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Development and optimization of a method for the analysis of low-molecular-mass organic acids in plants by capillary electrophoresis with indirect UV detection

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Abstract

Capillary zone electrophoresis was developed to analyze low-molecular-mass organic acids including oxalic, tartaric, formic, malic, citric, succinic, glutaric, acetic and lactic acid. The influences of some crucial parameters such as buffer concentration, pH value, surfactant concentration and separation temperature, on electrophoretic separation were investigated. Under the conditions of 15 m*M* phthalate containing 0.6 m*M* tetradecyltrimethylammonium bromide as the run buffer (pH 5.6); separation voltage −15 kV (−263.2 V/cm) and temperature 25 °C, a satisfactory separation of nine organic acids was accomplished within 7 min. The detection limits ($S/N=3$) ranged from 0.008 to 0.08 μg/ml and the quantification limits ranged from 0.01 to 0.1 μg/ml for electrokinetic injection. The method was successfully applied to analyze organic acids in different parts of *Var splendens* (an edible vegetable in China). The recoveries of organic acids in real samples ranged from 88 to 121%.

Keywords: Plant materials; *Var splendens*; Organic acids

1. Introduction

Low-molecular-mass (LMM) organic acids are common and natural constituents in many plants. Analysis of these organic acids has become increasingly important due to their role in the physiological activity of plants. Some organic acids such as citric, malic, succinic and oxalic acid are parts of Krebs cyclic pathway. They are intermediates in the most important metabolic pathway of carbohydrates, lipids and proteins [1]. Many of them are believed to play an important role in the cation transport, either nutrient or toxic, in xylem vessels. In addition, these organic acids respond to environment stimuli such as photoperiod, temperature, water and nutrition supply. For example, LMM organic acids in root exudation, mainly including acetic, aconitic, citric, formic, glycolic, lactic, malic, oxalic and succinic acids, are enhanced in many plant species under phosphorus deficiency [2]. Moreover, these acids in root exudates are related with the mobilization of soil mineral nutrients, e.g. nickel, aluminum, iron, zinc, copper

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and manganese [3–5]. The accumulation of organic acids in root cell compartments of metal tolerant plant species may indicate a more general character of the metal organic acid interrelationships in plants [6]. The success in clarification of these functions depends on the identification of organic acids in soil and different parts of plants.

For the simultaneous determination of organic acids in plant samples, many separation methods have been developed. As LMM organic acids are generally non-volatile, their determinations with gas chromatography (GC) require derivatisation. However, the derivatisation process is often quite tedious, time-consuming and retards the reproducibility of the analysis [7]. Currently, the most widely used methods for the determination of LMM organic acids in biological samples include high-performance liquid chromatography (HPLC) [8,9] and ion chromatography (IC) [10,11]. Both of these methods are not without limitations. For example, these two methods need large quantity elutant. Furthermore, organic substances in the plant matrix need to be removed by sample preparation assay limited column lifetime or complicated chromatogram may result. In addition, with both HPLC and IC, very little can be done to manipulate the selectivity as the analytical columns are only used with simple buffers or dilute acid. Therefore, it is difficult to optimize the separation for specific analysis from specific matrices.

Recently, capillary electrophoresis (CE) has complementary to chromatographic methods; due to providing several promising features like high separation efficiency, fast analysis and low consumption of chemicals. It has been widely used in the determination of LMM organic acids in food [12–16] and environmental samples [17,18]. In CE method, these acids are frequently migrated as anions in a co-electroosmotic mode with indirect UV detection. Generally, an electroosmotic flow modifier like tetradecyltrimethylammonium bromide is added to the run buffer in order to reverse the electroosmotic flow (EOF). Also compounds with UV-absorbing properties must be added in the run buffer to provide suitable conditions for indirect UV detection. Doble et al. [19] discuss several separation factors, including nature of the probe, the relative mobility of the probe and the analyte, the EOF modifier and the type of buffer, in CE of inorganic and LMM organic anions using indirect UV detection. Moreover, the separation selectivity could be enhanced by adding divalent cations to the run buffer [20,21].

