

APPLICATION OF HISTIDINE-BONDED PHASE CAPILLARY COLUMN TO ELECTROPHORETIC SEPARATION OF PLANT PHENOLS

Hui-Huang Chiang, Chia-Chann Shiue, Yi-Fen Pai, and
Chuen-Ying Liu*

Department of Chemistry, National Taiwan University,
Taipei 10617, Taiwan

ABSTRACT

A fused-silica capillary that is wall-modified, via chemically bonding histidine functional groups to the capillary wall, is described. With benzyl alcohol as neutral marker, the electroosmotic flow (EOF) of the bonded phase is reversed and the migration velocity increases slightly as the pH decreases. Changes in the type, concentration, and pH of the buffer, as well as the applied voltage, were performed to determine the optimum condition for the simultaneous separation of neutral and anionic plant phenols. A separation of (+)-catechin, gallic acid, caffeic acid, (–)-epicatechin, salicylic acid, quercetin, and *p*-coumaric acid could be obtained on a 100 cm × 75 μm I.D. column under acetate buffer (10 mM, pH 5.0) and an applied voltage of –20 kV with good peak shapes and efficiencies over the range 7.46×10^5 to 3.86×10^6 plates per meter. The amount of plant phenols in wines has also been determined with the established method. Quantitative analysis was performed by the standard addition method. The mechanism of the separation of the analytes is based on a combination of differences in electrophoretic mobility and solute/attached moiety interactions.

INTRODUCTION

Capillary electrochromatography (CEC) is a form of high performance liquid chromatography (HPLC) which uses an electric field to drive the eluent through

*Corresponding author. E-mail: cyliu@ccms.ntu.edu.tw

the column (1). The continuing interest in CEC is shown by the increasing number of papers that are being published on this technique. Most CEC experiments have been performed with packed columns. However, problems such as difficulties in packing the small LC packing materials and fabricating the frits still exist in preparing the CEC columns. Compared to packed columns, open-tubular columns do not require the tedious packing procedures and frit fabrication and, therefore, seem very promising in CEC applications (2–7).

Several studies indicate that catechins and polyphenols in general, from fruits, vegetables, tea, and red wine have prophylactic properties that are beneficial to human health. High performance liquid chromatography (HPLC) has been the most useful approach for the determination of catechins and caffeine in aqueous and biological samples (8,9). Capillary zone electrophoresis (CZE) has been used to analyze polyphenols in tea samples (10–12). Several micellar electrokinetic chromatographic (MEKC) methods have also been developed for the separation of plant phenols and the resolution of the analytes was shown to be much better than that with CZE (13–16). Most of these methods utilize borate or boric acid as selector for polyphenols with vicinal hydroxyl groups in conjunction with hydrophobic interactions with the surfactant to achieve separation of the analytes. However, poor consistency and reproducibility, as well as inefficient detection of some polyphenols, have been reported by Lee and Ong (12).

New stationary phases with high selectivities that adequately separate very similar analytes in complex matrix samples are continuously being sought. The isoelectric ampholyte property of histidine seems very promising in analytical application. Indirect photometric detection of anions in capillary electrophoresis, using dyes as probes and electrolytes buffered with histidine, has been reported by Johns et al. (17). A new buffer system containing histidine, crown ether, and lactic acid has also been reported by Fung et al. for the analysis of leachable and total trace metals in air particulate matter by capillary electrophoresis (18). Enantioseparation of underivatized amino acids by capillary electrophoresis, using copper(II)-(S)-3-aminopyrrolidine-L-histidine ternary complex as the chiral selector, has been reported by Zhao and Liu (19). In our previous work, histidine-functionalized silica was prepared and acted as the packing material of packed CEC for the separation of both organic and inorganic anions (20).

In this work, a fused-silica capillary that has been wall-modified via chemically bonding histidine functional groups to the capillary wall will be described for the separation of plant phenols.

EXPERIMENTAL

Apparatus

The open-tubular CEC experiments were performed with a SpectraPHORESIS 100 electrophoresis system (Thermo Separation Product, Fremont, CA, USA)

equipped with an UV absorbance detector (Spectra Focus Scanning CE detector) using PC 1000 software Ver. 3.0 for system control, data acquisition, and data analysis.

