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Simultaneous determination of inorganic anions, organic acids, amino acids and carbohydrates by capillary electrophoresis

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Abstract

A capillary zone electrophoretic method for the simultaneous analysis of inorganic anions, organic acids, amino acids and carbohydrates was developed with indirect UV detection using 2,6-pyridine dicarboxylic acid as the background electrolyte. This is the first paper to report their simultaneous analysis. Highly alkaline conditions were used in order to confer a negative charge not only on inorganic and organic anions but also on amino acids and carbohydrates, and to promote their migration towards the anode. Electroosmotic flow was reversed in the direction of the anode by adding cetyltrimethylammonium hydroxide to the electrolyte. Outstanding separations were obtained and electrophoretic mobilities of 82 compounds including nine inorganic anions, 23 organic acids, 18 amino acids and 32 carbohydrates were determined by the method. Under the optimized conditions 43 compounds were well separated in a single run. The relative standard deviations ($n=5$) of the method were better than 0.5% for migration times and between 0.8% and 4.9% for peak areas except for a few compounds. The detection limits for anions and amino acids were in the range from 6 to 12 mg/l and for carbohydrates from 23 to 35 mg/l with 300 mbar·s pressure injection (6 nl) at a signal-to-noise ratio of three. This method could be readily applied to the simultaneous determination of inorganic anions, organic acids, amino acids and carbohydrates in soy sauce, nutrient tonic and pineapple.

Keywords: Background electrolyte composition; Inorganic anions; Organic acids; Amino acids; Carbohydrates

1. Introduction

Inorganic anions, organic acids, amino acids and carbohydrates are important compounds in many fields such as chemistry, biochemistry, pharmacy, agriculture and food science where their analysis is essential, although they have been determined using a variety of different analytical techniques. Inorganic anions are usually analyzed using ion chromatography (IC), while organic acids are determined with HPLC or IC. The analysis of amino acids is performed by pre- or post-column derivatization HPLC methods with UV (UVD) or fluorescence detection (FLD) [1–3]. On the other hand the commonly used technique for underivatized carbohydrate analysis is HPLC with refractive index detection [4] or pulsed-amperometric detection [5,6]. For derivatized carbohydrates, HPLC–UVD [7,8], HPLC–FLD [9–11] or

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gas chromatography (GC) [12] have been employed. Since the analytical instrumentation, separation columns, mobile phases and detectors are different, at least three instruments and methods are necessary for the complete analysis. Therefore, an easy and rapid simultaneous analysis method for these compounds would be of great benefit.

Recently a GC method for simultaneous determination of trimethylsilyl derivatized organic acids and carbohydrates was developed [13], however, the GC method is not easily applied to inorganic anions and amino acids since these are nonvolatile compounds and are difficult to derivatize to achieve sufficient volatility.

Capillary electrophoresis (CE) is a powerful separation technique that can provide high resolution efficiency. Many CE methods have been developed for the analysis of inorganic anions, organic acids, amino acids or carbohydrates analysis. Since inorganic and organic anions have little or no UV absorbance, most published work on their analysis by capillary zone electrophoresis (CZE) utilizes indirect UV detection with various background electrolytes (BGEs) such as chromate [14–16], pyromellitate [16], phthalate [14,16], benzoate [14,16] and 2,6-pyridine dicarboxylic acid (PDC) [17]. The analysis of amino acids by CE has mainly been performed with derivatization methods with UV chromophore [18] or fluorophore reagents [19,20]. For carbohydrate analysis by CE, various derivatization techniques [21–24] have been employed because carbohydrates lack both charge and a strong chromophore in the UV range although a couple of underivatized carbohydrate analysis methods have also been reported [25–27].

Jones and Jandik [15] described the analysis of 30 inorganic and organic anions using a chromate buffer as the BGE in a CE-indirect UV method. The separation of 18 amino acids has been described using CZE with indirect UV detection at pH 11.0 [28]. As we have demonstrated previously, 17 underivatized monosaccharides including acidic, neutral and amino sugars and sugar alcohols can be fully separated and determined using CZE with indirect UV detection at pH 12.1 [27].

