citive current at the ensemble ($i_{c,u}$) is given by $i_{c,u} = \nu C_d A_a$, where A_a is the active electrode area (2).

Because A_a will always be less than A_g, $i_{c,u}$ will always be less than $i_{c,m}$. The theoretically expected improvement in detection limit at the ensemble can be calculated by dividing $i_{c,u}$ by $i_{c,m}$; the quotient is just the ratio of the active to geometric areas or the fractional electrode area calculated via eq 5. Thus, Table I suggests that the 3UME, 8UME, and 13UME should yield detection limits which are 0.09, 0.06, and 0.08 times the detection limit at the analogous macrosized electrode.

Figure 6 compares the voltammogram obtained at 3UME, for a dilute $Fe(CN)_6^{4-}$ solution, with the analogous voltammogram obtained at the macrosized electrode. The dashed lines in Figure 6 are the experimental background currents obtained at each electrode. In agreement with the above analysis background, currents at 3UME are lower, and as a result the signal to background ratio is higher. (The difference in current sensitivities in parts A and B of Figure 6 results because the preponderance of current at the macroelectrode is capacitive.) Figure 7 compares the $Fe(CN)_6^{4-}$ calibration curve obtained at 3UME with the analogous curve for the macrosized electrode; as anticipated, 3UME shows a lower detection limit.

To obtain a quantitative value for the magnitude of the improvement in detection limit, we define detection limit as that concentration that gives a faradaic signal which is equal to the background current. (Note that since we will ratio the detection limits at the electrodes, other definitions would yield identical results.) The detection limits obtained are 44 and 474 nM for 3UME and the macrosized electrode, respectively. The ratio of these detection limits is 0.09, which is identical with the anticipated ratio (vide supra and Table I).

CONCLUSIONS

We are currently investigating the electrochemical responses of ensembles prepared from membranes which were custom made for us by the Poretics Corp. The pore diameters and densities were tailored such that these membranes should yield ensembles with detection limits which are as much as 3 orders of magnitude lower than detection limits at conventional electrodes. We will report the results of these investigations soon.

Registry No. C, 7440-44-0.

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Quantitative Analysis of Low Molecular Weight Carboxylic Acids by Capillary Zone Electrophoresis/Conductivity Detection

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Low molecular weight carboxylic acids are separated and quantitated by capillary zone electrophoresis (CZE) with an on-column conductivity detector. The addition of 0.2–0.5 mM TTAB (tetradecyltrimethylammonium bromide) controls the electroosmotic flow so that all carboxylate anions pass through the detector. Unlike other CZE detection methods, conductivity detection shows a direct relationship between retention time and peak area. This confers on conductivity detection, in CZE, the unique advantage that use of an internal standard allows accurate determination of absolute concentrations in a mixture without separate calibration of response for each component.

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INTRODUCTION

Aqueous solutions of low molecular weight carboxylic acids have a significant environmental and biological importance from groundwaters to partially fermented juices and other biological fluids. Gas chromatography/mass spectrometry is the most widely used analytical technique in which these polar molecules are first derivatized to increase their volatility and then separated by gas chromatography followed by mass spectrometric analysis (*1*). However, preparation of the sample can be elaborate and time-consuming. Consequently, liquid chromatography (*2*) employing absorption, conductivity, or fluorescence detection is often preferred, especially that of ion-exchange chromatography (*3*). An alternative procedure is to use isotachophoresis in which the sample is introduced between leading and terminating electrolytes and separated by electrophoresis. An excellent review of this procedure for

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the analysis of mixtures of aqueous carboxylic acids has been given in 1985 by Boček et al. (*4*). More recently, Barth (*5*) has described the use of isotachophoresis in the quantitative determination of low molecular weight carboxylic acids in formation waters.

A variant of this procedure is the use of capillary zone electrophoresis (CZE) in which a narrow band of the sample is introduced into the capillary column and then subjected to electrokinetic separation (*6–8*). Examples of this are the work of Mikkers, Verheggen, and Everaerts (*9*) who reported the separation of malonate, adipate, acetate, propanoate, β-chloropropanoate, and benzoate as part of a 16-component sample, the work of Foret et al. (*10*) reporting the separation of formate, maleate, and glycolate, Hjertên et al. (*11*) presenting the separation of formate, acetate, propanoate, and 1-butanoate, and the work of Tsuda (*12*) reporting the separation of formate and acetate.

