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Capillary electrophoretic determination of organic acids with indirect detection

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Abstract

Methods for the determination of several organic acids commonly found in foods and beverages, including oxalic, citric, acetic, tartaric, malic, succinic, lactic, carbonic, aspartic, glutamic, ascorbic and gluconic acids, by capillary electrophoresis (CE) with indirect absorbance detection were developed. Several absorbance providers, including chromate, *p*-hydroxybenzoate, phthalate, terephthalate, trimellitate and pyromellitate, were investigated for their suitability as background electrolytes (BGEs). CE was performed in the negative voltage (reverse polarity, detector towards anode) mode. The effects of pH and various additives on CE separations were evaluated. The BGE and pH each played a major role in affecting the selectivity and resolution of CE. All analytes except malate and succinate could be baseline resolved in one run by performing CE with 5 m*M* trimellitate (as the BGE)–1 m*M* tetradecyltrimethylammonium bromide at pH 9.0 in less than 10 min. On the other hand, the CE separation of the tri- and dicarboxylic acids and hydroxydicarboxylic acids (the first five) could best be obtained at pH 5.5 in 5 min. The precision of the method for most monoprotic analytes is typically less than 1% for the migration time and 1–4% for the peak area ($n = 6$). The detection limit for most analytes is of the order of $2.0 \cdot 10^{-6}$ *M*. The new methods developed are rapid, sensitive and quantitative and can be readily applied to real food samples for quantitative analysis.

1. Introduction

Organic acids are commonly present in foods, beverages, medicines and a variety of samples of analytical interest. The simultaneous determination of several organic acid constituents in samples can best be accomplished by chromatographic techniques, e.g., gas chromatography (GC), ion chromatography (IC) or high-performance liquid chromatography (HPLC), as has been amply demonstrated in the past [1,2].

The determination of organic acids in food samples by capillary electrophoresis (CE) was first demonstrated [3] by using a commercial proprietary chemical reagent kit. The various advantages offered by the CE method include ease of sample preparation, low cost, automation and speed. A mixture of seven standards, including citric, tartaric, malic, succinic, acetic, lactic and butyric acids, can be separated in less than 15 min and detected (by the indirect UV absorbance method) at the 50 μg/ml level. Since not all analytes exhibit high UV absorptivity, the indirect absorbance method has been welcomed as a universal means for the detection of non-absorbing analytes. In the indirect absorbance method, an absorbing co-ion [background electrolyte (BGE)], which provides a background

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UV (or visible) absorbance, co-migrates with the mixture of analytes which do not absorb (at the wavelength that the detector was set for the BGE). When the analyte passes the detector, a negative absorbance peak appears. For analytes which do not absorb appreciably in the UV region, the indirect absorbance technique provides a universal detection scheme. Because for many analytes the indirect absorbance method affords good sensitivity, CE coupled with indirect UV absorbance detection has gained increasing popularity. CE methods have been successfully implemented for the determination of various organic acids in sugar refinery juices, fruit juices, foods and urine samples [4–7]. Various BGEs including chromate [4], benzoate [5], phthalate [6,7], 1,2,4-benzenetricarboxylate (trimellitate) [8,9], 1,2,4,5-benzenetetracarboxylate (pyromellitate) [10], 2,6-naphthalenedicarboxylate [11] and naphthalene mono-, di- and trisulfonates [12] have been investigated. Selectivity could be optimized to improve the CE resolution of organic acids via dynamic pH- and flow-gradient techniques [13]. The optimization of indirect UV detection and the injection technique for the CE of anions have also been addressed in several studies [14–21].

Previously, we have developed methods for the CE analysis of a mixture of twenty common amino acids with indirect absorbance detection [22]. In the present investigation, we employed the indirect detection method for the determination of anions, including acetate, lactate, succinate, malate, tartrate, glutamate, ascorbate, citrate, oxalate, gluconate, carbonate and aspartate, that are commonly found in foods and beverages. Several absorbance providers, including chromate, benzoate, *p*-hydroxybenzoate, phthalate, terephthalate, trimellitate and pyromellitate, were investigated for their suitability as background electrolytes (BGEs). CE could be performed by applying either positive voltage (towards the cathode) or reverse polarity; however, for the present analysis, we found that the reverse polarity mode gave better results. The effects exerted by pH and various additives on the selectivity and resolution of CE were evaluated. The new methods developed are rapid, sensitive and quantitative and can be readily applied to real food samples for the determination of these analytes.

2. Experimental

2.1. Chemicals

Various organic and inorganic acids and BGEs, as either free acids or sodium salts, and electroosmotic flow (EOF) modifiers, all of analytical or reagent grade, were obtained from several suppliers. Various brands of beverage and food samples were purchased from local market stores. Doubly deionized water prepared with a Milli-Q system (Millipore, Bedford, MA USA) or doubly deionized, distilled water was used exclusively to prepare all solutions.

