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Sensitive and selective method for the separation of organic acids by capillary zone electrophoresis

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Abstract

A simple and rapid method was developed for the separation and determination of a large number of organic acids by using capillary zone electrophoresis, with hydrostatic injection for 30 s and UV detection at 254 nm. Electropherograms were obtained with 4-hydroxybenzoate buffer (5 m*M*) adjusted to pH 4.75 at 25°C. The electroosmotic flow modifier (commercial solution) was added to the buffer at a concentration of 25 ml l^{-1}. Co-migration problems could be solved by modifying the pH of the electrophoresis buffer or by addition of Ca^{2+} to the buffer.

1. Introduction

Capillary zone electrophoresis (CZE) is the simplest form of capillary electrophoresis (CE). The separation mechanism is based on differences in electrophoretic mobilities which are dependent on the charge to mass ratio. CZE has grown increasingly popular in the field of analytical chemistry, and has been successfully used to detect and measure biomolecules [1], carbohydrates [2,3], milk and soy proteins [4] and organic acids in juices from beet sugar production [5].

CZE with indirect UV detection has been shown to be a good alternative to conventional chromatographic methods such as high-performance liquid chromatography (HPLC). Indeed, CZE requires no sample preparation, other than dilution, has a fast analysis time (less than 7 min) and consumes only limited amounts of reagents.

The objective of this work was to optimize the separation and determination of the greatest possible number of organic acids in culture filtrates of soil fungi.

2. Experimental

2.1. Apparatus

CZE was performed on a Waters Quanta 4000 capillary electrophoresis system equipped with a UV detector set at 254 nm, with an applied voltage (*V*) of 20 kV. A fused-silica capillary (Waters), 60 cm in length (L_t) with I.D. 75 μm, was used and the detector window was set at 52.4 cm (L_d). The wall of the capillary was covered with ionizable silanol groups. In order to

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ensure that the surface of the capillary was fully and uniformly charged, it was necessary to pretreat it. Before analysis, the capillary was pretreated for 10 min with 0.1 *M* sodium hydroxide solution, 10 min with deionized water and 30 min with running buffer until equilibrium was reached.

2.2. *Power supply and electroosmotic flow*

In a normal CZE system, the capillary is filled with a background electrolyte solution and the capillary ends are immersed in separate reservoirs containing this solution. The detector is located near the cathode and the electroosmotic flow (EOF) direction is from the anode to the cathode. The EOF, as a result of an applied voltage along the capillary, is the net flow of buffer solution in the direction of the negative electrode. Usually, the EOF rate is so strong that all analytes, even those with a negative charge, move to the cathode. However, when the electrophoretic mobilities of some anions are higher than the EOF mobility of the electrolyte, these anions will escape detection because they move backwards. This problem could be solved by reversing the polarity of the applied electric field. However, the organic acids with an electrophoretic mobility equal to or lower than the EOF mobility will not be detected. One way to avoid this problem is to add an appropriate surfactant to the electrolyte in order to reverse the direction of the EOF by reversing the charge of the capillary wall. Accordingly, a negative-voltage power supply was used and 25 ml of an electroosmotic flow modifier (Waters OFM Anion-BT commercial solution) was added per litre.

2.3. *Injection mode*

At one capillary end the background electrolyte reservoir is replaced by the sample reservoir only during sample injection. Two injection modes are usually possible under our practical experimental conditions: hydrostatic injection or electrokinetic injection (electromigration). Some workers have remarked that electrokinetic injection involves biases [6,7]. When a voltage is applied to the capillary length during electrokinetic injection, a larger effective volume of faster ions than slower ions will be injected, because of the different mobilities of the species in the sample solution [8]. Contrary to electrokinetic injection, hydrostatic injection effected by gravity does not discriminate between the ions, and the same effective sample volume of each ion is injected. In CZE with hydrostatic injection, as in HPLC analysis, there always exists a defined injection volume. Accordingly, the hydrostatic injection mode was used.