Differences in the respective molecular characteristics of this range of analytes would make the simultaneous determination of inorganic and organic anions, amino acids and carbohydrates using a single LC method somewhat problematic. However the fact that these analytes are charged or can be made to be charged would suggest that a CE separation method is possible. Given that some of the analytes have no chromophore and others only slight absorbance, development of an indirect UV detection method would appear to hold the most promise.

The aim of this work, therefore, was to establish a CE method for the simultaneous determination of underivatized inorganic anions, organic acids, amino acids and carbohydrates. We propose a CZE method with indirect UV detection using PDC and cetyltrimethylammonium hydroxide (CTAH) at pH 12.1 for their analysis. The method was optimized and then applied to the simultaneous analysis of anions, amino acids and carbohydrates in food, pharmaceutical and agriculture samples.

2. Experimental

2.1. Chemicals

Lactulose, mannuronic acid, mannosamine, *N*-acetylmannosamine and *N*-glycolylneuraminic acid (NGNA) were purchased from Sigma (St. Louis, MO, USA). *N*-acetylglucosamine was from Aldrich (Milwaukee, WI, USA). Xylitol, erythritol and CTAH solution (25% in methanol) were obtained from Tokyo Kasei (Tokyo, Japan). All other reagents were from Wako (Osaka, Japan). Inorganic and organic anion standards were prepared from their sodium salts or free acids. The chemicals used were of analytical or reagent grade. Water was purified with a Milli-Q purification system (Millipore, Bedford, MA, USA). Individual stock solutions of inorganic and organic anions at a concentration of 1 g/l and carbohydrates of 10 g/l were prepared in Milli-Q water. Those of Tyr, Cys-Cys, Asp, Trp, Leu, Ile and Phe were prepared at a concentration of 10 g/l in 0.1 *M* NaOH and other amino acids were in 0.01 *M* HCl. The working mixture standard was prepared by diluting stock solutions with Milli-Q water.

2.2. Instrumentation

All CE experiments were performed using a HP^{3D}Capillary Electrophoresis System from Hew-

lett-Packard (Waldbronn, Germany). The system comprises a CE unit with built-in diode-array detector and an HP^{3D}CE ChemStation for system control, data collection and data analysis.

2.3. Electrophoretic procedures.

Separations were carried out on fused-silica capillaries with 112.5 cm (104 cm effective length)×50 μm I.D.. The electrolyte solution was prepared containing 20 m*M* PDC and 0.5 m*M* CTAH which was used to reverse the direction of the electroosmotic flow (EOF) [29]. The electrolyte pH was adjusted to 12.1 with 1 *M* NaOH.

Prior to first use, a new capillary was pretreated with the run electrolyte for 20 min. Before each injection, the capillary was preconditioned for 4 min by flushing with the run electrolyte. The sample was injected with a pressure of 50 mbar for 6.0 s. The applied voltage was set at −30 kV and the capillary temperature was thermostated to 15°C. Detection was carried out with indirect UV detection using a diode-array detector. The signal wavelength was set at 350 nm with a reference at 230 nm.

3. Results and discussion

3.1. Separation of inorganic anions, organic acids, amino acids and carbohydrates

In order to achieve the simultaneous determination of inorganic and organic anions, amino acids and carbohydrates, a CZE method with indirect UV detection at highly alkaline pH was selected. Since most inorganic and organic anions, amino acids and carbohydrates have little or no UV absorbance, indirect UV detection was employed. The high-alkaline pH was used to ensure anionic ionization of all compounds and to promote migration toward the anode. At pH values higher than 12, carbohydrates are negatively charged since pK_a values of most carbohydrates are between 12 and 13 [27]. Amino acids are also anionic at this pH since their isoelectric points (p*I*) are from 2.98 to 10.76 [30].

The choice of the BGE is the most important in developing a method employing CZE with indirect UV detection [14,16,17]. In this work, PDC was selected as the BGE because it exhibits medium mobility [17] and therefore is well suited to analysis of both high mobility anions [17] and low mobility carbohydrates [27].

