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Separation of plant phenolic compounds by capillary zone electrophoresis

Jen-Fon Jen*, Yean-Hwa Hsu, Maw-Rong Lee

Department of Chemistry, National Chung-Hsing University, Taichung 402, Taiwan

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Abstract

A capillary zone electrophoretic method with on-line UV detection was used for resolving fourteen phenolic species within 13 min by using a 100 cm × 75 μm I.D. fused-silica tube with 0.01 *M* sodium borate buffer (pH 9.0) as electrolyte at 18 kV. The electrophoretic behaviour of phenolic species was investigated in order to optimize their separation as a function of the running buffer pH, concentration, solute injection method and time and applied voltage. Selectivity is strongly affected by the electrolyte concentration and pH. Quantitative aspects of capillary zone electrophoresis are also discussed.

Keywords: Phenolic compounds

1. Introduction

Because simple phenolic species are known to be products of vascular plant metabolism [1], and also contribute to the polymeric structures of tannins and lignin [2], the isolation and identification of plant phenolics have been determinant in many studies related to phenolic compounds [3–7]. Although the detrimental effect of plant phenolics on nutrient availability is well documented [8], they are considered to exhibit anti-tumoural and anti-viral activities and to act an inhibitors of mutagenesis and carcinogenesis in some oriental folk medicine [5–6].

Phenolics to be determined can be free or combined, and after chemical pretreatment have been quantitatively evaluated by GC–MS [9–11], HPLC–UV [8] or GC with electrochemical detection [12,13]. Unfortunately, these separations are often time-consuming or need derivatization of the solutes.

Recently, high-performance capillary electrophoresis (HPCE) has emerged as a powerful technique for the separation of many types of compounds sufficiently soluble in aqueous buffer solutions owing to its high-resolution separation capability and non-solvent, less electrolyte and less solute requirements [11,14–20]. It might be a potential tool for measuring phenolics in complex plant matrices at low concentration.

In this work, the suitability of CZE for the separation of phenolic species was investigated. For this purpose, the main parameters affecting the electrophoretic behaviour, including pH, buffer concentration and applied potential, were studied in order to achieve a satisfactory optimi-

* Corresponding author.

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zation for obtaining good resolution. To evaluate the applicability of the proposed method, in addition to the electrophoretic behaviour, detection limits, linear dynamic detection ranges and reproducibility were studied through the separation of several phenolics. A real sample of Taichung sanitary landfill leachates was analysed.

2. Experimental

2.1. Chemicals and reagents

Distilled, deionized water (DDW) was used to prepare all solutions. All chemicals used were of analytical-reagent grade. Borate running buffers (pH 9.0) were prepared by mixing equimolar solutions of $Na_4B_2O_7$ (Baker, Phillipsburg, NJ, USA) and H_3BO_3 (Riedel-de Haën, Hannover, Germany). Catechol and resorcinol were obtained from Janssen Chimica (Noisy le Grand, France), *o*- and *p*-cresol were from Riedel-de Haën, ferulic acid, gallic acid, *m*- and *p*-hydroxyphenylacetic acid, methylsuccinic acid, syringic acid, vanillic acid, acetovanillone, *p*-coumaric acid and resacetophenone from TCI (Tokyo, Japan) and vanillin from Merck (Darmstadt, Germany). Stock standard solutions were prepared at a concentration of 100 μg/ml, stored in brown glass bottles and kept at 4°C in a refrigerator for a maximum of 2 weeks. Fresh working standard solutions (4 μg/ml) were prepared daily by appropriate dilution of the stock standard solutions. All solutions were filtered through a 0.45-μm GN-6 grid filter (Gelman Science, Ann Arbor, MI, USA) and degassed prior to being used.

2.2. Apparatus

The HPCE instrument was a Prime system (Europhor, Toulouse, France). It includes a Vision I sampler and a Vision V power supply, and is equipped with a UVIS-206 detector (Linear, Reno, NV, USA). A 100 cm long (50 cm to the detector cell) ×75 μm I.D. fused-silica capillary column (Polymicro Technologies, Phoenix, AZ, USA) was used. All experiments were performed at suitable voltages at room temperature (25°C) controlled by an air conditioner. Samples were introduced pneumatically by pressure application at 0.75 p.s.i. for 2 s at the anode (positively charged) end. Solutes were monitored by on-line detection at 210 nm or in the range 200–360 nm for fast multi-wavelength scanning. Peak areas and electrophoretic mobilities were measured by using a PC with Chemlab (Taipei, Taiwan) software or a Shimadzu (Kyoto, Japan) C-R6A Chromatopac integrator.