Reagents and Chemicals

Fused silica (100 μm I.D.) capillaries were purchased from Resteck (Bellefonte, PA, USA). Most chemicals were analytical reagent grade from Merck (Darmstadt, Germany). Purified water (18 M Ω -cm) from a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to prepare solutions. All solvents and solutions for CEC analysis were filtered through a 0.45 μm PTFE membrane (Millipore) and degassed prior to use. Acetic acid, hydrochloric acid, acetone, and methyl alcohol (Merck, Darmstadt, Germany), sodium acetate and sodium hydroxide (Wako, Japan), 3-chloropropyltrimethoxysilane (Aldrich, Milwaukee, WI, USA), *m*-xylene (Janssen, Belgium), gallic acid, salicylic acid, (+)-catechin, (-)-epicatechin, caffeic acid, quercetin, *p*-coumaric acid, histidine, and dimethyl sulfoxide (Sigma, St. Louis, MO, USA), benzene and benzyl alcohol (Acros, Switzerland) were purchased from the indicated sources. All liquid reagents and solvents used in moisture-sensitive reactions were distilled and collected over type 4A molecular sieve. Wines (red and white) were purchased from the supermarket.

Preparation of the Bonded Phase Capillary Column

Fused-silica capillaries (100 cm \times 75 μm I.D.) were first flushed with 1 M NaOH (30 min), then pure water (15 min), 1 M HCl (30 min) and pure water (15 min). Before silanization, the capillaries were rinsed with methanol (5 min) and then dried in a gas chromatography oven at 110°C for 1 h under a nitrogen flow of 2.5 kg cm⁻². The pretreated capillary column was first filled with 3-chloropropyltrimethoxysilane in *m*-xylene (10%, v/v). Both ends of the capillary were sealed with septa, and the capillary was heated at the same condition overnight in a GC oven. Next, the column was flushed with *m*-xylene and reconnected to the GC oven and dried at 110°C under nitrogen flow. The coating solution, consisting of histidine in dimethyl sulfoxide (2%, w/v), was preheated at 160°C. The silylated capillary was filled with the coating solution at a temperature of 110°C, then sealed at both ends and heated in the GC oven at 150°C overnight. The resulting column was flushed successively with acetone and pure water at ambient temperature.

Electrophoresis Conditions

Before analysis, the coated capillaries were preconditioned with the running buffer. They were rinsed with methanol–water (1:9, v/v) and buffer between runs. Stock solutions of the analyte were prepared in methanol. The running buffer and

the sample solutions were degassed in an ultrasonic bath for 5 min prior to use. Samples were introduced electrokinetically at the cathodic end of the capillary column. The analytes were detected by monitoring their absorbance at 280 nm.

RESULTS AND DISCUSSION

Characterization of the Bonded Phase Capillary Column

Since the nature of the chemical group bound to the column surface, the degree of surface deactivation, and the phase thickness determine the dependence of the EOF on pH, and also the direction of EOF, quantitation of EOF was used to measure the effectiveness of bonding procedures (21). The EOF velocities of both bonded phase and the bare fused-silica were determined, according to the migration time of benzyl alcohol which served as the neutral marker. Here, the sample injection for the bare fused-silica was made from the positive end, while that for bonded phase was from negative end. Figure 1 shows the dependence of