Around pH 12 high mobility anions migrated toward the anode, whereas most amino acids and carbohydrates migrated in the opposite direction because the EOF is faster than those low mobility compounds. This problem could be overcome by reversing the direction of the EOF by adding CTAH to the electrolyte. In previous studies [17,27] we used cetyltrimethylammonium bromide (CTAB) for EOF reversal, however, in this work CTAH was chosen in order to avoid interference with the detection of bromide. The 112.5-cm total length capillary was chosen to optimize resolution, and initially the temperature was thermostated to 20°C.

In CZE, ionic species are separated based on their charge and size, therefore the electrolyte pH has a significant effect on the separation. Separations of 44 compounds were studied over the pH range 11.6–12.3. No inorganic and organic anions except PO_4^{-3} have pK_a values in this region thus their mobilities were nearly constant. However, as shown in Fig. 1 the effective mobilities of some amino acids and most carbohydrates were increased at higher pH due to ionization changes of the anions around their pK_a values. Although the mobilities of Leu, Ile and His and those of fructose and mannose were almost the same, most compounds were well separated at pH 12.1. Therefore the 20 m*M* PDC electrolyte with 0.5 m*M* CTAH at pH 12.1 was chosen as the optimum electrolyte. The effective mobilities of 82 compounds including nine inorganic anions, 23 organic acids, 18 amino acids and 32 carbohydrates were determined by this method and listed in Table 1.

The effective mobility, μ_e, for each anion was calculated using the following equation:

$$\mu_e = lL/t_aV - lL/t_{EOF}V \ [cm^2\ V^{-1}\ s^{-1}]$$

where l and L are the length of the capillary to the detector and the total length of the capillary, respectively, V is the applied potential, t_a is the migration time of the anion and t_{EOF} is the migration time of a neutral marker.

A standard solution of compounds of general interest was constructed to contain 43 components, including seven inorganic anions, five organic acids, 16 amino acids and 15 carbohydrates to be analyzed