The purpose of this paper is to develop and optimize a suitable CE method in analysis of LMM organic acids in plant samples. We discuss the influences of several crucial parameters, including buffer concentration, pH value, type and concentration of surfactant and temperature. The applicability of the method is demonstrated by analysis of these acids in different parts of *Var splendens* (an edible vegetable in China), e.g. root, stem and leaf.

2. Experimental

2.1. Instrumentation

All experiments were performed on a Beckman P/ACE MDQ capillary electrophoresis system (Beckman, Fullerton, CA, USA) equipped with a photodiode array detection system. The electropherograms were recorded and integrated by an IBM personal computer with 32 Karat software version 4.0 (Beckman). A fused-silica capillary (Beckman) with a total length of 57 cm and an I.D. of 75 μm was used. A detection window was created at 50 cm from the capillary inlet by removing the polyimide coating. Indirect UV detection was performed at a single wavelength of 254 nm. The hydrostatic injection mode (5 s, 20 p.s.i.) or the electrokinetic injection mode (20 s, −5 kV) was used for the injection of the standard solution or plant sample, respectively (1 p.s.i.=6894.76 Pa).

2.2. Chemicals

All organic acids, including oxalic, tartaric, formic, malic, citric, acetic, lactic, glutaric and succinic acids were from Beijing Reagents (China). Potassium hydrogenphthalate was obtained from Shanghai Reagent Factory (China). Dodecyltrimethylammonium bromide (DTAB), tetradecyltrimethylammonium bromide (TTAB), hexadecyltrimethylammonium bromide (CTAB) and octadecyltrimethylammonium chloride (OTAC) were purchased from Kanto Chemicals (Tokyo, Japan). All reagents were of analytical-reagent grade. Acetone was also from

Beijing Reagents and used as marker for the determination of electroosmotic flow. Water for preparation of sample and buffer solution was deionized by a Milli-Q purification system with a 0.2-μm fiber filter (Barnstead, CA, USA).

2.3. Procedure

Var splendens, which is a common vegetable in China, was purchased from the market. Root, stem and leaf were detached and smashed. A 10-g sample material was weighted and placed into a 50-ml beaker, adding 10 ml deionized water, incubating and homogenizing for 30 min on water-bath at about 50 °C. After cooling, the slurry was transferred quantitatively into a 25-ml volumetric flask. Finally, the samples were divided into small parts that were kept in a refrigerator. Before injection into the CE system, the samples were filtered through a 0.2-μm syringe filter.

New capillary were initially washed with methanol for 5 min, followed by deionized water for 2 min, 0.1 *M* HCl for 5 min, deionized water for 2 min, 0.1 *M* NaOH for 5 min and deionized water for 2 min. To achieve reproducible, all experiments were performed at 25 °C and were run in triplicate. Before each separation, the capillary was rinsed with 0.1 *M* NaOH for 2 min, deionized water for 2 min and run buffer for 2 min.

Electropherograms were obtained with a phthalate buffer, preparing with deionized water and adjusted to the appropriate pH with 0.1 *M* sodium hydroxide. Also, 0.6 m*M* TTAB was added, resulting in a reversed electroosmotic flow and necessitating the use of a reversed-polarity source. Stock solutions of organic acids were obtained by dissolving the organic acid in deionized water, and the concentration of all stock organic acid solution were 500 μg/ml. Standard solutions were prepared by dilution of stock solutions.

2.4. Calculation

Electroosmotic mobility, μ_{eo}, was determined by the measurement of the migration time of 5% acetone solution, t_{eo}, according to the following expression:

$$\mu_{eo} = (L_t L_d)/(V t_{eo})$$

where L_t is the total capillary length, L_d is the capillary length from injection inlet to the detector and V is the applied voltage.