2.2. Samples, buffers and pH adjustment

Solutions of oxalic, citric, tartaric, malic, succinic, carbonic, acetic, lactic, aspartic, glutamic, ascorbic and gluconic acids (or sodium salts) were prepared, each at 10^{-2} *M*. These solutions were mixed and diluted to 0.1 m*M* (pH adjusted to 7.0); the diluted mixture was used as the standard. Most real samples were diluted tenfold or more with 2 m*M* NaOH. After dilution, most real samples were near neutral, hence further pH adjustment was usually unnecessary. BGEs, including benzoic acid, *p*-hydroxybenzoic acid (PHBA), phthalic acid (PHA), terephthalic acid (TPA), trimellitic acid (TMA), pyromellitic acid (PMA) and sodium chromate, were prepared as 5 m*M* stock solutions; their pH values were adjusted to 5–10 with concentrated NaOH or HCl depending on the experiments. Cetyltrimethylammonium bromide (CTAB), dodecyltrimethylammonium bromide (DTAB), tetradecyltrimethylammonium bromide (TTAB) and diethylenetriamine (DETA) were employed as EOF modifiers and their concentrations were varied from 0.2 to 5 m*M*. The pH of the buffer was checked periodically and readjusted when necessary. All samples and buffers were filtered

through 0.2-μm membranes and degassed under vacuum for 10 min.

2.3. Electrophoretic procedures

CE experiments were carried out in a fully automated Spectra Phoresis Model 1000 instrument (Thermo Separation Products, Fremont, CA, USA) as described previously [23]. In most experiments, except in the electrophoretic mobility determination of BGEs, the detector wavelength was fixed at the optimum wavelength depending on the BGE used as specified in Table 1. In indirect detection, peaks in the electropherogram appear originally as negative peaks but are inverted to positive peaks by using the vendor's software. The separation capillaries (bare fused silica) from Polymicro Technologies (Phoenix, AZ, USA) were 75 μm I.D. (365 μm O.D.) × 43 cm (36 cm to the detector) for the determination of the mobilities of BGEs and 75 μm I.D. (365 μm O.D.) × 70 cm (63 cm to the detector) for the separation of analytes.

Procedures for capillary pretreatment, pre- and post-washings and a daily routine pre-washed sequence were similar to those reported previously [23]. Sample injection was effected in the hydrodynamic (HD) mode for 3 s. The separation run was carried out at −20 kV constant voltage (except in the mobility determination experiment, in which a positive voltage was applied), at 25°C constant temperature and with a current of ca. 20 μA. Between runs, the capillary was post-washed with deionized water for 5 min. Peak identification for each analyte was carried out by spiking with the known standard, and the peak with increased height was identified.

2.4. Electrophoretic mobility determination

The mobilities of various BGEs under the specified CE conditions were determined in buffer solution containing 10 m*M* sodium phosphate (pH 8.0). A mixture of all BGEs, 0.1 m*M* each in deionized water, containing 0.05% dimethyl sulfoxide (DMSO) as a neutral marker, was injected in the HD mode for 1 s. The CE voltage applied was +20 kV. Detection was effected by rapid scanning of absorbance from 200 to 350 nm, which allowed a positive identification of the background provider. The electroosmotic mobility, μ_{eo}, and the electrophoretic mobility of the BGE, μ_e, were calculated from the observed mobility, μ_{obs}, using the equations described previously [22].

3. Results and discussion

3.1. Selection of suitable BGE and EOF modifier

Among the twelve acid analytes of interest here, only ascorbic acid absorbs strongly in the UV region above 200 nm ($\varepsilon_{max} = 8.2 \cdot 10^3$ l mol^{-1} cm^{-1} at 254 nm). All other analytes have molar absorptivities from 25 to 180 l mol^{-1} cm^{-1} at 220 nm. When selecting a BGE suitable for CE, the mobility and the molar absorptivity of the BGE must both be taken into consideration. In theory, the BGE with a mobility matching those of the majority of the analytes would give a

Table 1
CE conditions for the various combinations of BGE–EOF modifier pairs

System	BGE	EOF modifier	pH	λ (nm)
Chromate	5 m*M* chromate	0.5 m*M* TTAB	8.0	265
PMA	2 m*M* PMA	2 m*M* DETA	9.0	220
TMA	5 m*M* TMA	1 m*M* TTAB	9.0	220
PHA	5m*M* PHA	0.5 m*M* TTAB	8.0	220
TPA	5 m*M* TPA	0.25 m*M* TTAB	9.0	240
Benzoate	5 m*M* benzoate	0.3 m*M* TTAB	8.0	220

