \varepsilon_{r,\mathrm{wall}} \xi_{\mathrm{wall}}}{\eta_{\mathrm{wall}}}\right) \qquad (1)$$

Where ε_0 is the vacuum permittivity; $\varepsilon_{r,\mathrm{wall}}$ and η_{wall} are, respectively, the relative dielectric constant and viscosity in the electric double layer and ξ_{wall} is the zeta potential at the capillary wall.

The effect of organic solvents on the magnitude of the effective mobility of the carboxylic acids and the overall magnitude of the EOF was tested by addition of six different solvents, 15% (v/v), to a 25 mM phosphate BGE of pH 6.0. Oxalic (pKa_1 1.27, pKa_2 4.27), formic (pKa 3.75), and acetic acid (pKa 4.76) were all effectively dissociated at this pH, since it exceeded their pKa's and were used as analytes for all calculations because they were well resolved and could be clearly identified in all six BGEs. The effective mobilities of the carboxylic acids and the corresponding EOF observed in BGEs containing various organic solvents, are summarised in Table 1. All organic solvents had a marked influence on the magnitude of the EOF. The EOF was calculated from the retention time of benzyl alcohol and decreased in the following sequence: water (phosphate BGE without organic solvent) $>$ EtOH $>$ PrOH $>$ MeOH $\approx$ ACN $\gg$ ethylene glycol. This suggested that acetonitrile and ethylene glycol could be useful for the optimisation of resolution. Masselter and Zemann[25] reported that the absolute viscosity and the permittivity of the solutions were altered due to the addition of organic solvent. Therefore, it is likely that the changes in EOF may result not only from changes in the zeta potential in these mixed solvents, but also from changes in other parameters, such as the solvent viscosity as suggested from Eq. (1). The effect of added organic solvents observed here is similar to that

Table 1. Effective electrophoretic mobilities of oxalic, formic and acetic acid together with EOF for six solvent mixtures at an organic solvent concentration of 15% (v/v) in 25 mM KH_2PO4 and 0.75 mM TTAB adjusted to pH 6.0.[a]

Solvent (15% (v/v))	μ_s (10^{-4} cm^2 V^{-1} s^{-1}) Oxalic	Formic	Acetic	μ_{eo}(10^{-4} cm^2 V^{-1} s^{-1}) EOF[b]
Water	4.81 ± 0.10	4.12 ± 0.04	2.88 ± 0.01	4.03 ± 0.01
Acetonitrile	4.19 ± 0.04	3.71 ± 0.03	2.48 ± 0.02	2.29 ± 0.06
MeOH	3.73 ± 0.01	3.20 ± 0.02	2.10 ± 0.06	2.24 ± 0.06
EtOH	3.21 ± 0.02	2.87 ± 0.02	1.80 ± 0.05	2.71 ± 0.04
PrOH	3.24 ± 0.05	2.89 ± 0.07	1.88 ± 0.05	2.56 ± 0.03
Ethylene glycol	3.67 ± 0.01	3.19 ± 0.01	2.23 ± 0.02	0.69 ± 0.01

[a] Error refers to one standard deviation of the mean ($n = 3$).

[b] Benzyl alcohol used as EOF marker.

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observed in previous studies examining the impact of organic solvent on the zeta potential.[26,27]

From Table 1, it can be seen, that the nature of the organic solvent (15% v/v) also influenced the effective mobility of the carboxylic acids, which decreased significantly in the order: water > ACN > ethylene glycol, MeOH > PrOH ≈ EtOH. The highest mobility was obtained in water, a moderate mobility was obtained in acetonitrile, and a lower mobility was obtained in all of the alcohols. This indicated that the separation selectivity could be manipulated by using solvent modification. The impact of the solvent on the effective mobility of solutes results from changes in the hydrodynamic volume of the solvated species and, also their acidity, leading to changes in the pKa.[4,25]