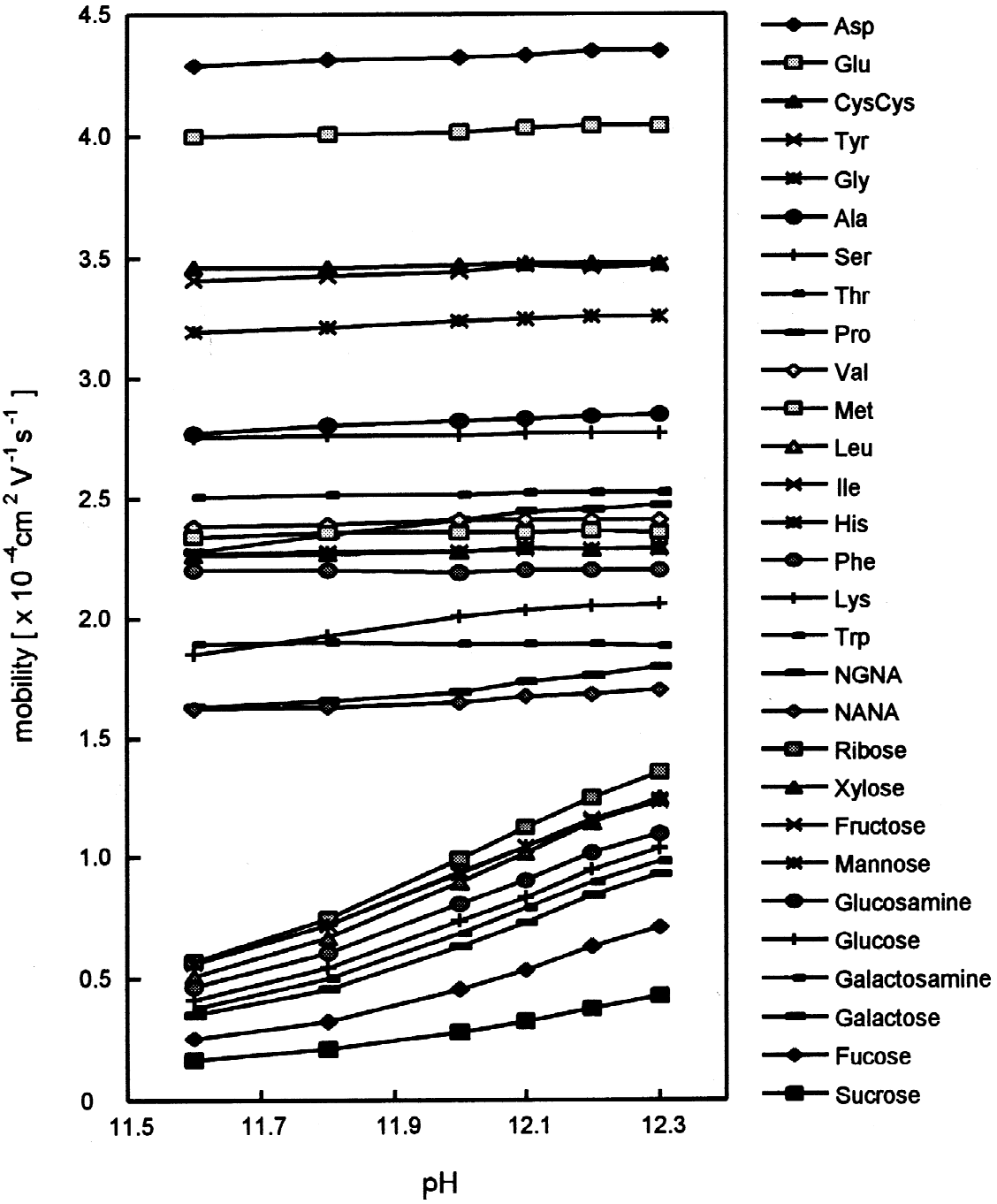

Fig. 1. Effect of electrolyte pH on amino acid and carbohydrate mobility. Conditions: capillary, fused-silica×112.5 cm (104 cm effective length) 50-μm I.D.; electrolyte, 20 m*M* PDC, 0.5 m*M* CTAH; applied potential, −30 kV; injection, 6 s at 50 mbar; temperature, 20°C; detection, signal=350 nm, reference=230 nm.

Table 1
Electrophoretic mobilities of anionic compounds at pH 12.1 (20°C)

Compound	Mobility ($\times 10^{-4}$ cm^2 V^{-1} s^{-1})	Compound	Mobility ($\times 10^{-4}$ cm^2 V^{-1} s^{-1})
Br^-	−7.181	Met	−2.389
Cl^-	−6.983	*n*-Hexanoate	−2.385
NO_2^-	−6.648	Galacturonic acid	−2.337
NO_3^-	−6.442	His	−2.310
SO_4^{-2}	−6.140	Leu	−2.300
Oxalate	−5.784	Ile	−2.300
Ascorbate	−5.409	Phe	−2.220
Malonate	−5.093	*n*-Heptanoate	−2.149
F^-	−4.990	Gluconate	−2.060
Formate	−4.911	Lys	−2.026
Citrate	−4.775	Trp	−1.910
$P_2O_7^{4-}$	−4.760	NGNA	−1.719
PO_4^{3-}	−4.677	*n*-Octanoate	−1.707
Tartarate	−4.584	NANA	−1.675
Succinate	−4.565	*N*-Acetylmannosamine	−1.221
Malate	−4.520	Ribose	−1.037
α-Ketoglutarate	−4.513	Fructose	−0.983
Asp	−4.418	*N*-Acetylglucosamine	−0.975
Glutarate	−4.196	Mannose	−0.966
Glu	−4.084	Xylose	−0.935
Adipate	−3.934	*N*-Acetylgalactosamine	−0.919
Acetate	−3.589	Ramnose	−0.904
Pyruvate	−3.540	Glucosamine	−0.846
Cys–Cys	−3.514	Mannosamine	−0.832
Glycolate	−3.495	Lactose	−0.774
Tyr	−3.493	Arabinose	−0.764
Gly	−3.260	Glucose	−0.761
n-Propionate	−3.111	Maltose	−0.731
Lactate	−3.041	Galactosamine	−0.725
BO_3^{3-}	−2.963	Lactulose	−0.676
Ala	−2.858	Galactose	−0.659
Ser	−2.795	Fucose	−0.485
n-Butyrate	−2.781	Sucrose	−0.291
Levulinate	−2.729	Raffinose	−0.284
Mannuronic acid	−2.674	Mannitol	−0.134
Pyroglutamate	−2.631	Trehalose	−0.121
n-Pentanoate	−2.578	Sorbitol	−0.118
Thr	−2.542	Galactitol	−0.104
Glucuronic acid	−2.497	Xylitol	−0.086
Pro	−2.450	Erythritol	−0.076
Val	−2.444	Inositol	−0.064