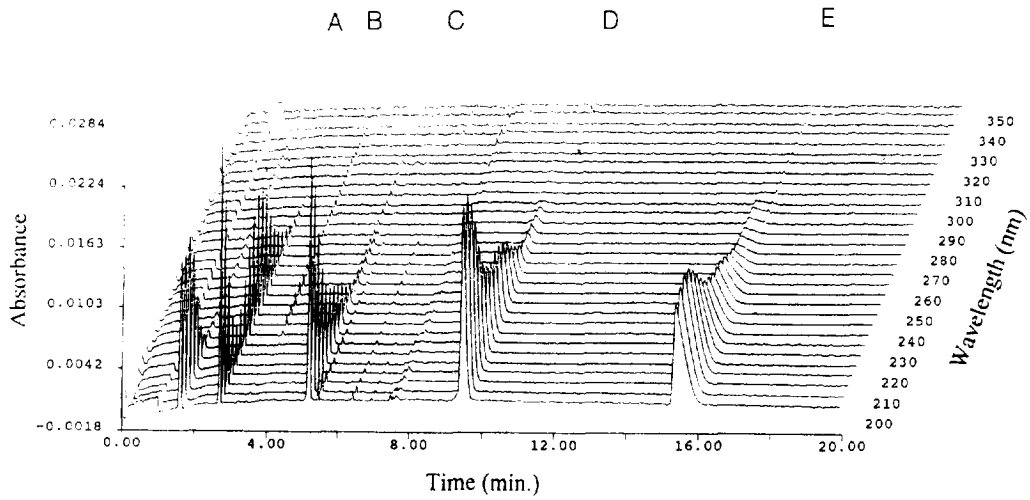

Fig. 1. Three-dimensional spectral view of CE separation of a mixture of four BGEs and the neutral marker. (A) DMSO; (B) PHBA; (C) PTA; (D) TMA; (E) PMA. Conditions as in the mobility determination (see Experimental for details).

better separation and resolution. The absorbance of the BGE should be high, and ideally should not overlap with those of the analytes. Fig. 1 shows a three-dimensional spectral scan of the CE of four selected BGEs to show their absorption spectral characteristics.

The electrophoretic mobilities for the various BGEs decreased in the following order: chromate > pyromellitate > trimellitate > terephthalate, phthalate > benzoate > *p*-hydroxybenzoate (Fig. 2, bottom). Chromate has the highest mobility, and is most suitable as the BGE for inorganic anions such as Cl^-, Br^-, NO_3^- and SO_4^{2-}. Its molar absorptivity is adequate in the region where the organic acids of interest are transparent. However, for the slower moving organic acids, performing CE using chromate as the BGE would result in poorer resolution and these acids would appear as trailing peaks. Other ionized BGEs carry charges from −1 (benzoate) to −4 (pyromellitate). Since their absorption characteristics are similar, they can be paired together to provide a wider range of mobilities that can match better with the mobilities of the present analytes (Fig. 2, top) Among the present analytes, tartaric, malic and succinic acids at alkaline pH have mobilities that are close to each other. On the other hand, the mobilities for the two amino acids, ascorbic and gluconic acid, are not significantly different at

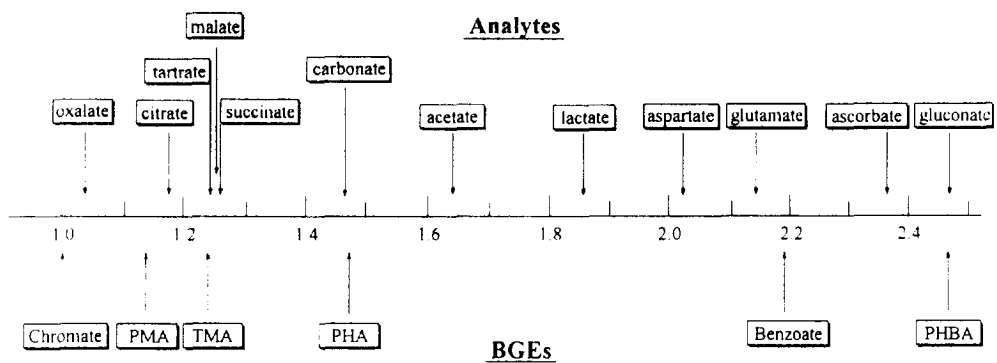

Fig. 2. Relative electrophoretic mobilities (with respect to chromate) of several BGEs and organic anions at pH 8.0.

acidic pH. Therefore, these analytes are most difficult to separate at the pH extremes. Trimellitate has a mobility that matches closest to those of the dicarboxylic acids; its transfer ratio is high, so it also offers better sensitivity [8]. Pyromellitate as the BGE would produce similar results to TMA. Phthalate, which has been a popular choice as a BGE in earlier studies, has a molar absorptivity that is not as high as that of PMA. Hence the sensitivity of phthalate is inferior to that of the latter. Terephthlate, although it has a higher molar absorptivity, suffers from a lack of resolving power for the analytes citrate, tartrate and malate.