We describe here a detailed study of the use of CZE for separating many different low molecular weight carboxylic acids. We use on-column conductivity detection (*13*) because this technique is suitable to the detection of low molecular weight carboxylic acids. Such carboxylic acids are strongly charged in aqueous solution at suitable pH values and have large mobilities. In general, these species are not readily detected by UV absorption.

Conductivity detection has a further advantage. By use of an internal standard we demonstrate that it is possible to make the quantitation of the components in a mixture on an absolute basis without reference to a detection response curve for each component. We illustrate this advantage by quantitating the concentration of lactate in yogurt.

EXPERIMENTAL SECTION

Instrumentation. The capillary zone electrophoresis system with an on-column conductivity detector is home-built. This apparatus has been fully described elsewhere (*13*). We use a polyimide-clad fused silica capillary 40–70 cm in length with an inside diameter of 75 μm (Polymicro Technology, Inc., Phoenix, AZ, and Scientific Glass Engineering, Inc., Austin, TX). A high-voltage power supply (0–30 kV with a reversible polarity output) is used (Hipotronics, Inc., Brewster, NY). Sample injection is by gravity. The capillary inlet is lifted 10 cm higher than the capillary outlet for 10 or 20 s. We prefer hydrostatic injection to electrokinetic injection for quantitative studies in order to avoid bias problems associated with the latter injection method (*14*).

Reagents and Samples. Reagent grade carboxylic acids (Aldrich and Sigma) were used as received. All solutions were prepared from distilled, deionized water (Model LD-2A coupled with a Mega-Pure Automatic Distiller, Corning Glassworks). In some cases, methanol (Chromar, HPLC grade) was added. Stock solutions of carboxylic acids were prepared at a concentration of 10 mM. Electrolyte solutions containing 2-morpholinoethanesulfonic acid (MES) or tris(hydroxymethyl)aminomethane (TRIS) were purchased from Sigma and BRL, respectively, and used without further purification. The electrolyte solutions were filtered through a 0.2-μm membrane (Acrodisc, Gelman Sciences, Inc., Ann Arbor, MI) and in some cases the surfactant tetradecyltrimethylammonium bromide (TTAB, Sigma) was added. The pH of the electrolyte is adjusted as needed.

Wine samples consisted of two types, a white wine (Geyser Peak Chardonnay) and a red wine (Charles Krug Cabernet Sauvignon). They were used as received except for dilution in electrolyte by a factor of 10–100.

Locally purchased plain, low-fat yogurt (Yoplait) was deproteinized by using acetonitrile with centrifugation and diluted with running buffer (10 mM MES/His, pH 6, 0.5 mM TTAB) to a series of final concentrations.

RESULTS AND DISCUSSION

Control of Electroosmotic Flow. In a typical CZE system, the detector is located near the cathode and the electroosmotic flow direction is from the anode to the cathode.

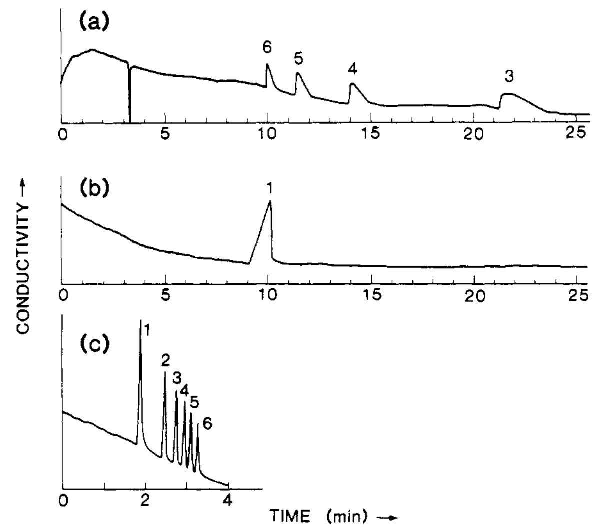

Figure 1. Electropherograms of a six-component mixture of carboxylic acids: 1, formate; 2, acetate; 3, propanoate; 4, butanoate; 5, pentanoate; 6, hexanoate. The conditions are 20 kV was applied to a 75 μm i.d. capillary, 42 cm long, 40 cm to the detector. The electrolyte was 10 mM MES/His at pH 5.9. (a) "Standard" CZE separation; (b) CZE separation with reversed electric field; (c) CZE separation as in b with 0.5 mM TTAB added to the electrolyte. The concentration of all sample components was 5 × 10^{-4} M.