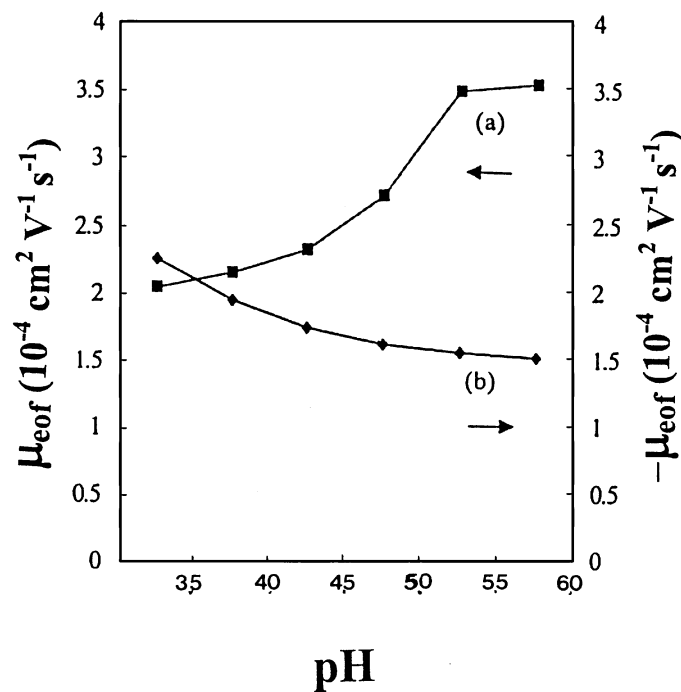
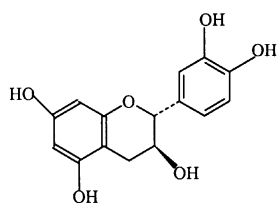


Figure 1. Effect of pH on electroosmotic flow mobility in various capillary materials. Curve a: Untreated fused-silica capillary; Curve b: Covalent surface modification with histidine. Column: 100 cm (80 cm to detector) \times 100 μm I.D.; Neutral marker: benzyl alcohol; Background electrolyte: acetate buffer (10 mM); Detection: UV at 280 nm. a. Column: bare fused-silica; Sample injection: electrokinetic (5 kV, 10 s); Applied voltage: 20 kV; b. Column: histidine-bonded phase; Sample injection: electrokinetic (-5 kV, 10 s); Applied voltage: -20 kV.

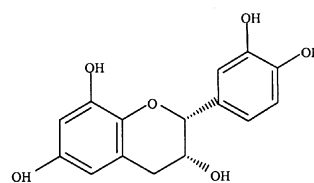
the EOF on pH in acetate buffer (10 mM). For the bare capillary, a sharp increase for the EOF with increasing pH was observed, due to a greater dissociation of the silanol group under higher pH. With binding of the histidine moiety to the capillary wall, a steady decrease for the EOF was indicated as the buffer pH increased. Since the isoelectric point (pI) of histidine (pK_{a1} , 1.82; pK_{a2} , 6.0; pK_{a3} , 9.2) is 7.59, cationic properties in acidic medium and the effective charge increase as the pH decreases are to be expected.

Separation of Plant Phenols

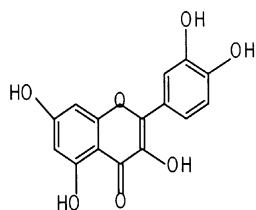
The prepared column was evaluated for the separation of plant phenols. Figure 2 shows the structure of the selected compounds.



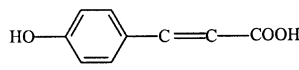
(+) catechin
($C_{15}H_{14}O_6$)



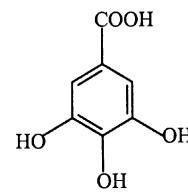
(-) epicatechin
($C_{15}H_{14}O_6$)



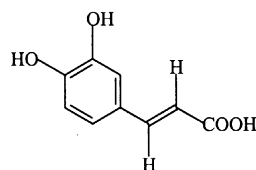
quercetin
($C_{15}H_8O_4$)



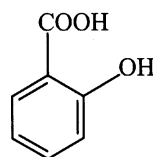
p-coumaric acid
($C_9H_8O_3$)



gallic acid
($C_7H_6O_5$)



caffeic acid
($C_9H_8O_4$)



salicylic acid
($C_7H_6O_3$)

Figure 2. Structures of the model compounds.