2.4. *Injection time*

Selection of the injection time was based on the relative standard deviations for peak area, which were found to decrease with increasing injection time [5]. Further, as described previously [9,10], to obtain high resolution in capillary electrophoresis the volume of the injected sample must be small compared with the capillary volume. With a capillary that had a volume of about 3 μl (as here), 5–50 nl was the injection volume required to avoid distortion by overloading [9,10]. In this work, samples were introduced hydrostatically into the capillary for 30 s, and about 30 nl were injected.

2.5. *Samples*

Stock standard solutions of carboxylic acids were prepared at a concentration of 10 m*M*, using deionized water obtained with a Milli-Q system (Millipore). Before storage at 4°C, all solutions were sterilized by filtration through a 0.2-μm membrane.

3. Results and discussion

Optimization of the parameters was carried out by investigating the influence of electrolyte composition, pH and temperature on the mobilities. In accordance with the aims of this work, the initial selection of the background electrolyte was based on literature data ([11], Waters com-

munications) and on our experimentation. Electropherograms were obtained with a 4-hydroxybenzoate buffer, prepared with deionized water (Milli-Q), and adjusted to the appropriate pH with sodium hydroxide at 25°C. The electrolyte solution was filtered through a 0.45-μm membrane. This buffer and the OFM concentration (as noted under Experimental) were selected to optimize the separation of most organic anions, especially current organic acids from microbe metabolism.

The electrophoretic mobility of different acids and the EOF are partly dependent on the buffer viscosity. The viscosity is dependent on temperature; therefore, precise temperature control is important. As the temperature increases, the viscosity decreases, and both the EOF and electrophoretic mobility increase. The mobility of most ions increases by 2% per degree. Further, the pH of some buffers is known to be temperature sensitive. For all these reasons, the electrolyte buffer pH was adjusted to 25°C (controlled temperature). However, because we could not operate at an elevated controlled temperature (near 25°C), an alternative was to reduce the buffer concentration from 20 to about 5 m*M*. Thus, 4-hydroxybenzoate buffer was used at 5 m*M*. However, a decrease in the buffer concentration also reduces the peak efficiency by decreasing the focusing effect. At a fixed buffer concentration, variations of pH modify the focusing effect and also the measured peak areas. Fig. 1 shows the resulting effect of electrophoresis buffer pH on the peak areas of some acids. The response depends on the species studied. However, in general, when the buffer pH increases from 4 to 6, the corrected peak areas also increase.

Fig. 2 shows the resulting electropherograms for an organic acid mixture. The effect of electrolyte pH on organic acid separation was tested. In order to make an accurate comparison between different analytical conditions, each electrolyte pH must be adjusted at a fixed temperature (25°C under our conditions). The net electric charge of an organic anion is pH dependent, and the selectivity of the separation is affected by the buffer pH. Indeed, when the molecular masses of the organic acids are approximately the same, acids with lower pK_a values are eluted faster than those with higher values. At a fixed buffer pH, species mobility is partly a function of the charge to mass ratio, which depends on the pK_a values. Table 1 gives the pK_a values of eleven organic acids and their charge to mass ratio. In Fig. 2a, it is apparent that most acid standards cannot be separated with an electrolyte pH of 7.25 at 25°C; only gallic acid was separated at this buffer pH. A better separation can be achieved by decreasing the pH of the