by this method. However, resolution between Pro and Val was poor and also Leu, Ile and His could not be resolved. In order to improve their separation, the effect of capillary temperature was studied at 15, 20, 30, 40°C. Optimum resolution was obtained at 15°C (Fig. 2). Lee and Lin [28] reported that using their CZE method with indirect UV detection Leu and Ile could not be resolved and Arg was not observed. The p*I* value of Arg is 10.76, however, the pK_a value of the guanidine group which is the side chain of Arg has a p*K*a of 12.48 [30]. We therefore presumed that Arg was not observed due to its being neutral at the operating pH of 12.1.

As shown in Fig. 2, Tyr and Trp were detected as negative peaks. In this method a signal wavelength of 350 nm with reference at 230 nm was employed

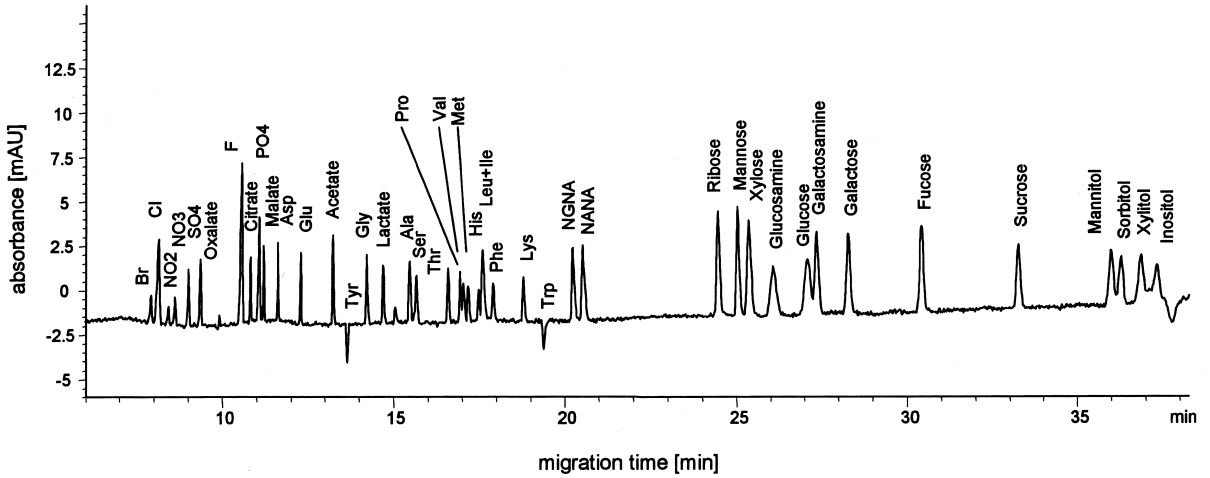

Fig. 2. Separation of inorganic and organic anion, amino acid and carbohydrate standard mixture. Concentrations: Cl^-, 110 mg/l; carbohydrates, each 200 mg/l; others, each 50 mg/l. Conditions: electrolyte, 20 m*M* PDC, 0.5 m*M* CTAH, pH 12.1; temperature, 15°C. Other conditions as in Fig. 1.

in order to visualize compounds as positive peaks. A decrease of adsorption at 230 nm produced by the presence of anions is recorded as a relative increase of the signal at 350 nm since the 230 nm signal is used as the reference wavelength. This greatly facilitates integration. However, since Tyr and Trp have a UV absorbance at 230 nm, this is recorded as an increase in the reference wavelength and therefore a negative peak for the signal detection wavelength.