Usually, the electroosmotic flow rate is so strong that all analytes, even those with a negative charge, move past the detector to the cathode. However, when the electrophoretic mobilities of some anions are higher than the electroosmotic flow mobility of the bulk electrolyte, these anions will escape detection. The electrophoretic mobilities of some small or multicharged carboxylic acids under conditions where they are mostly ionized (pH $\gg$ pK_a) are often higher than the electroosmotic flow mobility. For example, the electrophoretic mobility of formate ion is 5.7 × 10^{-4} $cm^2\ s^{-1}\ V^{-1}$ whereas the electroosmotic flow mobility of the electrolyte MES/His, 10 mM, pH 6.0, is only about 4.2 × 10^{-4} $cm^2\ s^{-1}\ V^{-1}$. This means that under normal conditions the formate ion moves toward the anode and is not detected. To illustrate this effect, we injected a six-component mixture of carboxylic acids and made a "standard" CZE separation (Figure 1a). We observe only four broadened peaks, corresponding to hexanoate, pentanoate, butanoate, and propanoate. Under these conditions, both formate and acetate escape detection. Actually, the acetate anion has nearly the same flow rate as the electrolyte, but their directions are opposed.

It might be thought that this problem could be solved simply by reversing the polarity of the applied electric field. Figure 1b shows the resulting electropherogram of the same six-component sample. It is apparent that only the formate anion is detected and its peak is badly broadened. The other carboxylic acids are not detected because their electrophoretic mobilities are equal to or lower than the electroosmotic flow mobility.

However, this problem can be overcome by simultaneously reversing the polarity of the applied electric field and the intrinsic direction of the electroosmotic flow. One way to accomplish this is to add an appropriate surfactant to the electrolyte. Previously, both Terabe et al. (*15*) and Tsuda (*12*) have reported a reversed electroosmotic flow direction when cetyltrimethylammonium bromide (CTAB) is added. We followed a similar procedure by adding 0.5 mM TTAB to our electrolyte. Figure 1c shows the resulting electropherogram of the six-component carboxylic acid mixture. All carboxylic acids are fully resolved and their peaks are sharp. Moreover, the retention times are much less than in Figure 1a,b because the electrophoretic mobilities of the carboxylic acids and the

Table I. Relationship of Electroosmotic Mobility to TTAB Concentration

concn of TTAB, mM	electroosmotic mobility, 10^{-4} cm^2 s^{-1} V^{-1}
0.0	4.47
0.1	3.78
0.2	1.79
0.3	0.53
0.4	−0.30
0.5	−0.98
1.0	−2.19

Table II. Reproducibility Study of Retention Times and Peak Areas

	% coefficient of variation		
	absolute peak area	relative peak area	retention time
formate	7.0	2.5	0.9
acetate	6.6	2.3	1.6
propanoate	6.1	3.3	1.2
butanoate	8.3	4.6	1.3
pentanoate	6.2	2.5	1.4
hexanoate	6.9	4.3	1.4

electroosmotic flow mobility are in the same direction.

The electroosmotic flow direction and rate depend on the concentration of TTAB, as shown in Table I. Evidently, the TTAB attaches to the inner surface of the capillary and influences the electroosmotic flow in an opposite sense to that of the untreated surface. Fused silica has an excess of negative charges because of the ionization of the silanol groups. TTAB is a cationic surfactant. As Table I shows, with a sufficient concentration of TTAB in the electrolyte, the electroosmotic flow direction is actually reversed. In this case, as the electroosmotic flow rate increases, the retention time decreases but the resolution also decreases. Therefore, we find that there is an optimal concentration of TTAB (about 0.2–0.5 mM) for minimizing the retention time while maximizing the resolution.

Quantitation. Twelve consecutive runs of the six-component carboxylic acid mixture were made to examine the reproducibility of our measurements of peak areas and retention times. Table II summarizes the results. The coefficients of variation in the absolute peak areas are seen to be less than 9%. Some of this variation can be traced to lack of control of the time in the manual injection of the sample causing the injection of different amounts of the sample. However, the effect of this variation can be removed by computing relative peak areas normalized to the sum of the areas of all peaks. With this procedure, we find that the coefficients of variation of each component are less than 5%. The coefficients of variation for the retention times are less than 2%.