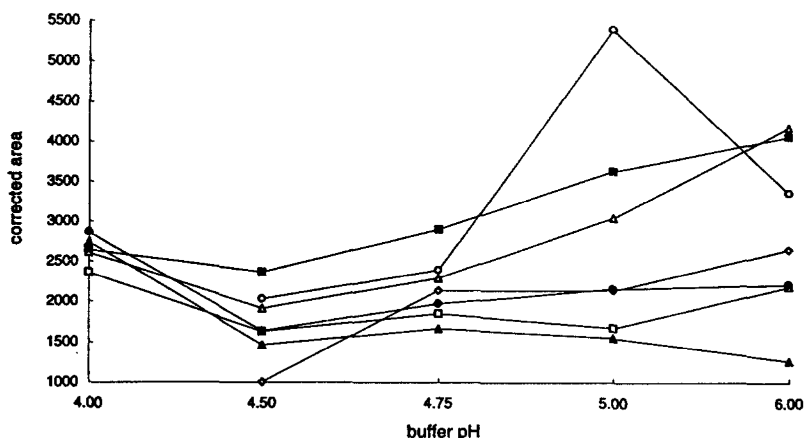
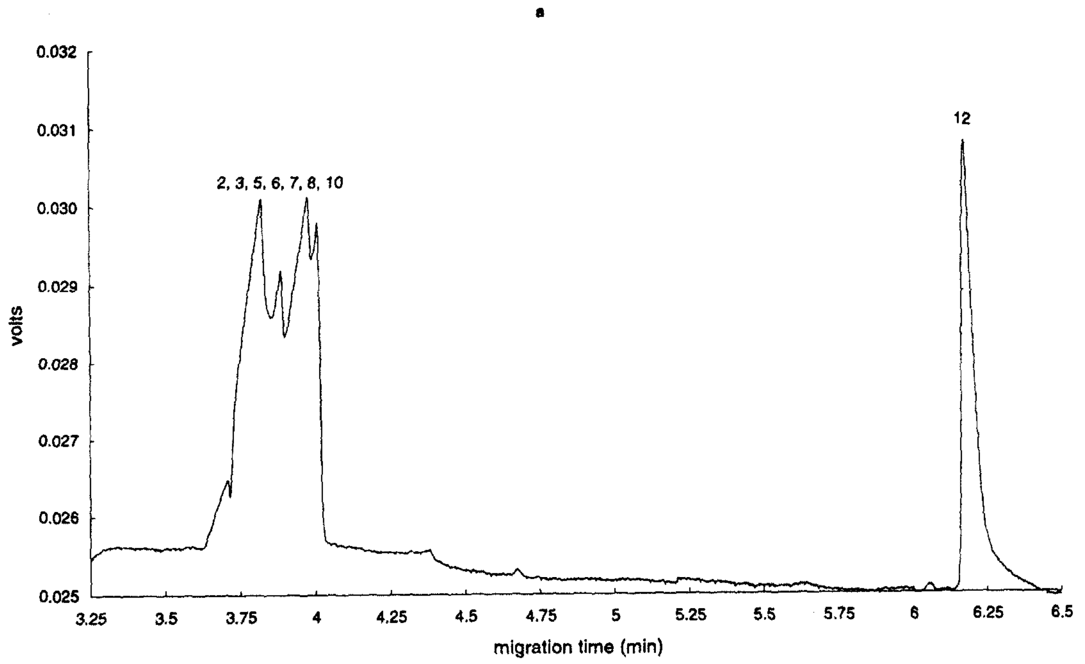


Fig. 1. Effect of buffer pH on measured peak areas which depend on the focusing effect. Each pH was adjusted at 25°C, after addition of 2.5% (v/v) OFM. Analytes: ▲ = Formic acid (0.1); ■ = fumaric acid (0.1); ○ = citric acid (0.2); ◇ = L-malic acid (0.1); □ = D-lactic acid (0.1); △ = succinic acid (0.1); ● = D-gluconic acid (0.1 m*M*).