In CE of anions, a negative voltage is applied and the analytes migrate towards the anode in the opposite direction to the EOF. The migration time of anions can be shortened significantly by decreasing the EOF with the addition of cationic surfactants (EOF modifiers), which neutralize the negative surface charges on the bare fused-silica capillary. Both TTAB and CTAB were fairly effective (see below), but TTAB gave slightly better results. DTAB could also be used, but it has a higher critical miscelle concentration and thus required a higher concentration to be effective, which caused a higher current. PMA formed white precipitates with cationic surfactants (e.g., TTAB). In this case, hexamethonium hydroxide [10] or diethylenetriamine [7] must be substituted as the EOF modifier to be paired with PMA. Among the various combinations of BGE–EOF modifier pairs investigated (Table 1), the combined use of TMA as the BGE and TTAB as the EOF modifier appears to give the best separation.

3.2. CE Separation of organic acids

Fig. 3a shows a typical electrophoregram of a mixture of the twelve organic acids (0.1 m*M* of each analyte) in 5 m*M* TMA–1 m*M* TTAB at pH 5.5. The faster migrating species, tri- and dicarboxylates, could well be separated in less than 6 min. However, at this pH, glutamate and ascorbate could not be baseline resolved and citrate appears as a smaller and broader peak. On the other hand, at alkaline pH (9.0), with other CE conditions being the same, all except the three dicarboxylic acids, tartrate, malate and succinate, can be baseline resolved in less than 9.5 min (Fig. 3b). At, pH 9.0 the migration times of the analytes increase in the following order: (1) oxalate, (2) citrate, (3) tartrate, (4) malate, (5) succinate, (6) carbonate, (7) acetate, (8) lactate, (9) aspartate, (10) glutamate, (11) ascorbate and (12) gluconate. Note that the order of migration times also changes for citric acid at the two pHs. At acidic pH, the uncharged carbonic acid migrates too slowly to appear in the electropherogram. The migration time of the ion is strongly dependent on the electric charges that it carries and its mass. The tri- and diprotic acids migrate faster than all other acids because they carry more negative charges than the other analytes. Albeit carrying more negative charge, citrate trails behind oxalate, presumably because of its higher mass (see Table 2). Tartrate migrates faster than malate, and the latter is faster than succinate, since malate and tartrate carry one and two hydroxyls, respectively, while succinate has none. The faster mobility may be attributed to the presence of hydroxyl groups. Among the monocarboxylic acids, carbonate, acetate and lactate migrate in ascending order in accordance to their mass. Being zwitterions, aspartate and glutamate trail behind owing to the positive charges carried on the amine group. Ascorbate and gluconate move the slowest because of their higher masses. Note that because the mobility of TMA (as the BGE) is between those of citrate and succinate, the peak shape of these analytes appears to be symmetrical. However, those analytes which move slower than TMA have trailing peaks.

3.3. Effect of pH

The electrophoretic mobility of acids is strongly dependent on the pH of the running buffer, as contrasted in Fig. 3a and b. Table 2 gives the pK_a values for the BGEs and the analytes of interest which serve as a guide for manipulating the extent of ionization of these analytes by adjusting the pH. The effect of pH on the relative migration time of the twelve acids is