Figure 1(a) and (b) show the change in EOF and the observed mobility of two organic acids with increasing acetonitrile or MeOH content. For both solvents, the magnitude of the EOF and the observed mobility of the acids decreased with increasing organic solvent content. The mobility of oxalic acid decreased to $3.4 \times 10^{-4}\,\mathrm{cm^2\,V^{-1}\,s^{-1}}$ in 20% (v/v) methanol and to only $4.0 \times 10^{-4}\,\mathrm{cm^2\,V^{-1}\,s^{-1}}$ when 20% (v/v) acetonitrile was added. In contrast, changes in the magnitude of the EOF were observed upon solvent addition. For example, the EOF decreased from 4.03 to 1.43 upon addition of 20% methanol, and for acetonitrile the EOF was significantly decreased when the solvent content was increased from 10 to 20%. Compared to methanol, acetonitrile has less influence on the adsorption of water on the surface of the modified capillary, because acetonitrile is a polar molecule with a low permittivity in solution.[4] Acetonitrile, therefore, has less affinity for the EOF modifier and less influence on the modified surface.[4,25] These results indicate that organic solvent addition can be used to manipulate the selectivity of the carboxylic acids. As an example, in Fig. 2(a) nine carboxylic acids, some possessing similar pKa's, are successfully separated in the co-EOF mode with a 25 mM phosphate BGE containing 15% (v/v) acetonitrile and 0.75 mM TTAB at pH 6.0, as described our previous reports.[6,14] The same electropherogram under identical condition, but using a BGE with no added acetonitrile, is shown in Fig. 2(b) for comparison. Clearly, the addition of acetonitrile has increased the separation selectivity of all peaks with only a moderate increase in analysis time.

Effect of Ion-Pairing Reagent

Buffer additives, such as CTAB, TTAB, and MTAB have been extensively used to reverse the direction of the EOF and manipulate selectivity.[28] In

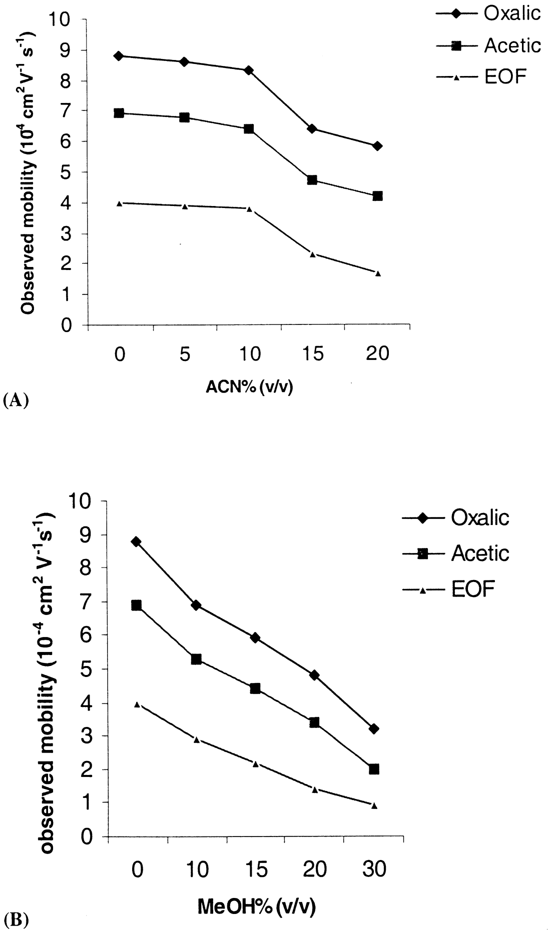

Figure 1. Influence of organic solvent on the observed mobility of oxalic or acetic acid and the corresponding EOF, as determined from the retention time of the EOF marker, benzyl alcohol in (A) ACN or (B) MeOH. Conditions: capillary, fused silica capillary 50 µm × 80 cm (L: 72.5 cm); electrolyte, 25 mM potassium phosphate at pH of 6.0, 0.75 mM TTAB; applied potential, −20 kV; Hydrostatic injection: 10 cm for 30 s, UV detection at 185 nm. Capillary temperature, 25°C.