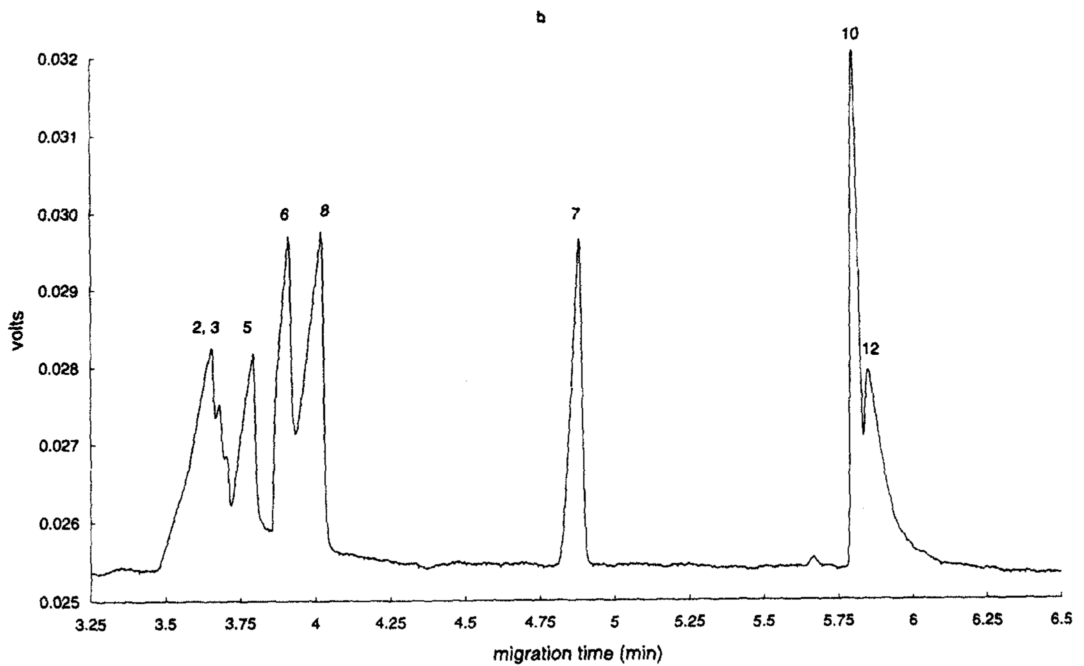


Fig. 2.