3.2. Method validation

The reproducibility, linearity and sensitivity of the method were determined. The R.S.D. ($n=5$) of the method for the 43 compounds were better than 0.5% for migration times and between 0.8% and 4.9% for peak areas except for a few compounds. Only some areas of NO_2^-, NO_3^-, ribose and four sugar alcohols were higher than 5%. NO_2^- and NO_3^- showed the smallest peak dimensions since they have UV absorbance below 230 nm and therefore are difficult to integrate. Although the reason is unclear, ribose and sugar alcohols showed decreasing peak area during the runs.

Linearity and sensitivity were studied for SO_4^{2-}, citrate, lactate, Glu, Val, Lys, fructose, glucose, galactose and sucrose which are representative of the inorganic and organic anions, amino acids and carbohydrates. The calibration curves for all these compounds were linear at 20, 50, 100, 200, 500 and 1000 mg/l with correlation coefficients better than 0.9996. The detection limits for anions and amino acids were in the range from 6 to 12 mg/l and for carbohydrates were from 23 to 35 mg/l with 300 mbar·s pressure injection (6 nl) at a signal-to-noise ratio of three.

3.3. Application to the analysis of soy sauce, nutrient tonic and pineapple

The developed method was applied to the simultaneous determination of inorganic and organic anions, amino acids and carbohydrates in food, pharmaceutical and agriculture samples. Fermentation products such as soy sauce contain many kinds of inorganic and organic anions, amino acids and carbohydrates, and their analysis is important because the measurement of the concentrations of them can help track metabolic products of fermentation and correlate its flavor trends. Fig. 3 shows a typical electropherogram of a soy sauce analysis using the described method. A well-defined electropherogram was obtained without interference from other matrix compounds. Six inorganic and organic anions (Cl^{-1}, citrate, succinate, malate, acetate, lactate), 16 amino acids and 2 carbohydrates (glucose, galactose) were identified by matching their migration times with those of a standard solution. Sample preparation was

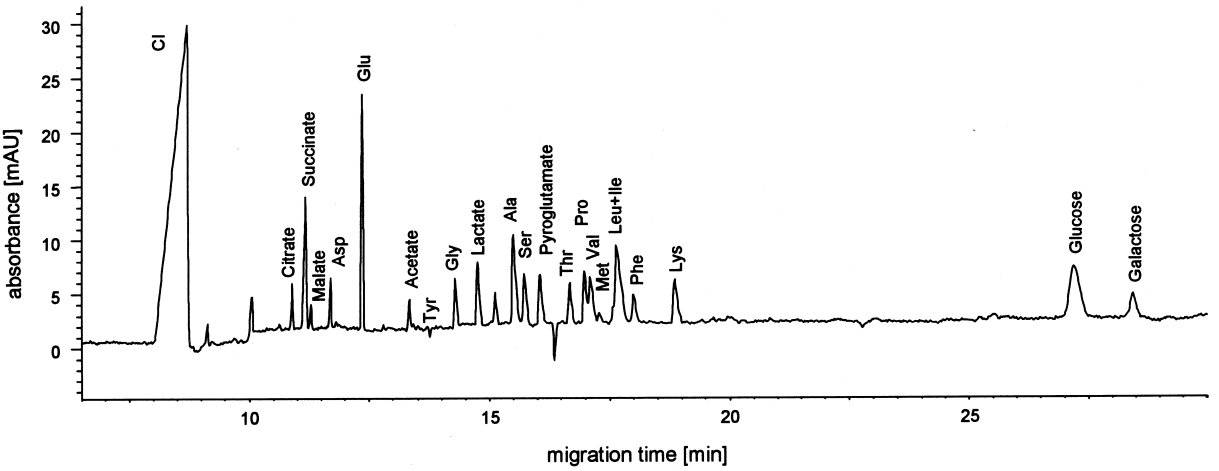

Fig. 3. Electropherogram of inorganic and organic anions, amino acids and carbohydrates in soy sauce. Conditions as in Fig. 2.