2.3. Procedure

The capillary column was first conditioned with the separation buffer (3 min), immediately prior to injection. The column was washed with 0.1 *M* NaOH (3 min), then rinsed with water (3 min) between runs.

3. Results and discussion

3.1. Optimization of capillary zone electrophoresis

The conditions for the efficient separation of phenolics by CZE were investigated. According to the theory of CZE resolution derived by Jorgenson and Lukacs [21], optimum resolution can be achieved by controlling the electrophoretic mobilities of the solutes or the electroosmotic flow (EOF), whereas the electrophoretic mobility and the EOF coefficient can be modified by using various running buffers, including pH, type and concentration of electrolytes. Therefore, factors that affect the migration behavior of phenolics such as electrolyte pH and concentration, applied voltage, injection time and stability were studied thoroughly.

3.2. Time and stability of hydrodynamic injection

Because the amount of sample loaded is nearly independent of the sample matrix [22], hydrodynamic injection was used in this study. It is well known that the length of the sample plug

affects the efficiency and the peak resolution of the electropherogram [23–25]. Because the detection sensitivities of separated species are limited owing to the short light-path of the capillary, an appropriate sample plug is required to improve detection. Therefore, the effect of injection time on detection sensitivity and separation efficiency was investigated. As reported earlier [23–25], the number of theoretical plates decreases on increasing the injection time. Although a long injection time would increase the detection sensitivity, the resolution deteriorated when the sample was injected for 3 s. Therefore, and injection time of 2 s was preferred and adopted throughout the study. In order to test the stability of the hydrodynamic injection system, a solution containing 1 μg/ml of trimethylphenylammonium chloride was injected into the CZE system for 2 s at 0.75 p.s.i. applied pressure and monitored at 210 nm. The same experiment was repeated fifteen times. Fig. 1 shows the stability of the peak areas for the series of injections. The observed reproducibility of 1.72% (R.S.D.) with hydrodynamic injection is acceptable.

3.3. Selection of electrolyte pH and concentration

The choice of the running buffer is extremely important for the achievement of a proper CE

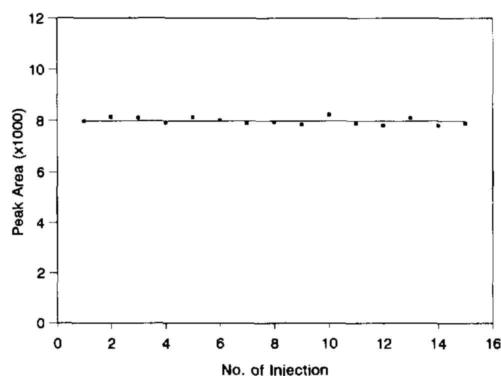

Fig. 1. Stability of the peak area with hydrodynamic injection. Sample, 1 μg/ml trimethylphenylammonium chloride; injection, 2 s at 0.75 p.s.i. applied pressure; average area, 7981 ($n = 15$), R.S.D. 1.72%.

separation. The sensitivity of the EOF to pH requires the use of buffers that maintain a constant pH. In running a CZE experiment, the selectivity can mostly be altered through changes in the buffer pH. Hence the electrolyte pH is one of the key parameters in a CZE separation. The influence of pH on the CE separation of 14 phenolics was studied with acetate buffers at pH 4 and 5, phosphate buffers at pH 6, 7 and 10, borate buffers at pH 8 and 9, at a 0.01 *M* concentration of each salt. Among these seven sets of conditions, migration at pH 9–10 provided better resolution and selectivity of the phenolics. Because a higher buffer pH might lead to polymerization or autoxidation of phenolic compounds, a borate ($pK_a = 9.24$) buffer at pH 9.0 was selected as the electrolyte in the CZE system.

The influence of the buffer concentration on the mobilities of phenolics and their resolutions were studied from 0.005 to 0.04 *M* borate buffer at pH 9.0. The results indicated that the mobilities of phenolics decreased when a more concentrated borate buffer was used. This can be explained by the increase in the double layer thickness on the capillary wall when a high-concentration buffer is used, which decreases the effective charge at the wall and limits coulombic interactions of solute with the walls. However, there was no significant change in selectivity except for the resolution of resacetophenone and cresol, which was worse in 0.04 *M* borate buffer owing to lengthening of the migration time and peak broadening. Conversely, the mobilities of phenolics are faster with a lower concentration buffer (e.g., 0.005 *M*), leading to a lower resolution electropherogram. Therefore, 0.01 *M* borate buffer (pH 9.0) electrolyte was used to achieve optimum resolution.