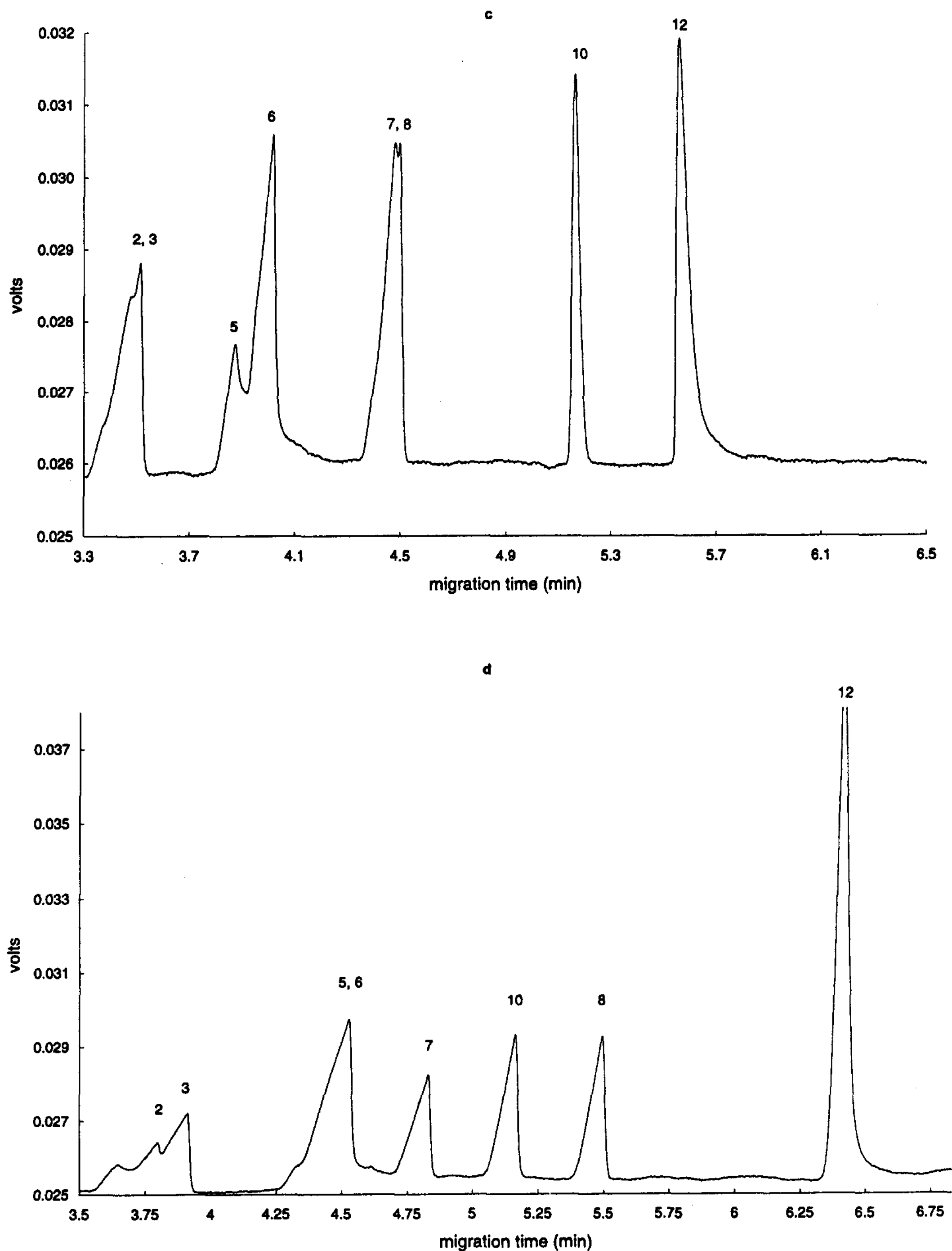


Fig. 2. Separation of eight-component organic acid mixture. pH of electrolyte: (a) 7.25 at 25°C, with 2.5% (v/v) OFM; (b) 6.00 at 25°C, with 2.5% (v/v) OFM; (c) 5.00 at 25°C, with 2.5% (v/v) OFM; (d) 4.00 at 25°C, with 2.5% (v/v) OFM. Peaks: 2 = formic acid (0.1); 3 = fumaric acid (0.1); 5 = citric acid (0.2); 6 = L-malic acid (0.1); 7 = D-lactic acid (0.1); 8 = succinic acid (0.1); 10 = D-gluconic acid (0.1); 12 = gallic acid (0.5 m*M*). Absorbance was measured at 254 nm.

Table 1
pK_a values of sixteen organic acids, and their relative charge to mass ratio at pH 4.75 at 25°C

Compound	M_r	pK_a at 25°C	Charge/mass ratio
Oxalic acid	90.04	$pK_1 = 1.27$, $pK_2 = 4.275$	0.0222
Formic acid	46.02	$pK = 3.75$	0.0217
Fumaric acid	116.07	$pK_1 = 2.03$, $pK_2 = 4.54$	0.0173
Pyruvic acid	88.1	$pK = 2.49$	0.0113
Maleic acid	116.07	$pK_1 = 1.97$, $pK_2 = 6.24$	0.0086
L-Malic acid	134.09	$pK_1 = 3.40$, $pK_2 = 5.13$	0.0074
Citric acid	192.12	$pK_1 = 3.13$, $pK_2 = 4.76$, $pK_3 = 6.39$	0.0052
D-Lactic acid	90.08	$pK = 3.83$	0.0111
Succinic acid	118.09	$pK_1 = 4.21$, $pK_2 = 5.64$	0.0084
DL-Aspartic acid	133.1	$pK_1 = 1.99$, $pK_2 = 3.90$	0.0150
D-Gluconic acid	196.16	$pK = 3.60$	0.0050
Acetic acid	60.05	$pK = 4.76$	0.0166
L-Ascorbic acid	176.12	$pK = 4.17$	0.0056
Shikimic acid	174.2	$pK = 4.21$	0.0057
Propionic acid	74.08	$pK = 4.87$	0
n-Butyric acid	80.1	$pK = 4.83$	0