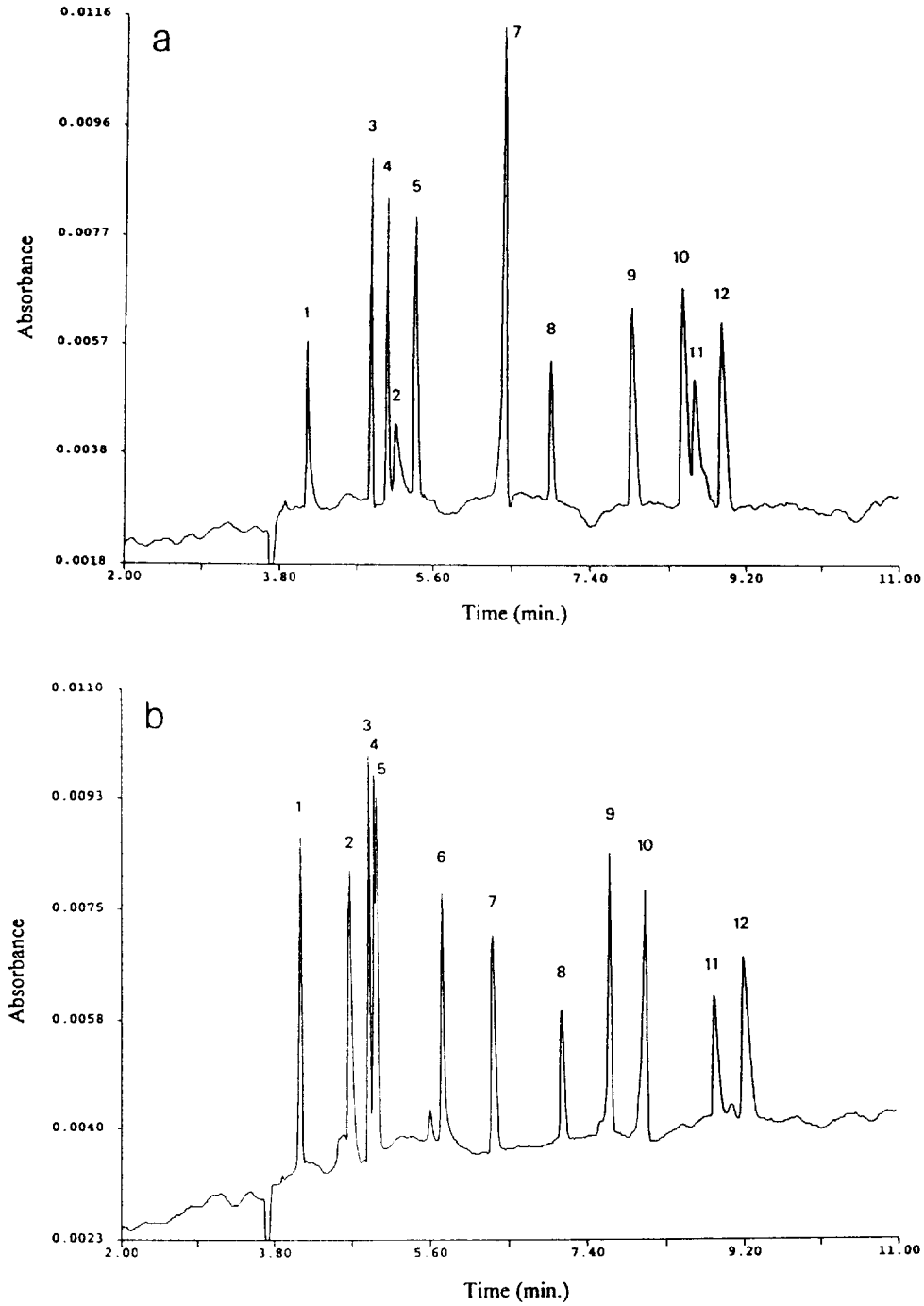

Fig. 3. Electropherograms of a mixture of the twelve anions (0.1 m*M* each) in 5 m*M* TMA–1 m*M* TTAB at (a) pH 5.5 and (b) pH 9.0. Migration order (peaks): 1 = oxalate; 2 = citrate; 3 = tartrate; 4 = malate; 5 = succinate; 6 = carbonate; 7 = acetate; 8 = lactate; 9 = aspartate; 10 = glutamate; 11 = ascorbate; 12 = gluconate.

Table 2
Molecular masses and pK_a values of the various BGEs and analytes

Acid	M_r	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}
Benzoic	122.1	4.19			
p-Hydroxybenzoic	138.1	4.48	9.32		
Phthalic	166.1	2.89	5.51		
Terephthalic	166.1	3.51	4.82		
Trimellitic[b]	210.1	2.42	3.71	5.01	
Pyromellitic[b]	254.2	1.87	2.72	4.30	5.52
Oxalic	90.1	1.23	4.19		
Citric	192.1	3.14	4.77	6.39	
Tartaric	150.1	2.98	4.34		
Malic	134.1	3.40	5.11		
Succinic	118.1	4.16	5.61		
Carbonic	62	6.35	10.33		
Acetic	60.1	4.76			
Lactic	90.1	3.08			
Aspartic	133.1	2.09	3.86	9.82	
Glutamic	147.1	2.19	4.25	9.67	
Ascorbic	176.1	4.10	11.79		
Gluconic	196.2	3.76			

[a] From Ref. [31], except where indicated.
[b] From Ref. [32].

shown in Fig. 4. At acidic pH, the pH affects the di- and triprotic acids most significantly. At pH 5.5, citric acid dissociates only two protons and migrates behind tartrate and malate but ahead of succinate because of its heavier mass (Fig. 3a). As the pH is raised above the pK_{a3} of citric acid, citrate carries an extra negative charge and migrates ahead of tartrate and malate. Thus, a better separation can be obtained at pH 5.5 for the fast-moving polyacids. On the other hand, at alkaline pH (9.0), a better resolution could be achieved for carbonate and the slower moving amino acids, ascorbic and gluconic acid.