Effect of Buffer pH

Catechins have been reported to be chemically unstable in an alkaline environment (16). By considering the pI value of histidine and the ionization of the plant phenols, a pH range of 3–6 seems a better choice for the separation of these analytes. Hence, phosphate and acetate buffer were tested as possible background electrolytes. Peak tailing was observed for most of the compounds when phosphate was used as the running buffer. The use of acetate buffer improved the peak shape. A good match in conductivity for the analyte and the BGE might be the reason. With acetate buffer (10 mM) and applied voltage of -20 kV, good resolution for the caffeic acid/salicylic acid/(+)-catechin/(–)-epicatechin/gallic acid/queracetin/*p*-coumaric acid mixture was provided over the pH range of 4~5. However, the polyphenols showed several broadened peaks at pH above 5.5, especially for the analytes with slower migration. The influence of pH values on the electrophoretic mobilities of plant phenols is shown in Fig. 3.

An increase in the pH range from 4 to 5 showed a significant increase in the electrophoretic mobility for caffeic acid and salicylic acid. Buffer pH had only a

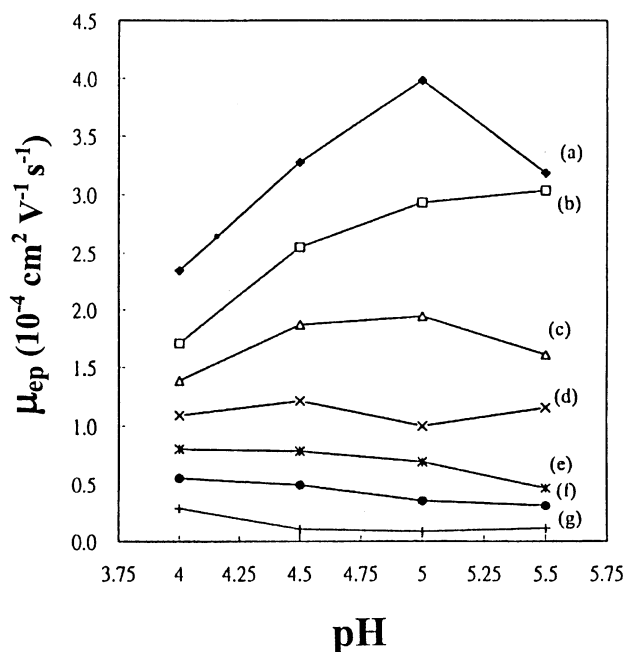


Figure 3. Influence of pH on the electrophoretic mobility for the separation of plant phenols. Column: histidine-bonded phase, 100 cm (80 cm to detector) \times 100 μ m I.D.; Background electrolyte: acetate buffer (10 mM); Sample injection: electrokinetic (-5 kV, 10 s); Applied voltage: -20 kV; Detection: UV at 280 nm. (a) caffeic acid, (b) salicylic acid, (c) (+)-catechin, (d) (–)-epicatechin, (e) gallic acid, (f) queracetin and (g) *p*-coumaric acid.

minor influence on the electrophoretic mobility of (+)-catechin, (–)-epicatechin, gallic acid, quercetin, and *p*-coumaric acid. Moreover, a significant decrease in the electrophoretic mobility for caffeic acid and (+)-catechin was noted at pH values above 5, while salicylic acid exhibited a different behavior.

Effect of Buffer Concentration

The acetate buffer concentration was tested in the range 2~20 mM. An increase of the buffer concentration decreases the thickness of the electric double layer at the liquid-solid interface (22). This will reduce the EOF. An increase in acetate concentration over the range of 5~10 mM showed an increase in electrophoretic mobility for caffeic acid, salicylic acid, (+)-catechin, (–)-epicatechin, and gallic acid but not for quercetin and *p*-coumaric acid (Fig. 4). The last two had a higher affinity for the bonded phase. The concentration of 10 mM provided good resolution of almost all the compounds. Higher concentrations did not increase the resolution. The effect of Joule heating might be more pronounced.