electrolyte solution (Fig. 2b–d). The electric field strength (E V cm^{-1}) was constant with an applied voltage of −20 kV. At a fixed buffer concentration, when the buffer pH increases, the resulting current increases (Table 2). However, more current increases the heat produced, owing to Joule heating [12–14]. The heat is more or less readily dissipated across the silica capillary wall. Consequently, under our experimental conditions, when the pH increases the resolution decreases. A liquid cooling system [15] would be the most effective means of heat removal and thermal control. We might adjust the electrophoresis buffer pH in order to maintain both high resolution and a substantial detection level.

Fig. 3 shows the resulting electropherogram for an eighteen carboxylic acid mixture analysed

Table 2
Resulting current (μA) with different buffer pH, 5 m*M* 4-hydroxybenzoate and 2.5% (v/v) OFM, with an applied voltage of −20 kV

Buffer pH	Current (μA)	Buffer pH	Current (μA)
4.00	3.8	6.00	6.1
4.50	4.1	7.25	7.0
4.75	4.8	8.50	7.4
5.00	5.2		

with an electrolyte at pH 4.75; complete separation was obtained. The differences in species mobilities are greatly influenced by the pH of the electrolyte solution. Table 3 gives the electrophoretic mobilities [apparent migration rate = $(L_d/t)/(V/L_t)$] of different organic acids separated in Fig. 3. The actual mobility (electroosmotic mobility) of a species is the sum of the EOF rate and the electrophoretic rate and is expressed in 10^{-4} cm^2 s^{-1} V^{-1}. The EOF rate is determined by measuring the migration time of a neutral marker molecule such as water. Therefore, to perform reproducible analyses, the EOF must be carefully controlled.

Some organic acids have very similar structures or the same electrophoretic behaviour, and their mobilities are not sufficiently different to allow good resolution. Under the above-defined analytical conditions, citrate and isocitrate migrate together, as do fumarate and tartrate. The relative mobility of ions can be influenced by changing their charge state through selective complexation. This principle has been used by different workers [16–18]. To optimize the separation of the fumarate–tartrate and citrate–isocitrate pairs, cationic complexation has been found to be effective. Fig. 4 shows the resulting effect of addition of calcium chloride to the