We have also investigated how the peak areas change with the concentration of the carboxylic acids. The good correlation ($r > 0.99$) indicates that this relationship is linear and suggests that this method can be employed for the quantitative analysis of a mixture containing carboxylic acids. In order to expand the low concentration range, Figure 2 was plotted as a log–log function. The concentration range shown in Figure 2 is from 2×10^{-5} to 1×10^{-3} M. If desired, this concentration range can be extended in both directions by changing the concentration of the background electrolyte. In this manner we can detect carboxylic acid components with concentrations as low as 1×10^{-6} M with a 2:1 signal-to-noise ratio. Figure 3 shows the electropherogram of seven straight-chain monocarboxylic acids at 1×10^{-6} M. The buffer solution is composed of 1 mM Cl^- adjusted with TRIS to pH 7.1. Because the conductivity of the carrier ion (Cl^-) in the background electrolyte exceeds that of the monocarboxylic acids, the signal response is negative going.

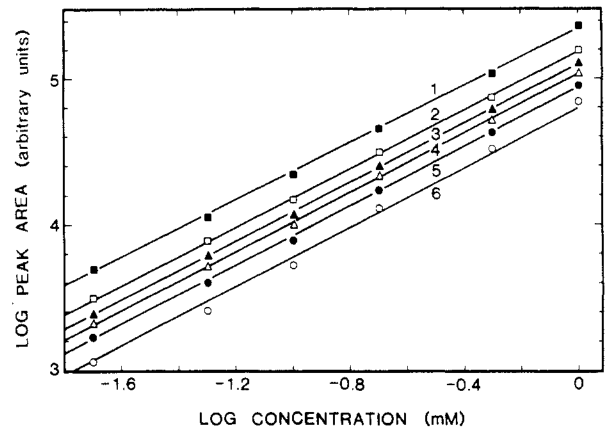

Figure 2. Plots of log (peak area) vs log (concentration) of carboxylic acids in a six-component mixture. Numbers correspond to those shown in Figure 1.

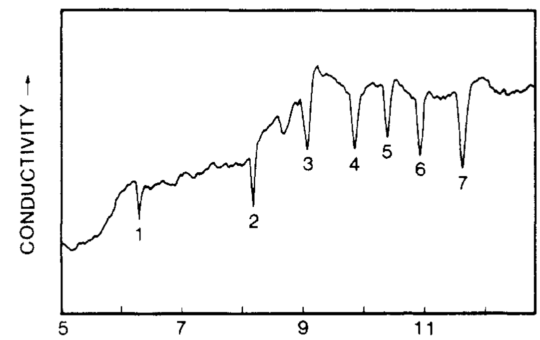

Figure 3. Electropherogram of a seven-component mixture of carboxylic acids: 1–6 correspond to those shown in Figure 1; 7, heptanoate.

Table III. Comparison of Calculated Mobilities with Literature Values (in 10^{-4} cm^2 s^{-1} V^{-1})

	experimental value	literature value (*15*)
formate	5.8	5.7
acetate	4.1	4.2
propanoate	3.5	3.7
butanoate	3.2	3.4
pentanoate	2.9	
hexanoate	2.7	

Measurement of Mobilities. The apparent migration rate of a species is the sum of the electroosmotic flow rate and the electrophoretic rate. The electroosmotic flow rate is readily determined by measuring the retention time of a neutral marker molecule, in our case, simply by detecting the water peak. This information may then be used to calculate the ionic mobility from the observed retention time of a species. Table III lists the calculated mobilities for the six carboxylic acids studied in Table II and it compares them to their literature values (*16*). The agreement gives us confidence that this method can be used to measure the mobility of many species that we can detect by our on-column conductivity system.