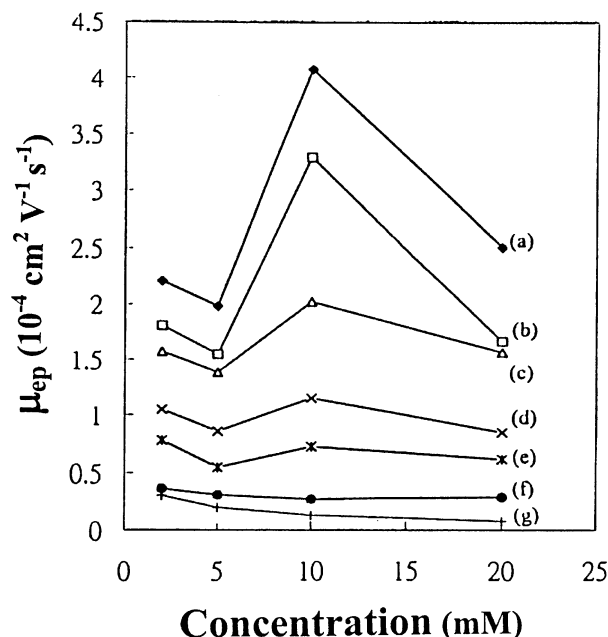


Figure 4. Influence of buffer concentration on the electrophoretic mobility for the separation of plant phenols. Column: histidine-bonded phase, 100 cm (80 cm to detector) \times 100 μ m I.D.; Background electrolyte: acetate buffer (pH 5.0); Sample injection: electrokinetic (–5 kV, 10 s); Applied voltage: –20 kV; Detection: UV at 280 nm. (a) caffeic acid, (b) salicylic acid, (c) (+)-catechin, (d) (–)-epicatechin, (e) gallic acid, (f) quercetin and (g) *p*-coumaric acid.

Separation Mechanism

Gallic acid, caffeic acid, salicylic acid, and *p*-coumaric acid are in the anionic form under the experimental conditions, while all the other phenols are in the neutral molecular form (Table 1). Since the bonded phase provided a cationic property on the capillary surface, it could be behaving as an anion exchanger. The charge-to-size ratio decreases in the order: salicylic acid > gallic acid > caffeic acid > *p*-coumaric acid > (–)-epicatechin ≈ (+)-catechin > quercetin. In view of the sorption selectivity of organic ions on an ion exchanger, van der Waals forces and hydrophobic interactions can often be important factors in the sorption mechanism in addition to the electrostatic force. Quercetin, (+)-catechin, and (–)-epicatechin have five –OH groups, which would form more bonding sites with the bonded phase than any of the other analytes. Quercetin has an additional carbonyl group. Formation of more hydrogen bonds than catechins would be expected. Gallic acid, salicylic acid, caffeic acid, and *p*-coumaric acid all have three to four conjugated double bonds in addition to the carboxylate anion. *p*-Coumaric acid has a linear structure and, hence, the least steric hindrance towards interaction with the capillary surface. Gallic acid has one more –OH group than caffeic acid. More hydrogen bond formation would occur with the bonded phase. Salicylic acid has an –OH group in the *o*-position, which might lead to a greater steric hindrance for bonding with the bonded phase than gallic acid. In the electrophoretic mode, compounds with greater charge-to-size ratio would migrate faster. In this case, the elution order was caffeic acid > salicylic acid > (+)-catechin > (–)-epicatechin > gallic acid > quercetin > *p*-coumaric acid. Based on the above discussion, both electrophoretic and anion exchange mechanisms might be the predominant role in the separation mechanism, although other complicated interactive force may also be involved.

Effect of Column Size

With variation of the column diameter under acetate buffer (10 mM, pH 5.0) and an applied voltage of –20 kV faster separation for the smaller capillary was indicated (Fig. 5). But, the overall performance of the 100 μm I.D. capillary is superior than that of the smaller capillary (75 μm I.D.) (Table 2). A greater thickness of the coating for the latter might be the reason.

Column Stability

A strong adsorption between the analytes and the capillary wall of the bonded phase was observed, especially at the lower BGE concentration. This might

Table 1. Chemical and Physical Properties of the Model Compounds

Analyte	pK_a^a (25°C, $\mu = 0$)	Effective Charge			
		pH 4.0	pH 4.5	pH 5.0	pH 5.5
(+)-Catechin (MW, 290)	8.64; 9.41; 11.26	0	0	0	0
Gallic acid (170)	4.27; 8.68; 11.45	0.351	0.630	0.843	0.945
Caffeic acid (180)	4.62; 9.07	0.193	0.431	0.706	0.883
(-)-Epicatechin (290)	8.72; 9.49; 11.23; 13.40	0	0	0	0
Salicylic acid (138)	2.81; 13.4	0.939	0.980	0.994	0.998
Quercetin (302)	b	b	b	b	b
<i>p</i> -Coumaric acid (164)	b	b	b	b	b