Similarly, the observed mobility of organic acids, μ_{obs}, was calculated from the migration time of the organic acids, t, according to the following relation:

$$\mu_{obs} = (L_t L_d)/(Vt)$$

The electrophoretic mobility, μ_{ep}, equal to the difference of μ_{eo} and μ_{obs}:

$$\mu_{ep} = \mu_{obs} - \mu_{eo}$$

The electroosmotic and electrophoretic mobilities were directed towards the anode and considered as positive values.

3. Results and discussion

3.1. Effect of electrophoretic run buffer concentration

Eqs. (1) and (2) predicted that electroosmotic mobility (μ_{eo}) and electrophoretic mobility (μ_{ep}) on a bare capillary decreased with increasing electrophoretic run buffer concentration:

$$\mu_{eo} = \frac{\varepsilon_0 \varepsilon_r \zeta}{\eta} \quad (1)$$

$$\mu_{ep} = \frac{2}{3} \cdot \frac{\varepsilon_0 \varepsilon_r \zeta}{\eta} \quad (2)$$

where η is the viscosity, ζ the zeta potential, ε_r the dielectric constant, and ε_0 the permittivity of vacuum ($8.85 \cdot 10^{-12}$ C^2 N^{-1} m^{-2}) [22]. But in reversed flow, the mechanism was more complex as higher run buffer concentrations increased the adsorption of TTAB onto the capillary wall [23]. The influence of electrophoretic run buffer concentration on EOF was investigated using acetone as a μ_{eo} marker. μ_{eo} decreased with increasing the concentration of electrophoretic run buffer. Its result indicated the shrinking of electric double layer dominated over the increased TTAB adsorption. The electrophoretic mobility (μ_{ep}) was also reduced as a function of the electrophoretic run buffer concentration for each of

the organic acids, which result from decreased ζ and higher buffer conductivity. When applying voltage, the high conductivity resulted in a lower electric field. Then sample ions would move slower than those in the low concentration buffer. As co-electroosmotic mode was used, the observed electrophoretic mobility (μ_{obs}) was given by the sum of μ_{eo} and μ_{ep}. The migration time was proportional to concentration. When the electrophoretic run buffer was 5 m*M*, the resolution between malic acid and citric acid was very low. Increasing the electrophoretic run buffer concentration improved the separation of organic acids, but at the expense of longer run times. The baseline separation of malic acid and citric acid could be achieved at 15 m*M*. Although resolution was little enhanced at 20 m*M*, the optimal electrophoretic run buffer concentration chosen was 15 m*M*, due to the shorter migration times at this concentration.

3.2. Effect of electrophoretic run buffer pH

The pH of the electrophoretic run buffer must be carefully controlled, as it not only influenced the EOF, but also the electrolytic dissociation equilibrium of organic acids, ultimately affecting resolution. The cationic surfactant (TTAB) was added in the run buffer and then bound to the capillary surface, resulting in positive charge density and reverse EOF. As the electrophoretic run buffer pH was increased, more Si–OH groups were ionized with no concomitant increase in TTAB binding because of surface saturation. As a result, the net surface charge density turned more and more negative and the EOF decreased as the pH of electrophoretic run buffer increased [24]. On the other hand, the increase of ionic strength caused by pH increase also reduced EOF (Fig. 1). The influence of pH on electrophoretic mobility of organic acids was also shown on Fig. 1. The migration time prolonged as increasing of pH, with changing the elution order of citric acid. At pH lower than 6.0, the organic acids present different ionization degrees, they have two to three ionization constants with pK_a values 3.1; 4.8; 6.4 for citric acid, 3.46; 5.10 for malic acid, 3.22; 4.81 for tartaric acid [25]. According to CE methods, the separation of organic acids was achieved corresponding with their charge to mass ratio. So elution order was tartaric, malic and citric acid, and a good resolution can be attained when pH value was 5.6. The tricarboxylic citric acid ionized at higher pH, which increased its charge. Therefore, citric acid migrated faster than tartaric and malic acid as shown in Fig. 1.