Use of an Internal Standard for Absolute Quantitation. The use of internal standards for quantitation in CZE was previously reported by Tsuda et al. (*17*) and by Fujiwara and Honda (*18*) using UV absorption. We present here the use of an internal standard in CZE with an on-column conductivity detector. The distinct advantage of conductivity detection is that the response of the detector is directly related to the ionic mobility of the species being detected. Since the

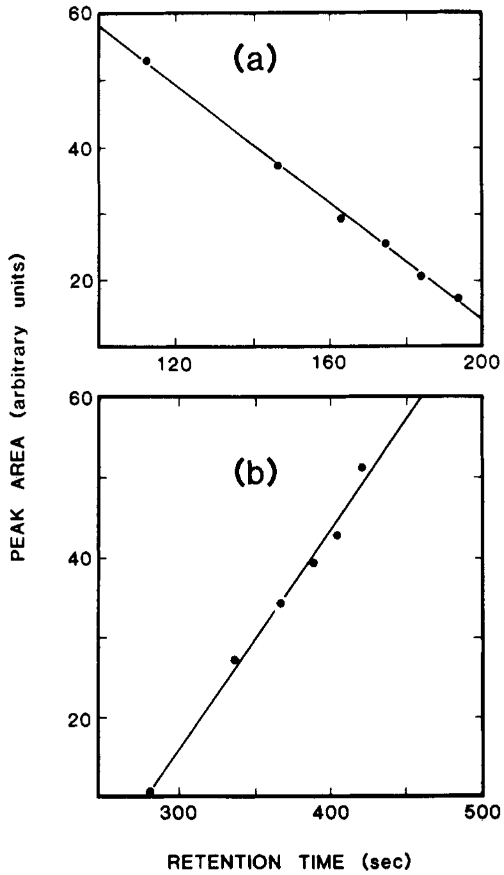

Figure 4. Plot of peak area vs retention time for six carboxylic acids. Numbers correspond to those shown in Figure 1. (a) In 10 mM MES/His buffer, pH 6, 0.5 mM TTAB; (b) in 5 mM Cl^- buffer adjusted to pH 7.1 with TRIS, 0.5 mM TTAB.

retention time of a species is also related to the ionic mobility, we should expect that the peak area correlates with the retention time. This relationship was explored for the six different carboxylic acids studied with two different electrolyte systems: (1) with a carrier ion (MES) whose mobility is lower than the mobilities of all six carboxylic acids, and (2) with a carrier ion (Cl^-) whose mobility is higher than all six carboxylic acids. We find a correlation coefficient of $r > 0.98$ in both cases (see Figure 4). Thus, this confirms the possibility of using the response from one species at a known concentration—an internal standard—to calibrate the response of all others present in a mixture on an absolute basis. This conclusion has important practical consequences; it shows that conductivity detection in CZE may be superior for many purposes compared to other detection systems which need to be calibrated for each species detected.

An example is the quantitation of carboxylic acids in dairy products. In particular, we have examined lactate in yogurt (Yoplait, plain, low-fat) using both an external standard, that is, a calibration curve (based on the detector response to varying lactate concentrations) and an internal standard. For the latter, we chose butanoate. The yogurt was deproteinized and serially diluted from 1:39 to 1:639. Then aliquots were mixed with equal volumes of 1.0 mM butanoate, leading to a final dilution of 1:79 to 1:1279 for the yogurt sample and 0.5 mM butanoate for all samples.

The range of peak area ratios, of lactate to butanoate, varies from 3.5 to 0.22 using the MES/His buffer with 0.5 mM TTAB (pH 6.0). The concentration of lactate can be calculated by

$$[\text{lactate}] = [\text{butanoate}]\frac{A(\text{lactate})}{A(\text{butanoate})}\,\frac{t(\text{lactate})}{t(\text{butanoate})}$$

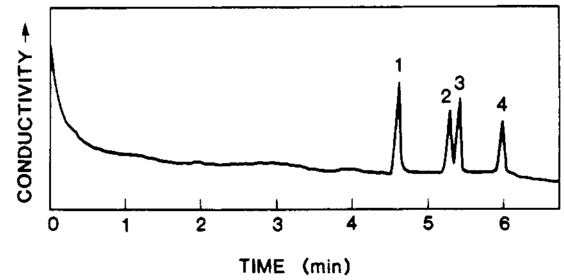

Figure 5. Electropherogram of a four-component mixture of carboxylic acids: 1, fumarate; 2, methylene succinate; 3, maleate; and 4, methyl maleate. The conditions are 25 kV was applied to a 75 μm i.d. capillary, 67 cm long, 66 cm to the detector. The electrolyte was 10 mM MES/His containing 0.5 mM TTAB. The concentration of all sample components was 5×10^{-5} M.