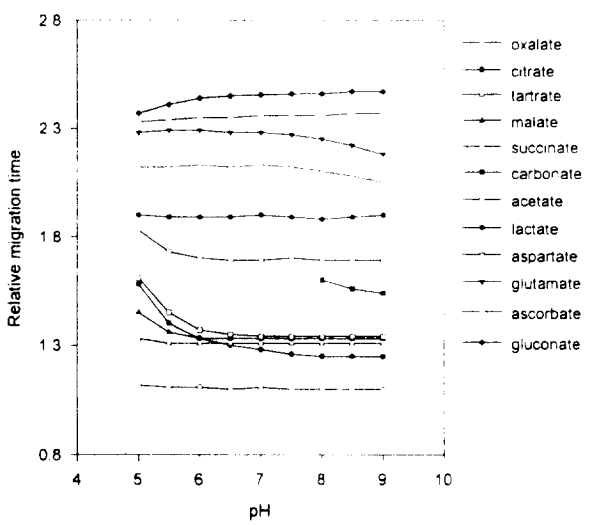

Fig. 4. Effect of pH on the relative migration times of the organic acids. Other conditions as in Fig. 3b.

3.4. Effects of EOF modifiers

Since in the CE of anions the analytes migrate towards the anode in the opposite direction to the EOF, adding cationic surfactants or alkylamines could neutralize the negative surface charges on the bare fused-silica capillary, thus reducing the EOF (as these compounds are often called EOF modifiers). Further, when enough modifiers are added to the running buffer, the direction of the EOF could be reversed, further facilitating the migration of anions. The migration time of anions can thus be shortened significantly. For TTAB, a concentration as low as

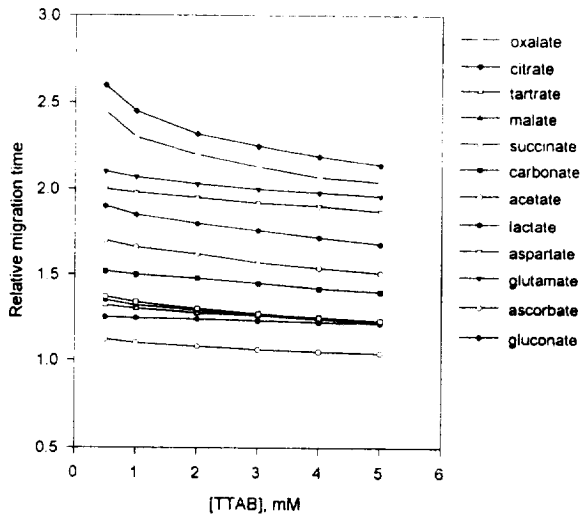

Fig. 5. Effect of TTAB concentration on the electrophoretic mobility of the organic acids in 5 m*M* TMA (pH 9.0). Other conditions as in Fig. 3b.

0.1 m*M* could reverse the EOF. On the other hand, when the concentration of the surfactant exceeds its critical micellar concentration (CMC), the effect of the surfactant concentration on modifying the EOF is less pronounced [22]. The effect of the concentration of TTAB from 0.5 to 5 m*M* on the relative migration time of the various analytes is shown in Fig. 5. It is apparent that when [TTAB] is above 2 m*M*, the separation for tartrate, malate and succinate become very poor. On the other hand, although at 0.5 m*M* [TTAB] the separation for these three anions is better, the relative migration time is much longer and the baseline starts to drift. Therefore, we chose 1 m*M* TTAB as a compromise. However, in samples in which ascorbate and gluconate are both absent, the CE conditions could be set at a lower pH and a lower concentration of EOF modifier to optimize the separation of the diprotic acids.

3.5. Precision and linearity

The precisions [expressed in terms of relative standard deviation (R.S.D.)] of the present method for the various analytes are summarized in Table 3. The R.S.D.s for the migration times are typically less than 0.3% for all except three analytes (<1%). The R.S.D.s for the peak areas typically are less than 3%, except for citrate (3.8%). The R.S.D.s for the peak heights are slightly better (about 2%), except for citrate (5.4%). The linearity of the present method was investigated by analysing standard solutions containing a mixture of twelve analytes with known concentrations ranging from $1.0 \cdot 10^{-5}$ *M* to 1.0 m*M*. From the electropherograms, either the peak area or the peak height is plotted against the concentration of the analyte to obtain the calibration graph for each analyte. Earlier studies have shown that in CE with indirect absorbance detection, the linearity of the cali-