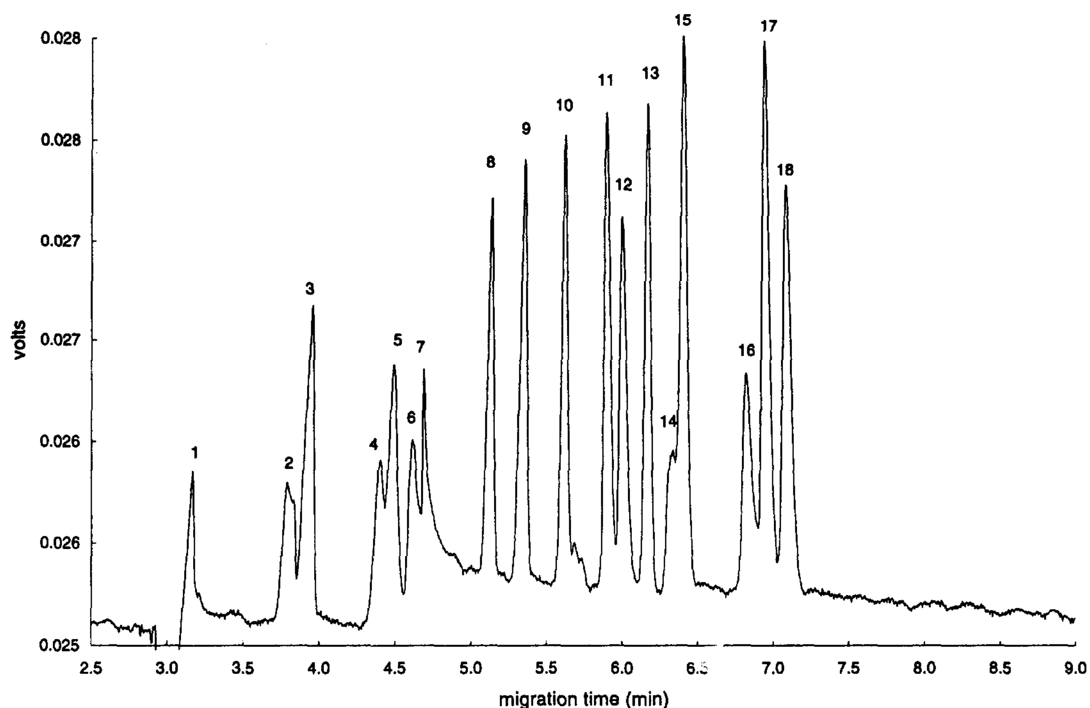


Fig. 3. Capillary electropherogram of eighteen-component organic acid mixture. 4-Hydroxybenzoate buffer at pH 4.75 at 25°C, with 2.5% (v/v) OFM. Peaks: 1 = oxalic acid (0.2); 2 = formic acid (0.1); 3 = fumaric acid (0.1); 4 = pyruvic acid (0.15); 5 = maleic acid (0.1); 6 = L-malic acid (0.1); 7 = citric acid (0.25); 8 = D-lactic acid (0.1); 9 = succinic acid (0.1); 10 = DL-aspartic acid (0.1); 11 = D-glucuronic acid (0.1); 12 = D-gluconic acid (0.1); 13 = acetic acid (0.1); 14 = L-ascorbic acid (0.1); 15 = shikimic acid (0.1); 16 = gallic acid (0.2); 17 = propionic acid (0.1); 18 = *n*-butyric acid (0.1 m*M*).

Table 3

Calculated electrophoretic mobilities of eighteen organic acids under the following conditions: 5 m*M* 4-hydroxybenzoate solution containing 2.5% (v/v) OFM, adjusted to pH 4.75 at 25°C, with an applied voltage of −20 kV

Compound	Electrophoretic mobility (10^{-4} cm^2 s^{-1} V^{-1})	Compound	Electrophoretic mobility (10^{-4} cm^2 s^{-1} V^{-1})
Oxalic acid	8.26	DL-Aspartic acid	4.65
Formic acid	6.91	D-Glucuronic acid	4.44
Fumaric acid	6.61	D-Gluconic acid	4.36
Pyruvic acid	5.94	Acetic acid	4.24
Maleic acid	5.82	L-Ascorbic acid	4.14
L-Malic acid	5.67	Shikimic acid	4.09
Citric acid	5.58	Gallic acid	3.84
D-Lactic acid	5.09	Propionic acid	3.77
Succinic acid	4.88	*n*-Butyric acid	3.69