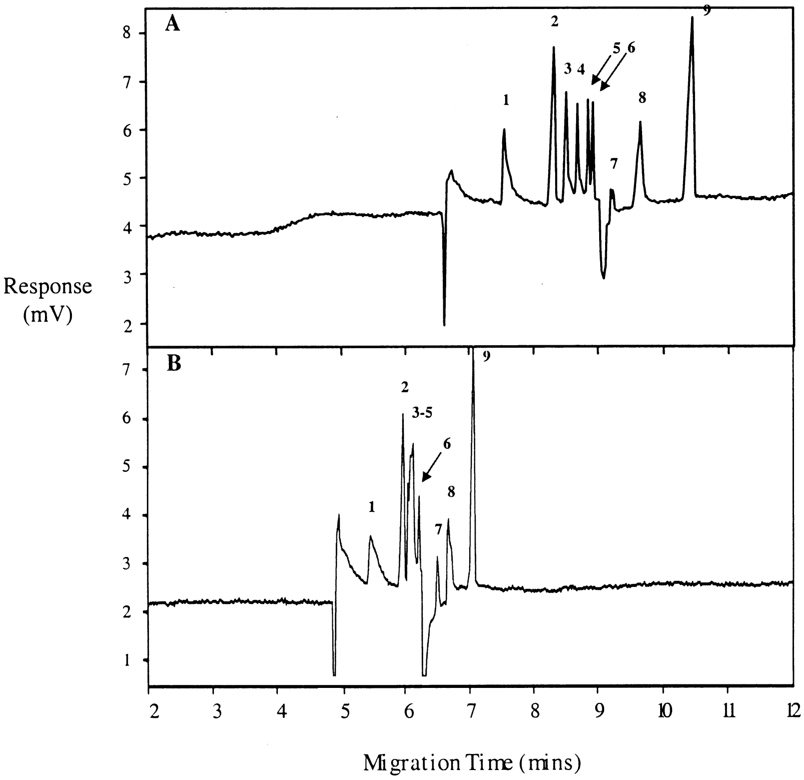

Figure 2. Electropherograms of nine organic acids (0.25 mM) obtained in a 25 mM KH_2PO_4 and 0.75 mM TTAB at pH 6.0 containing (A) 15% (v/v) acetonitrile and (B) no added acetonitrile. Peak identity: 1, oxalic; 2, formic; 3, fumaric; 4, malonic; 5, tartaric; 6, malic; 7, citric; 8, maleic; 9, acetic. Other conditions as in Fig. 1.

contrast to these cationic surfactants, only a few CE studies have discussed the role of ion-pairing reagents in improving selectivity.[16,17,22] The addition of ion-pairing reagents can yield changes in selectivity due to ion-pair interactions between the ion-pairing reagent and the analyte, resulting in alteration of the zeta potential at the capillary wall and, consequently, alteration in the EOF.[22] To demonstrate that alkylammonium salts could be used to improve the selectivity through ion-interaction with organic anions and the EOF, the separation of nine carboxylic acids was investigated in 25 mM phosphate BGE containing 15% ACN at pH 6.0 under co-CE mode. As shown in Fig. 3, the observed mobilities of all of the organic acids increased with the concentration

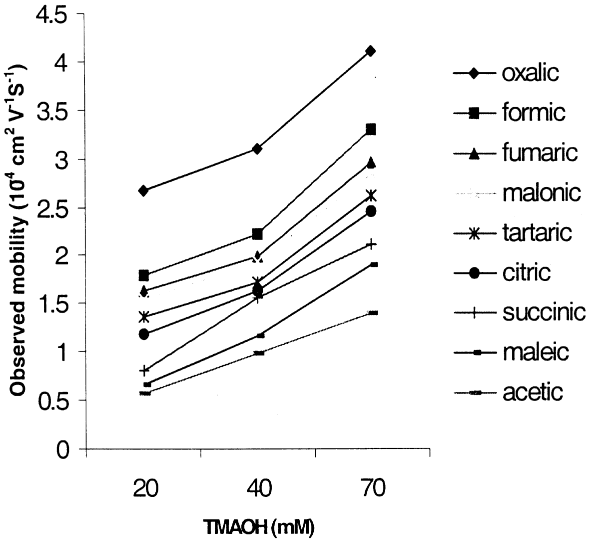

Figure 3. Influence TMAOH concentration in the BGE on the observed mobility of organic acids. Conditions: capillary, fused silica capillary 50 μm × 40 cm (L: 32.5 cm); electrolyte, 25 mM potassium phosphate at pH of 6.0, 15% ACN; applied potential, −20 kV; Hydrostatic injection: 10 cm for 30 s, UV detection at 185 nm. Capillary temperature, 25°C.