Table 3
Precision and linearity for the various analytes

Acid	R.S.D. (%)			Linearity		
	t_m	Area	Height	$a \times 10^5$	$b \times 10^4$	R^2
Oxalic	0.19	1.6	1.8	2.49	−0.04	0.9996
Citric	0.89	3.8	5.4	2.48	−1.04	0.9995
Tartaric	0.22	1.4	1.0	2.01	0.09	0.9992
Malic	0.20	2.0	1.2	1.97	0.013	0.9990
Acetic	0.22	2.7	1.7	1.55	0.27	0.9979
Lactic	0.24	1.4	1.4	1.32	0.12	0.9993
Aspartic	0.63	2.6	1.9	2.33	0.13	0.9995
Glutamic	0.73	2.5	2.3	2.83	0.15	0.9990
Ascorbic	0.26	2.9	1.9	2.73	−0.07	0.9998
Gluconic	0.30	2.3	1.8	2.50	0.2	0.9990

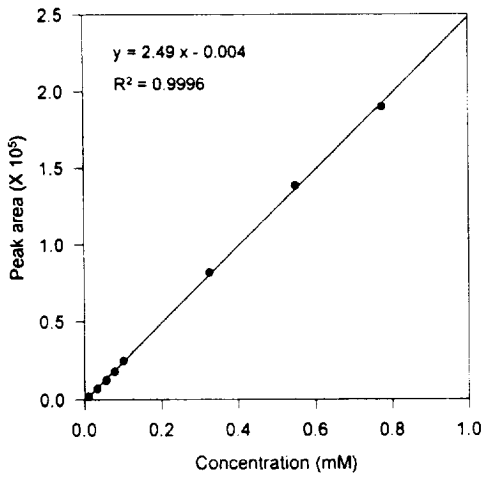

Fig. 6. Linearity of the calibration graph for oxalic acid (0.01–0.78 m*M*). Other conditions as in Fig. 3b.

bration spans only two orders of magnitude [24,25]. A typical calibration graph for the CE determination of oxalate is shown in Fig. 6. The data points from the calibration graphs were subjected to least-squares regression analysis and the slope a, intercept b and correlation coefficient R^2 for the various analytes are given in Table 3. The linearity of the present method for most analytes is good, with correlation coefficients better than 0.999.

3.6. Analysis of real beverage samples

To demonstrate their practical applications, we applied the present CE methods to determine various organic acids in real beverage samples. These samples are classified into four types: "sports drink", nutrients-added drink, fruit juice (natural or artificial flavoured) and tea. Electropherograms of the four representative beverage samples are shown in Fig. 7. The concentrations of the organic acids of interest for the four samples are summarized in Table 4. Sports-drinks generally contain high concentrations of salts, chloride being a predominant anion. Citrate, lactate and ascorbate are also commonly found in these drinks. The contents of nutrients-added drinks vary; besides citrate, malate and ascorbate, the amino acids aspartate and glutamate are also commonly found. In most juices,

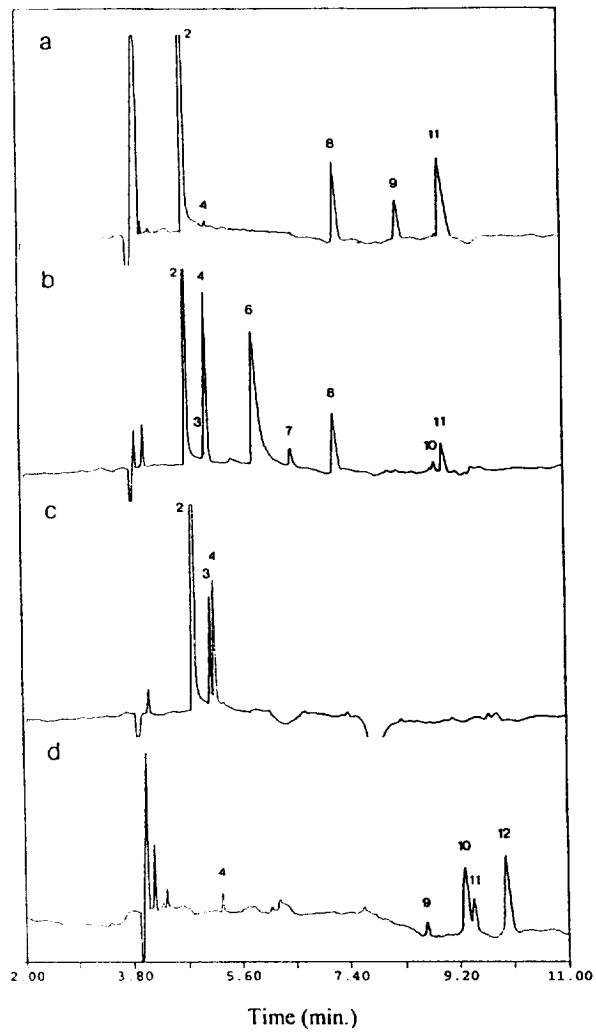

Fig. 7. Electropherograms of organic acids in real beverage samples: (a) sports-drink; (b) nutrients-added drink; (c) fruit juice; (d) tea. Other conditions as in Fig. 3b.