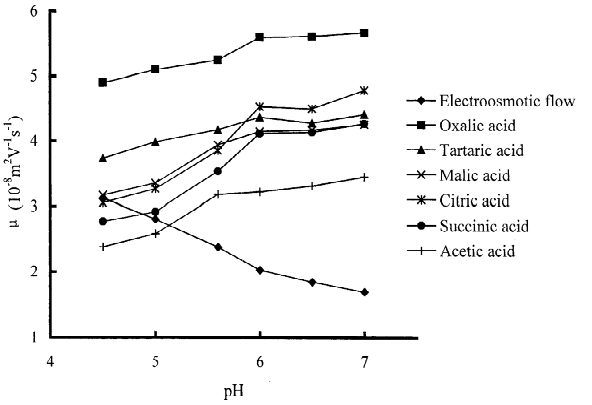

Fig. 1. Effect of pH on the mobility. Conditions: fused-silica capillary, 57 cm (length to detector 50 cm); buffer 15 m*M* phthalate containing 0.4 m*M* TTAB; voltage: −15 kV; detection 254 nm; temperature 25 °C; hydrodynamic injection 20 p.s.i. for 5 s.

3.3. Effect of surfactant

Four surfactants, DTAB, TTAB, CTAB and OTAC, were investigated. The effects of different surfactants on μ_{eo} were illustrated in Table 1. Electroosmotic flow towards the anode was considered as positive values. With exception of DTAB, the others can successfully reverse the EOF and had similar μ_{eo}, which resulted from the saturation of the capillary walls with the surfactant at 0.4 m*M*. For three other surfactants, the similar resolutions were obtained, and the solubility decreased with increas-

Table 1
Electroosmotic mobility, μ_{eo}, employed different surfactants

	DTAB	TTAB	CTAB	OTAC
μ_{eo} (10^{-8} m^2 V^{-1} s^{-1})	−3.61	2.53	2.55	2.57

Conditions: buffer: 15 m*M* phthalate–0.4 m*M* surfactant (pH 5.6), other conditions as in Fig. 1
Electroosmotic flow towards the anode was considered as positive values and that towards the cathode was considered as negative values.

ing of hydrophobic carbon chain. So TTAB was selected as the best surfactant.

The separation of organic acids was little affected by the concentration of TTAB. The electroosmotic mobility increased when varying the TTAB concentration from 0.2 to 0.4 m*M*, and it remained constant for higher TTAB concentration. For the capillary walls were saturated, free surfactant cations increased with increase in surfactant concentration, which potentially bind to the organic acid molecule, changing its net charge and thus its electrophoretic mobility. The best concentration (0.6 m*M*) was chosen, since the sensitivity was reduced when the concentration was too high.

3.4. Effect of separation temperature

The temperature significantly affected the migration times of organic acids. A remarkable decrease of migration time for increasing temperature from 15 to 35 °C has been recorded. These decreases were primarily due to the viscosity that was related to the temperature. Although higher temperature is to shorten the analysis time, the resolution reduced because of higher Joule heating. Therefore, the optimal temperature necessary to attain the highest level of resolution and shortest analysis time was concluded to be 25 °C.

3.5. Quantification

As shown in Table 2, calibration curve for each acid was established from six concentration levels of the mixing sample, which showed linearity within two orders of magnitude between peak area and sample concentration. In order to improve the limit of detection (LOD), electrokinetic injection was applied. Compared with hydrodynamic injection, electrokinetic injection gave worse linearity because the electric field being influenced by the sample conductivity affected the calibration curve. While difficult in practice to employ quantitatively, electrokinetic injection can provide impressive trace enrichment. Thus, electrokinetic injection was suitable for analysing trace organic acids in plant. The relative standard deviation (RSD) of the peak area was calculated based on five duplicate injections of a standard sample. The LOD were obtained at a 3:1 signal-to-noise ratio. The high reproducibility and low LOD indicated that the method was reliable for analyzing organic acids.