of TMAOH in the range 20–70 mM. This could be attributed to both the differences in interaction between carboxylic acid and TMA$^+$, and increases in ionic strength in the buffer. In addition, the EOF value reduced when TMAOH, added to the buffer due to TMA$^+$, adsorped on the capillary wall.[22] A similar result was achieved using ion-pairing reagent, added to the buffer to improve the selectivity of anionic metal complex.[20] The greatest differences in mobilities were observed using 70 mM TMAOH, and an optimum separation of nine carboxylic acids is shown in Fig. 4. Compared to the electropherogram obtained using TTAB as an organic additive [Fig. 2(a)], the electropherogram obtained here, using TMAOH, has a shorter separation time. Peak resolution was also significantly improved and this led to significant changes in selectivity.[19,21,29] However, the detection sensitivity of carboxylic acids was significantly reduced due to an increase in the background of the UV absorbance, when the concentration of TMAOH was increased. In this work, the EOF was substantially reduced by the addition of

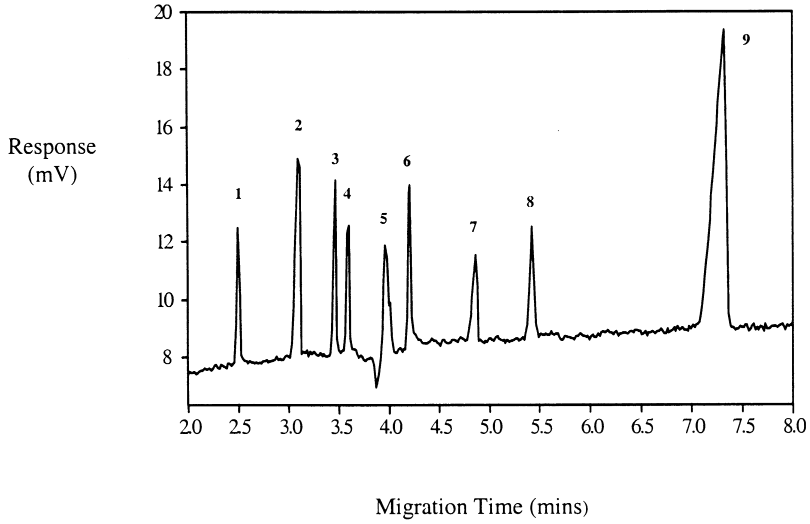

Figure 4. Typical electropherogram of nine organic acids (0.25 mM) obtained in a 25 mM KH_2PO_4 with 70 mM TMAOH ion-pairing reagent added and 15% (v/v) acetonitrile at pH 6.0. Peak identity: 1, oxalic; 2, formic; 3, fumaric; 4, malonic; 5, tartaric; 6, citric; 7, succinic; 8, maleic; 9, acetic. Other conditions as in Fig. 3.

TMAOH in the concentration range 20–70 mM. The increasing ionic strength of the BGE can partially be responsible for decreasing the EOF, as has been reported elsewhere.[30]

In general, ion-pairing complex formation between TMAOH and the carboxylic acids would lower their effective charge and increase their effective size, both of which would result in a lowering of mobility as the concentration of TMAOH increased. Such interactions have been well documented by Weldon et al.[16–18] for the separation of phenolic acids and, recently, by Liu et al.[20] for the separation of metal complexes using cationic alkylammonium salt. However, this is the opposite of the effect observed in Fig. 3, and suggests that ion-pairing alone is not solely responsible for the improved separation selectivity of these carboxylic acids in this BGE. While being an important ion-pairing reagent, TMAOH is also cationic and, at the concentrations used here, would certainly have strong interactions with the capillary wall, as the EOF modifier during CE analysis. The influence of TMAOH on the zeta potential is not well defined in the current literature and points to the need for further research in this area. However, the reasons for good separation are more of a theoretical interest and, in practice, the method described here can be used to successfully improve the selectivity of carboxylic acids with a similar pKa value in under 10 min, and even the reduce the sensitivity.