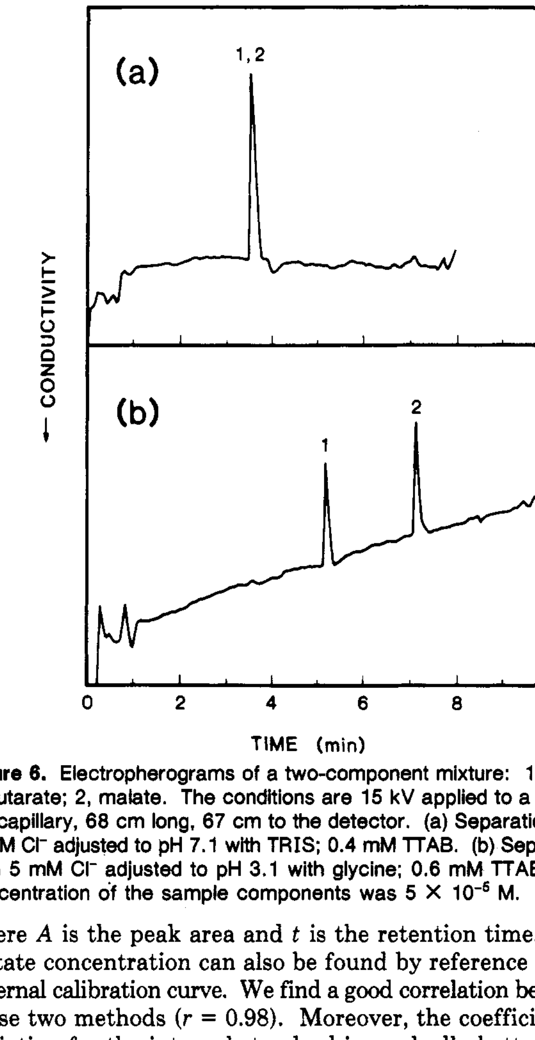

Figure 6. Electropherograms of a two-component mixture: 1, α-ketoglutarate; 2, malate. The conditions are 15 kV applied to a 75 μm i.d. capillary, 68 cm long, 67 cm to the detector. (a) Separation with 5 mM Cl^- adjusted to pH 7.1 with TRIS; 0.4 mM TTAB. (b) Separation with 5 mM Cl^- adjusted to pH 3.1 with glycine; 0.6 mM TTAB. The concentration of the sample components was 5×10^{-5} M.

where A is the peak area and t is the retention time. The lactate concentration can also be found by reference to the external calibration curve. We find a good correlation between these two methods ($r = 0.98$). Moreover, the coefficient of variation for the internal standard is markedly better than the external standard because errors introduced by the variation in the injection volume do not affect the results based on the use of an internal standard. Further improvements are possible, such as employing more than one internal standard.

Strategies for Separating Similar Carboxylic Acids. Some carboxylic acids have very similar structures, but the mobilities, under suitable conditions, are sufficiently different to allow full resolution. An example is the four unsaturated dicarboxylic acids, fumaric and maleic, cis and trans isomers of $HO_2CCH{=}CHCO_2H$, and their related acids, methylenesuccinic (itaconic) acid ($CH_2{=}C(CO_2H)CH_2CO_2H$), and methylmaleic (citraconic) acid ($HO_2CC(CH_3){=}CHCO_2H$). As Figure 5 shows, base-line resolution is achieved by using 25 kV applied to a 75 μm i.d. capillary, 67 cm long, 66 cm to the