citrate is omnipresent, and in grape juice, tartaric acid is present in high concentration. Examination of the ingredient labels (quantitative data are unavailable) on the containers generally showed good agreement with the analyses by the CE method. The determination of vitamin C (ascorbic acid) is of special interest to the food and pharmaceutical industries because vitamin C is present in beverages, juices and medicines [26–29]. A common problem associated with the determination of vitamin C is the oxidation

Table 4
Contents of the organic acids of interest found in representative beverage samples

Acid	Sample 1	Sample 2	Sample 3	Sample 4
Citric	13	9.5	13	0.42
Tartaric	0	0.13	1.5	0
Malic	0.014	3.2	1.9	0.057
Acetic	0	0.22	0	0
Lactic	1.1	2.8	0	0
Aspartic	0	0	0	0.051
Glutamic	0.54	0.023	0	0
Ascorbic	2.2	0.61	0	0.42
Gluconic	0	0	0	0.90

Samples: 1 = sports-drink; 2 = nutrients-added drink; 3 = artificial flavoured grape juice; 4 = honey tea. Concentrations in m*M*.

problem. Once dissolved, ascorbic acid is readily oxidized to dehydroascorbic acid, catalysed by air or light exposure [30]. Solutions of ascorbic acid should be freshly prepared and kept tightly capped in an amber-coloured bottle; solutions can be maintained for 12 h without obvious oxidation problems. Only the unoxidized form of ascorbate absorbs strongly in the UV region; therefore, the indirect absorbance detection method was used to monitor the oxidation reaction of ascorbic acid. Fig. 8 shows CE analyses of ascorbic acid samples under various conditions. A freshly prepared ascorbate solution gives only a single peak (profile a). Even when kept in the dark, the solution is 45% oxidized after 18 h (profile b) After the solution has been exposed to air and light for 3 h, almost 80% of the ascorbate is oxidized (profile c). After 24 h, a negative peak appears. Hence the CE method is a valuable aid for checking freshness and for quality assurance.

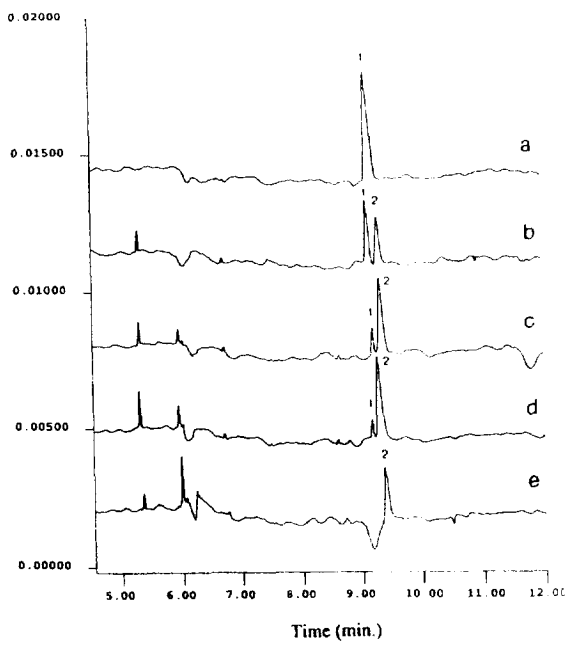

Fig. 8. Electrophoretic monitoring of the photocatalysed oxidation of 0.1 m*M* ascorbate: (a) fresh; (b) in the dark after 18 h oxidation; exposed to light and air after (c) 3 h, (d) 9 and (e) 24 h. Other conditions as in Fig. 3b.

4. Conclusion

Organic acids, commonly found in beverages and juices (either naturally or artificially added), could be determined readily by CE with indirect absorbance detection. The CE separation was best performed with reverse polarity (towards the anode) using TMA (5 m*M*) as the BGE and TTAB (1 m*M*) as the EOF modifier. For the simultaneous determination of all analytes, the optimum pH is 9.0, at which all peaks except for malate and succinate are baseline resolved within 9.5 min. Alternatively, an acidic pH (5.5) could be used to improve the resolution of the diprotic acids but at the expense that glutamate and ascorbate peaks would not be baseline resolved. The CE methods developed here show very good

precision and linearity and could be readily applied to analyses of real food samples.

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