Analytical Performance of the Method

The optimal new BGE containing both organic solvent and ion-pairing reagent was 25 mM KH_2PO4, containing 15% (v/v) acetonitrile and 70 mM TMAOH at pH 6.0. Both anions and organic acids were well resolved at 185 nm and exhibited sharp symmetrical peaks. No relation between migration order and pKa_1 was found between mono, di, and tricarboxylic acids. Anions commonly present in soil and water, such as Cl^-, $NO_2{}^-$, $NO_3{}^-$, $SO_4{}^{2-}$, did not interference with the separation of the examined carboxylic acids. Calibration plots were obtained by plotting peak area vs. concentration for three of the main carboxylic acids in root exudates (oxalic, citric and acetic acid). The calibration curves, so derived, were linear in the concentration range of 0.4–1 mM with correlation coefficients in the range 0.979–0.996. The detection limits ($S/N = 3$) ranged from 4–25 μM and the reproducibility in peak area (RSD% $n = 5$), from injecting a 0.25 mM standard mixture, ranged from 3–4%, as shown in Table 2.

The proposed method was used to determine the concentration of oxalic, citric, and acetic acid in plant exudates nutrient solution. A typical electropherogram, as shown in Fig. 5, illustrates that an excellent resolution between the analytes was obtained simply with direct injection of the root exudates. The proposed co-EOF method has high selectivity for the carboxylic acids of interest. Spiking with 0.1 mM standard mixtures showed 92–108, 93–103, and 91–106% recoveries for oxalic, citric, and acetic acids, respectively.

Table 2. Performance of the proposed method for the analysis of oxalic, citric and acetic acid.

Organic acid	Regression line	Regression coefficient	DL[a] (μM)	Reproducibility[b] (%)
Oxalic	$y = 23704x - 297.22$	0.996	25	3.8
Citric	$y = 30072x - 427.54$	0.993	15	2.9
Acetic	$y = 132741x + 48742$	0.979	4	3.2

[a] DL-Detection Limit (Signal/Noise = 3).

[b] RSD, $n = 5$, for peak area at 0.25 mM.

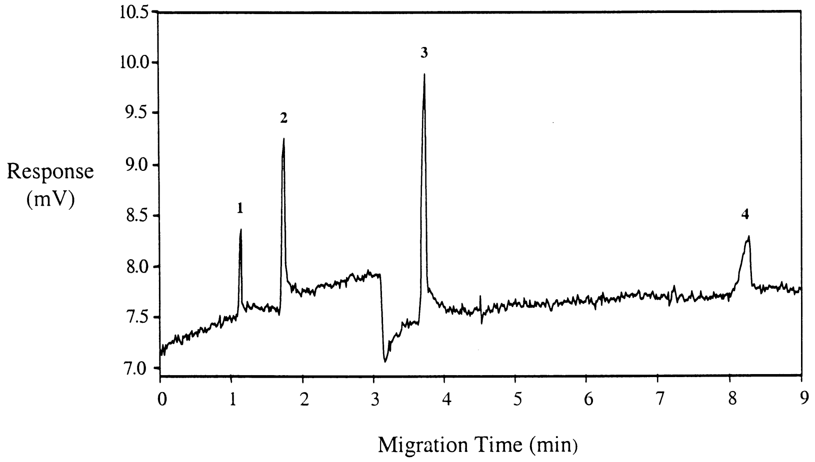

Figure 5. Typical electropherogram obtained from plant exudate samples spiked with 0.1 mM oxalic, citric, and acetic acid. Peak identity: 1, FeEDDHA; 2, oxalic; 3, citric; 4, acetic. Other conditions as in Fig. 4.

CONCLUSIONS

In this work, we have demonstrated that separation selectivity in the co-EOF mode can be manipulated by the addition of both organic solvent and ion-pairing reagent. Organic solvent influences both the electrophoretic mobility of the solutes and the EOF, while the ion-pairing reagent interacts with the solute to form complexes and alters the electrophoretic mobility, and decreases the EOF by increasing the ionic strength of the BGE. Consequently, both organic solvent and ion-pairing reagents can be used to modify selectivity. However, the addition of a high concentration of ion-pairing reagent reduces detection sensitivity, but it satisfies the requirement in our samples. The proposed co-EOF method, which used direct UV detection, is useful for the determination of the carboxylic acids in plant exudates. This method is currently being used to study the phytoremediation of heavy metals, such as Cu^{2+}, from soil, and to monitor the interactions of Cu^{2+} with oxalic, citric, and acetic acids that are commonly exuded from plant roots.

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