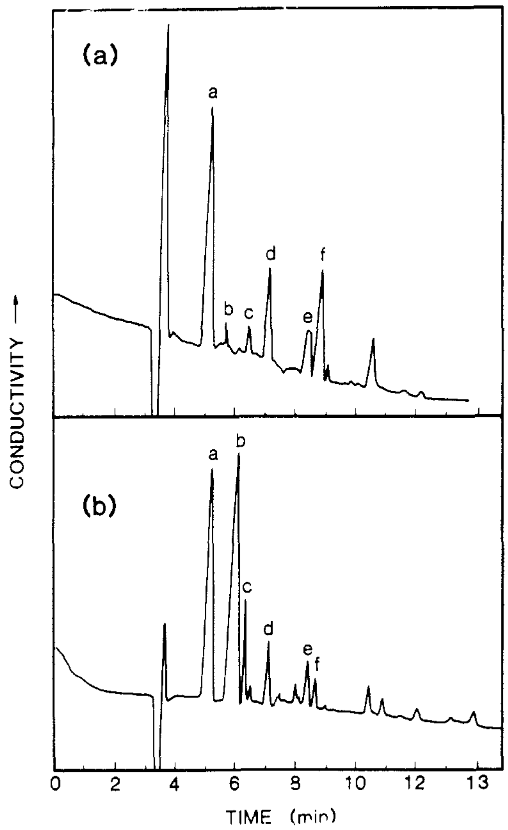

Figure 7. Electropherograms of wine: (a) Cabernet Sauvignon; (b) Chardonnay. Each sample is diluted 1:39 with 7 mM MES/His (pH 6) electrolyte containing 0.5 mM TTAB and 30% methanol to aid separation. The peaks are as follows: a, tartarate; b, malate; c, citrate; d, succinate; e, acetate; f, lactate. Capillary dimensions and run voltage are the same as shown in Figure 5. Reprinted with permission from Gordon, M. J.; Huang, X., Pentoney, S. L., Jr.; Zare, R. N. *Science* **1988**, *242*, 224–228. Copyright 1988 by the AAAS.

detector. The electrolyte was 10 mM MES/His containing 0.5 mM TTAB. The concentration of all sample components was 5×10^{-5} M. On the other hand, the resolution of some similar carboxylic acids proves to be a severe challenge.

Everaerts, Beckers, and Verheggen (*19*) have put forward three major methods for separating similar species in isotachophoresis. These are separation according to differences in mobility, separation according to different pK_a values, and separation according to interaction with different electrolytes. These ideas also apply to capillary zone electrophoresis.

For example, butanoate and pentanoate have almost the same pK_a values, 4.81 and 4.82, respectively (*20*). In order to separate them, a pH higher than the pK_a values is preferred. At high pH, both acids are almost completely ionized and the difference between mobilities can be utilized. As shown in Figure 1, when the pH is about 6, base-line resolution can be achieved. On the other hand, the mobilities of α-ketoglutarate ($HO_2CCH_2CH_2COCO_2H$) and malate ($HO_2CCH_2CHOHCO_2H$) are so close at a high pH, that it is very difficult to separate them. In this case, the difference between pK_a values should be utilized. At a low pH of about 3, the difference in the mobilities of the two carboxylic acids, whose un-ionized and ionized forms are in different ratios, is sufficiently large so that they can be resolved (see Figure 6).

The use of organic solvents is also helpful for some separations. At pH 6, malate and citrate ($HO_2CCH_2COHCO_2H$-CH_2CO_2H) are difficult to separate in aqueous media. However, with 30% (w/w) methanol in the electrolyte solution, complete separation can be achieved. We applied this method to two wine samples containing both malic and citric acids. Electropherograms of wine samples are shown in Figure 7. In this work, other electropherograms were run with the samples spiked with standards to confirm the identities of six of the most important organic acids as indicated by others using ion chromatography (*21–23*). As with any complex mixture, there is always the possibility that any single peak may represent the coelution of more than one component. This would occur when, in a given set of conditions, several species have almost the same electrophoretic mobility. It is usually possible to resolve the presence of more than one species from the original single peak by changing conditions, such as the pH and solvents. The differences in mobility are greatly influenced by pH. Additional peaks are clearly observable but no attempt was made to identify the unlabeled peaks. On the basis of the short time for analysis, the sensitivity, the ease of operation, and the ability to quantitate on an absolute basis using an internal standard, CZE with an on-column conductivity detector may be advantageous in a number of applications involving carboxylic acid mixtures.

Registry No. Formic acid, 64-18-6; acetic acid, 64-19-7; propanoic acid, 79-09-4; butanoic acid, 107-92-6; pentanoic acid, 109-52-4; hexanoic acid, 142-62-1; methylene succinic acid, 97-65-4; maleic acid, 110-16-7; methylmaleic acid, 498-23-7; α–ketoglutaric acid, 328-50-7; tartaric acid, 87-69-4; citric acid, 77-92-9; succinic acid, 110-15-6; lactic acid, 50-21-5.

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