3.6. Application to real samples

The method was used to determine the organic acids in root, stem and leaf of *Var splendens*. Typical electropherograms were shown in Fig. 2. Nine organic acids can be well separated without the interference of inorganic ions (Fig. 2I). A remarkable difference of organic acids content in three parts was observed. The migration times of organic acids in real samples increased because a great deal of ions in samples influence the separation (Fig. 2III). The reproducibility of migration time was very good; the relative standard deviation for triplicate, successive analyses was less than 0.6% (Table 3).

Table 2
Quantification data (conditions as in Fig. 2)

Organic acid	*a*	*b*	*r*	Linear range (μg/ml)	RSD (%) ($n = 5$)	LOD (μg/ml)
Formic	28864	410.01	0.9987	0.02–1.0	0.78	0.01
Tartaric	15262	146.14	0.9991	0.01–1.0	0.82	0.008
Malic	15619	293.60	0.9990	0.02–1.0	0.48	0.01
Citric	8347	841.10	0.9974	0.10–1.0	0.11	0.08
Succinic	18740	370.07	0.9992	0.02–1.0	0.55	0.01
Acetic	23717	572.97	0.9993	0.02–1.0	0.96	0.01
Glutaric	21397	−987.71	0.9957	0.05–1.0	0.43	0.03
Lactic	21694	323.93	0.9971	0.02–0.5	1.04	0.01

Calibration curves are expressed as regression lines ($y = ax + b$), where y is integrated peak area and x is concentration of organic acid (μg/ml). a is slope, b is intercept and r is relative coefficient. RSD is relative standard deviation. LOD is limit of detection at 3:1 signal-to-noise ratio.