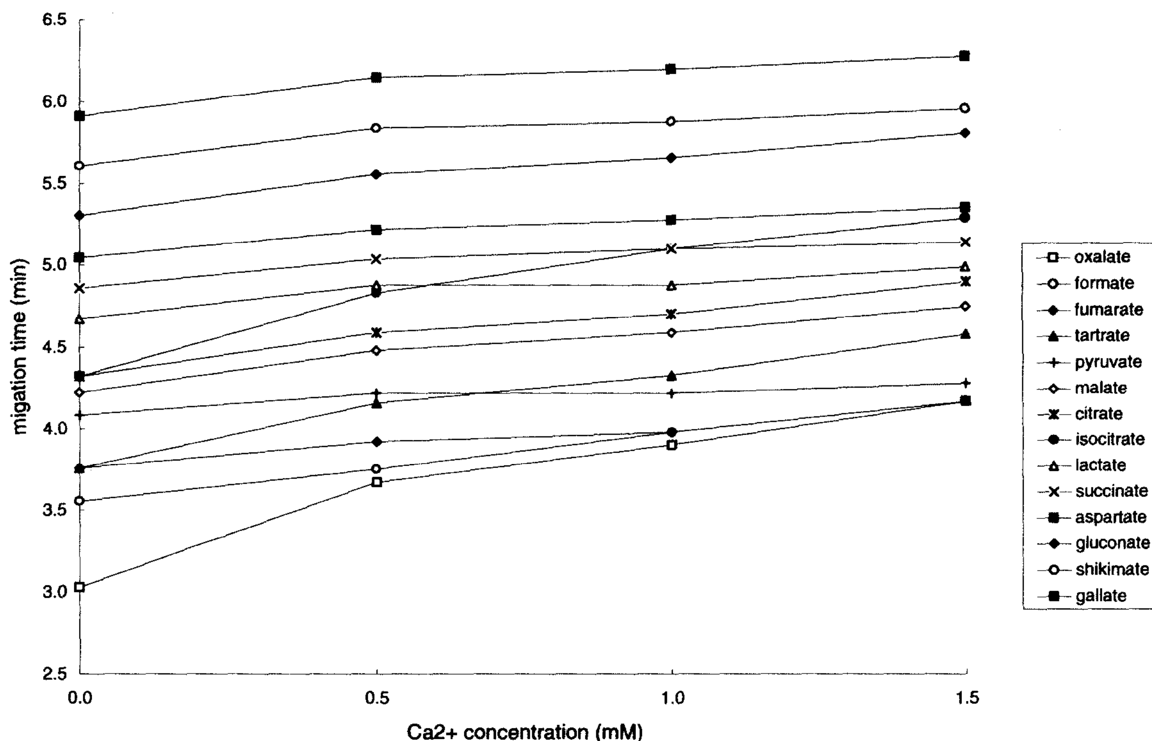


Fig. 4. Effect of Ca^{2+} concentration (calcium chloride addition) on the separation of organic acids. Conditions as in Fig. 3, except for the addition of calcium.

electrolyte buffer on the separation of fourteen organic acids. The buffer pH was adjusted to 4.75 after addition of calcium chloride. With 0.5–1.5 m*M* Ca^{2+} ($CaCl_2 \cdot 6H_2O$) added to the buffer, all organic acids are more or less retarded. Only oxalate, tartrate and isocitrate are significantly more retarded than the other acids tested. As shown in Fig. 4, a more effective separation was obtained with about 0.4 m*M* Ca^{2+}. The same results, for the separation of tartrate have been reported by Lalljie et al. [5] in work on the determination of organic acids in sugar refinery juices.

The separation and determination of oxalic acid are too difficult, if not impossible, using this method because the small oxalic acid signal could be partly or even totally obscured by the response of the accompanying inorganic anions such as chloride, sulfate, nitrite and nitrate, in a more complex medium than a standard acid mixture. This problem can be solved by using another electrolyte buffer; separation between inorganic anions and oxalic acid is obtained with 5 m*M* chromate buffer at pH 6.0 (results not shown).

4. Conclusions

Analysis performed with 0.4 m*M* Ca^{2+} and 2.5% (v/v) OFM added to 4-hydroxybenzoate buffer adjusted to pH 4.75 at 25°C gave optimum separation between all the organic acids tested.

For organic acids from plant or fungal metabolisms, the proposed method using CZE provides good separation in a relatively short time, with a limited amount of sample. However, a considerable amount of time may have to be spent carefully selecting buffers, additives and analysis conditions in order to identify other additional species. From the separations reported here, it is apparent that CZE will become

a powerful complement to HPLC for the analysis of complex mixtures and the separation of organic acids from plant or microbe metabolism.

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