3.4. Effect of applied voltage

In most CZE, the ideal separation is generally obtained by applying a voltage as high as possible. This would lead to the best separation efficiency in the shortest time. However, this benefit is limited by the dissipation of the heat generated during the electrophoretic process due

to the Joule effect. The mobility of the analytes is then increased owing to the change in the buffer viscosity induced by heat dissipation. In this study, a better separation seemed to be achieved with a lower applied voltage, with an increase in migration times and broadening of the peaks, compromising the separation efficiency. As shown in Fig. 2, the migration times of phenolics are significantly influenced by the applied voltage. A compromise was made between a poorer separation at high voltage and a longer time of analysis at too low a voltage by adopting 18 kV for the separation of phenolics.

3.5. Electrophoretic separation of phenolics

The electropherogram of fourteen phenolics obtained under the proposed conditions is shown in Fig. 3. It is clear that all phenolic species give sharp and symmetrical peaks except for the peak of resacetophenone, which was overlapped by an unknown species. Peaks were assigned not only by comparing the migration times with those of standards, but also by fast multi-wavelength scanning of the peaks better 200 and 360 nm with

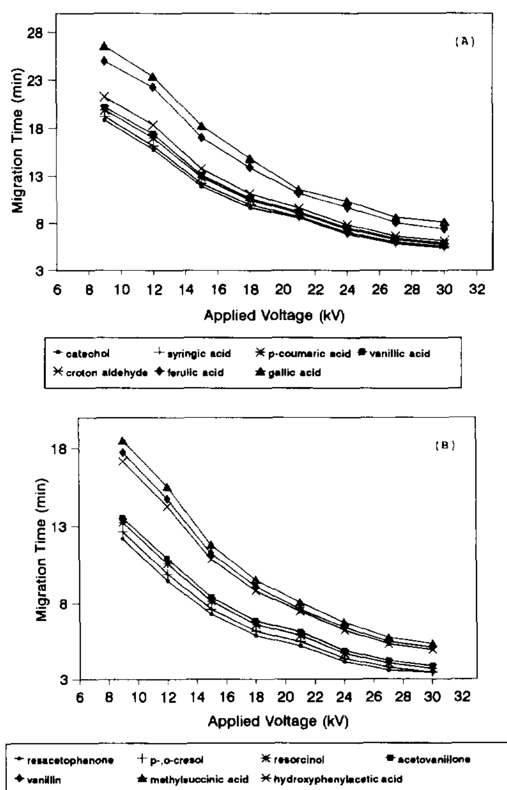

Fig. 2. Influence of applied voltage on the migration time of phenolics. CZE conditions: Electrolyte, 0.01 M H_3BO_3–0.01 M $Na_4B_2O_7$ (pH 9.0); column, 100 cm long (50 cm effective length) × 75 μm I.D. fused-silica capillary; injection, hydrodynamic for 2 s at 0.75 p.s.i. applied pressure.