simple and consisted only of diluting the soy sauce 1:50 with Milli-Q water, and centrifugal filtering through a Millipore M_r 30 000-cutoff filter to remove proteins and peptides. Satisfactory reproducibilities were obtained for all compounds with R.S.D. values ($n=5$) for migration times better than 0.3% and for peak areas between 0.6 and 5.4%.

The rise in demand for nutrient tonics has prompted pharmaceutical companies to increase production and to develop new varieties. Fig. 4 illustrates the analysis of a new nutrient tonic which contains eight different essential amino acids. The sample was diluted with water (1:10) before injection. The calculated concentrations of amino acids were in good agreement with the label values, which were written in the product description (Table 2). The product description does not mention the carbohydrate content, however, fructose, glucose and sucrose were detected and their concentrations were determined as 4.3 g/l, 4.4 g/l and 149 g/l, respectively. The RSD ($n=5$) values for all analytes were excellent between 0.03% and 0.51% for migration times and better than 3.7% for peak areas.

This method was also applied to the analysis of organic acids and carbohydrates in pineapple. In the agriculture industry, technical experts are trying to

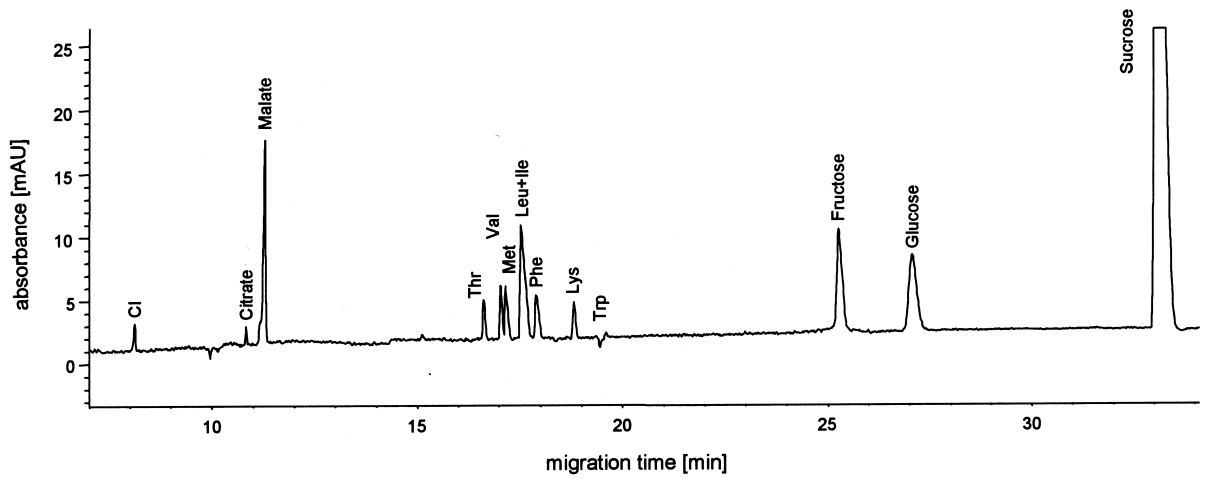

Fig. 4. Electropherogram of inorganic and organic anions, amino acids and carbohydrates in nutrient tonic. Conditions as in Fig. 2.