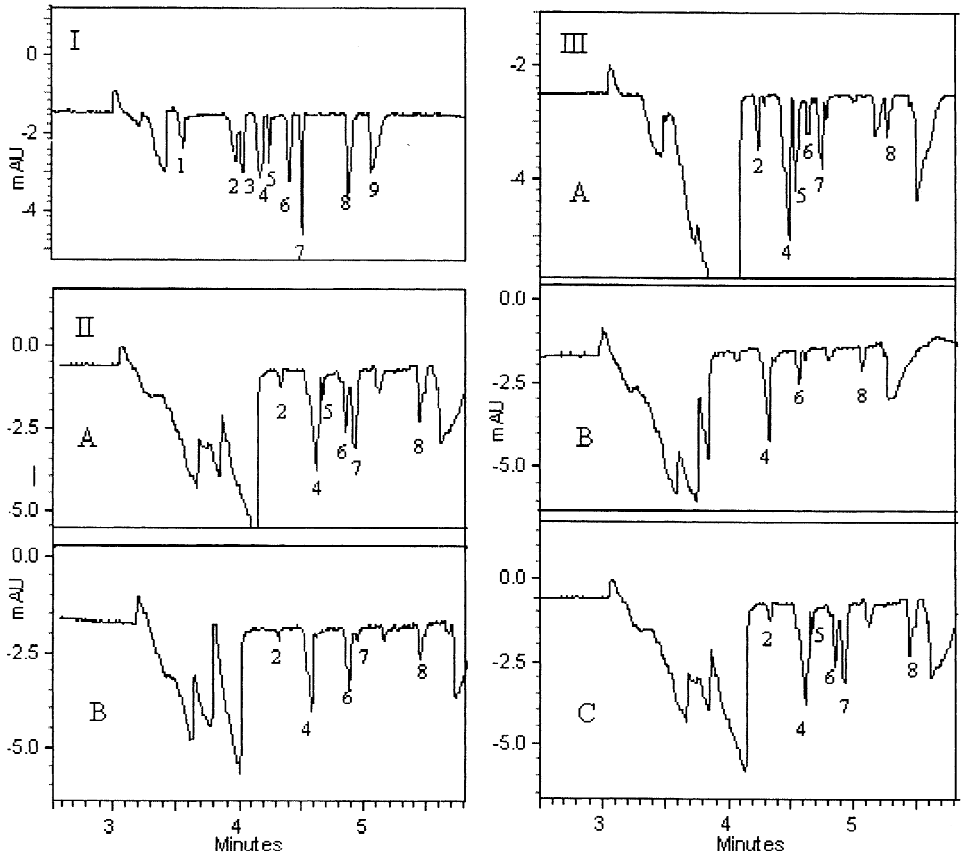

Fig. 2. Electropherograms of nine organic acids. Conditions: fused-silica capillary, 57 cm (length to detector 50 cm); buffer 15 m*M* phthalate, 0.6 m*M* TTAB, pH 5.6; separation voltage: −15 kV; detection 254 nm; temperature 25 °C; electrokinetic injection 20 s, $V_{inj} = -5$ kV. (I) Electropherogram of standard sample. (II) Electropherograms of organic acids in *Var splendens* leaf for storage several days. (A) The fresh sample. (B) Storage for 2 days (storage temperature <0 °C). (III) Electropherograms of organic acids in different parts of *Var splendens*. (A) Root. (B) Stem. (C) Leaf. Peaks: 1=oxalic acid, 2=formic acid, 3=tartaric acid, 4=malic acid, 5=citric acid, 6=succinic acid, 7=glutaric acid, 8=acetic acid, 9=lactic acid.

However, the organic acid contents were reduced during storage. For example, after storage for 2 days, the contents of formic, malic, succinic, glutaric, acetic acids in leaf were reduced to 0.02, 0.82, 0.36, 0.09, 0.11 μg/g, respectively, and that of citric acid was lower than the LOD. The unreliability of organic acids determinations was related to sample instability, not to the analytical method (Fig. 2II). In fact, some of the soil microorganisms, or their associated enzymatic activity can survive during the sample preparation process. Many aerobic microorganisms can break-down LMM organic acids in their anabolism. Furthermore, the survived enzyme, such as succinic dehydrogenase, malic enzyme, which widely existed in most plants and plays a very important role in the Krebs cycle, also can decomposed these acids. The key step in achieving good performance was to analyse three times immediately after sample preparation because longtime storage made sample have ample time to develop enzymatic activity. Typical results for six organic acid determinations were summarized in Table 3. The recoveries of formic and citric acid were not good enough, due to their low contents and decomposition.

Table 3
Result for the determination of organic acids in *Var splendens* by CE (conditions as in Fig. 2)

Sample		Formic	Malic	Citric	Succinic	Glutaric	Acetic
Root	t_R (mean, min)	4.263	4.506	4.564	4.658	4.767	5.292
	t_R repeatability (RSD,%)	0.39	0.28	0.37	0.25	0.31	0.28
	Content (μg/g)	0.15	1.44	1.49	0.06	0.37	0.20
	Recovery (%)	107	105	105	116	95	110
	RSD (%)	3.8	2.1	3.7	4.2	1.8	3.6
Stem	t_R (mean, min)	N.D.	4.345	N.D.	4.578	N.D.	5.085
	t_R repeatability (RSD,%)	–	0.14	–	0.13	–	0.18
	Content (μg/g)	–	1.24	–	0.12	–	0.09
	Recovery (%)	–	98	–	121	–	109
	RSD (%)	–	1.8	–	4.4	–	2.4
Leaf	t_R (mean, min)	4.339	4.610	4.659	4.842	4.920	5.433
	t_R repeatability (RSD,%)	0.36	0.56	0.45	0.49	0.33	0.56
	Content (μg/g)	0.06	1.43	0.28	0.42	0.66	0.54
	Recovery (%)	113	107	95	88	105	94
	RSD (%)	2.6	1.4	2.4	3.1	2.0	2.9

t_R: migration time.
N.D.: no detectable amount.
RSD: relative standard deviation.

4. Conclusion

The analysis of organic acids in different plant parts with CE is a difficult task due to their low contents and unstable samples. This method quantified six organic acids, including formic, malic, citric, succinic, glutaric and acetic acids in *Var splendens*, and it is simple, rapid and does not require any complicated sample preparation. When using electrokinetic injection, the limits of determination are lower than the relevant concentrations of these acids in plant extracts. Hence, this method is suitable for quick determination of organic acids in plant samples.

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