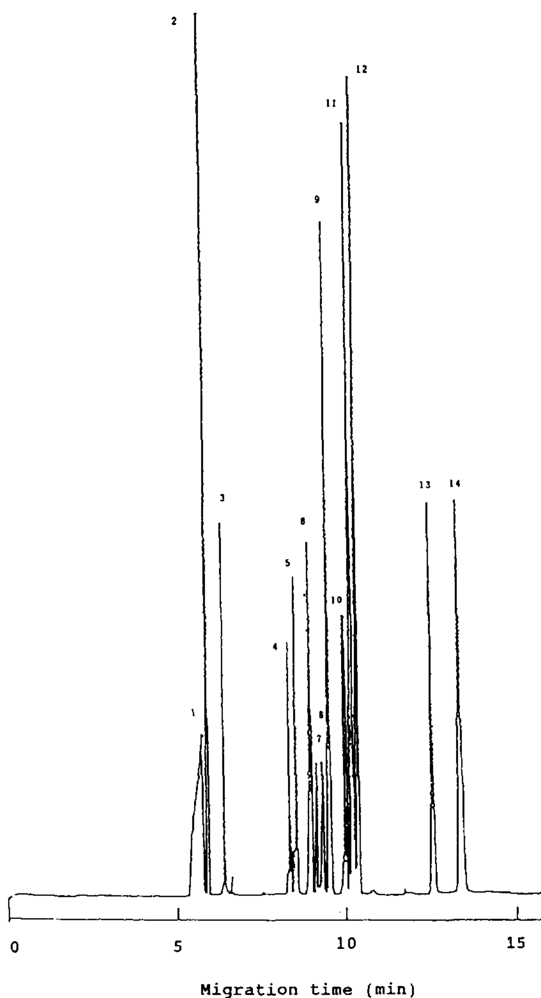

Fig. 3. Electropherogram of fourteen phenolics. CZE conditions: electrolyte, 0.01 M H_3BO_3–0.01 M $Na_4B_2O_7$ (pH 9.0); column, 100 cm long (50 cm effective length) × 75 μm I.D. fused-silica capillary; applied voltage, 18 kV; injection, hydrodynamic for 2 s at 0.75 p.s.i. applied pressure. Peaks: 1 = reacetophenone; 2 = p-, o-cresol; 3 = resorcinol; 4 = acetovanillone; 5 = p-, m-hydrophenylacetic acid; 6 = vanillin; 7 = methylsuccinic acid; 8 = catechol; 9 = syringic acid; 10 = p-coumaric acid; 11 = vanillic acid; 12 = crotonaldehyde; 13 = ferulic acid; 14 = gallic acid.

Table 1
Calibration data for four phenolics in landfill leachate[a]

Compound	Linear concentration range (μg/ml)	Correlation coefficient	Regression equation[b]
Cresol	1.0–83[c]	0.979	$y=3430x+2500$
Resorcinol	0.1–80	0.985	$y=2220x+6400$
Methylsuccinic acid	0.1–80	0.997	$y=2150x+1760$
Crotonaldehyde	0.9–69[c]	0.982	$y=1600x+3400$

[a] CZE conditions: 0.05 *M* borate buffer at pH 9.0 with an applied voltage of 10.5 kV.
[b] y = peak area (μVs); x = concentration (μg/ml).
[c] Concentration derived from ppm (v/v) × density.

a UV–photodiode array detector in order to check on the absorption of the analytes at 210 nm. As shown in Fig. 3, the peaks of the fourteen phenolics are obviously well separated. It is worth mentioning that the isomers of *p*- and *o*-cresol and *p*- and *m*-hydroxyphenylacetic acid were not separated under the proposed conditions.

3.6. Determination of phenolics in landfill leachate

The applicability of the method to the determination of phenolics in real samples was tested by analysing a landfill leachate sample. Because the matrix of this leachate sample was complicated, serious interferences occurred in the separation of phenolic species with the earlier conditions. Because of the flexible characteristics of the CZE technique, appropriate conditions can be easily set up by adjusting the electrolyte and the applied voltage for the separation of some target species. Thus, the electrophoretic conditions were adjusted to prevent interferences and achieve the separation. After a series of tests, optimum conditions for the separation of four species in landfill leachates were defined; 0.05 *M* borate buffer at pH 9.0 with an applied voltage of 10.5 kV was used. Under these conditions, quantification of the phenolics was achieved. Calibration data are given in Table 1.

As observed, the linear dynamic ranges of the calibration graphs differ by two orders of magnitude at the μg/ml level, with poorer correlation coefficients than in GC or LC. They are 0.979, 0.985, 0.997 and 0.982 for cresol, resorcinol, methylsuccinic acid and crotonaldehyde, respectively.

Detection limits, listed in Table 2, were measured at a signal-to-noise ratio of 3. The detection limits are obviously higher than in GC or LC owing to the shorter light-path in on-line detection. After being sequentially filtered with a 1-μm glass-fibre filter and a 0.45-μm PVDF

Table 2
Detection limits of phenolics using CZE

Compound	Detection limits[a] (μg/l)	Compound	Detection limits (μg/l)[a]
Resacetophenone	40	Catechol	200
Cresol	20	Syringic acid	200
Resorcinol	40	*p*-Coumaric acid	100
Acetovanillone	100	Vanillic acid	30
Hydroxyphenylacetic acid	100	Crotonaldehyde	30
Vanillin	100	Ferulic acid	40
Methylsuccinic acid	40	Gallic acid	40

[a] Instrumental detection limits.

filter, the leachate sample was injected hydrodynamically into the CZE column. Experiments were performed under the proposed conditions. The measured concentrations were 98.7 ± 3.4, 1.7 ± 0.2, 2.3 ± 0.2 and 57.6 ± 1.8 μg/ml ($n = 5$) for cresol, resorcinol, crotonaldehyde and methylsuccinic acid, respectively. On adding 20 μg/ml of these four phenolic to the leachate sample, the CZE method gave recoveries of 97.2% (R.S.D. 3.17%), 101.5 (R.S.D. 2.33%), 104.2 (R.S.D. 3.62%) and 98.1% (R.S.D. 2.96%) ($n = 5$) for cresol, resorcinol, crotonaldehyde and methylsuccinic acid, respectively.

4. Conclusions

This study has demonstrated the applicability of the CZE–UV method to the separation of fourteen plant phenolics in aqueous samples. The rather high UV detection limits of the phenolics could be improved by analysing lower concentration samples. However, the present performance is acceptable for determining phenolics in landfill leachates.

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