Table 2
Comparison of amino acid concentrations in a nutrient tonic by CE and actual contents

Compound	Concentration (g/l)	
	CE	Content
Thr	0.60	0.66
Val	0.80	0.85
Met	1.20	1.38
Leu+Ile	3.47	3.35
Phe	1.20	1.18
Lys	0.80	0.83
Trp	0.25	0.27

develop a new crossbreed of fruits. The taste of crossbred crops were characterized by analyzing the content of organic acids and carbohydrates. In pineapple for example, if the concentrations of citrate and malate are high, the taste tends to be sour. On the other hand, if the concentrations of carbohydrates are high, the taste is sweet. Determination of these compounds used to be performed by two techniques: HPLC for organic acids; and high-performance anion-exchange chromatography with pulsed-amperometric detection (HPAEC–PAD) for carbohydrates. However, the method described here enabled the simultaneous separation of both organic acids and carbohydrates in less than 18 min. In order to reduce the analysis time, a shorter length of capillary was used for this sample because pineapples contain fewer compounds than the 43-component standard. Squeezed pineapple juice was diluted 50-fold prior to injection. Fig. 5 shows a typical electropherogram of the analysis. Peaks were identified and the concentrations of citrate, malate, fructose, glucose and sucrose were calculated as 0.51%, 0.10%, 1.3%, 1.3% and 4.2%, respectively.

4. Conclusions

A simple and reliable CE method for the simultaneous determination of inorganic and organic anions, amino acids and carbohydrates has been developed. Compared with other developed techniques, this method has a big advantage: (1) most anionic compounds including amino acids and carbohydrates can be simultaneously analyzed without derivatization, (2) a well-defined electropherogram is obtained without other matrix interference, (3) sample preparation is minimum. And it provides excellent reproducibility, good linearity and appropriate sensitivity. Its utility was demonstrated by the analysis of soy sauce, nutrient tonic and pineapple. These results indicate that the proposed method can be useful for research and routine simultaneous analysis of inorganic and organic anions, amino acid and carbohydrate analysis in many application areas.

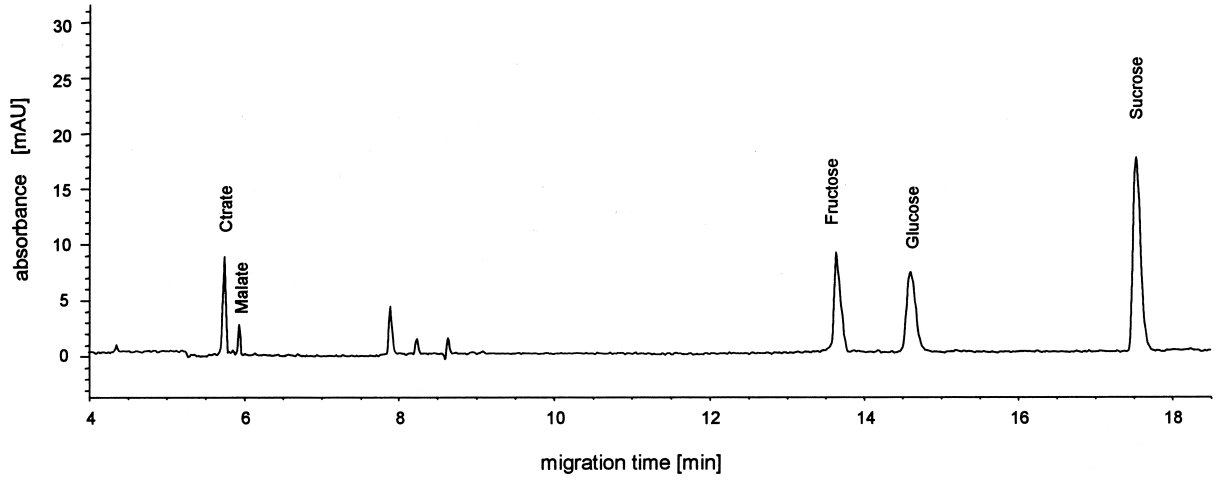

Fig. 5. Electropherogram of organic acids and carbohydrates in pineapple. Conditions: capillary, fused-silica×80.5 cm (72 cm effective length) 50 μm I.D.; applied potential, −25 kV; temperature, 20°C. Other conditions are the same as in Fig. 2.

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