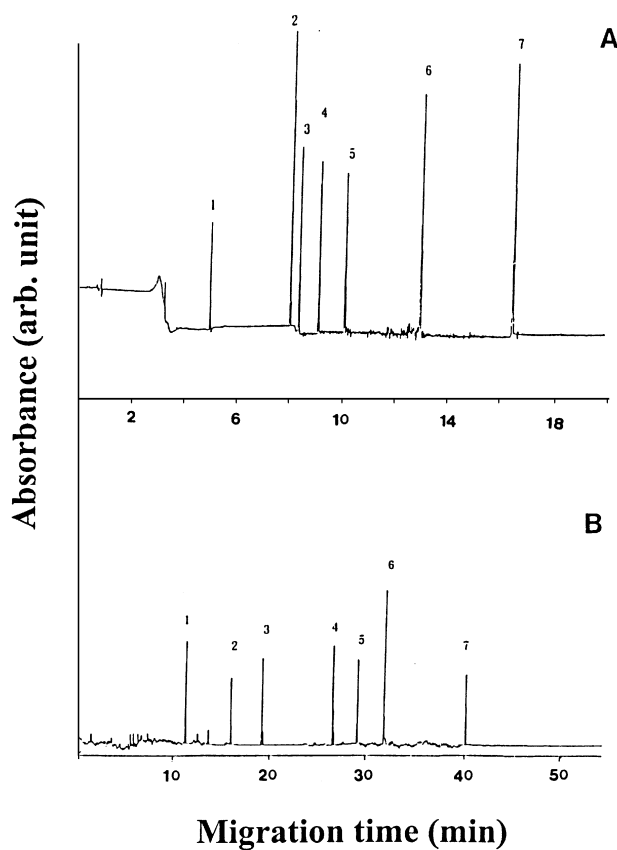
^aData from Refs. (26,27).^bData not available.

Figure 5. The effect of column dimensions on the separation efficiency. Background electrolyte: acetate buffer (10 mM, pH 5.0); Sample injection: electrokinetic (−5 kV, 10 s); Applied voltage: −20 kV; Detection: UV at 280 nm. Peak identification: 1. caffeic acid, 2. salicylic acid, 3. (+)-catechin, 4. (−)-epicatechin, 5. gallic acid, 6. quercetin and 7. *p*-coumaric acid. Column: histidine-bonded phase, A. 100 cm (80 cm to detector) × 100 μm I.D. B. 100 cm (80 cm to detector) × 75 μm I.D.

Table 2. Reproducibility and Efficiency for the Separation of Plant Phenols with the Histidine-Coated Capillary^a

Compound	Migration Time (\pm RSD, $n = 5$)	Theoretical Plate (m^{-1})	Plate Height (μm)
Caffeic acid	12.16 (1.6%)	7.46×10^5 (5.93×10^5) ^b	1.07 (1.35) ^b
Salicylic acid	17.23 (2.3%)	2.08×10^6 (1.06×10^6)	0.38 (0.75)
(+)-Catechin	19.22 (0.64%)	2.11×10^6 (4.18×10^5)	0.38 (1.91)
(-)-Epicatechin	27.11 (0.87%)	2.66×10^6 (7.17×10^5)	0.30 (1.12)
Gallic acid	29.32 (0.69%)	3.86×10^6 (1.31×10^6)	0.21 (0.61)
Quercetin	32.67 (1.03%)	1.48×10^6 (7.38×10^5)	0.54 (1.08)
<i>p</i> -Coumaric acid	40.52 (0.98%)	2.20×10^6 (9.17×10^5)	0.36 (0.87)

^aColumn: Histidine-bonded phase (100 cm \times 100 μm I.D.); Sample injection: -5 kV, 10 s; Background electrolyte: acetate buffer (10 mM, pH 5.0); Applied voltage: -20 kV; Sample concentration: 0.1 mM for each; Detection: UV-Vis at 280 nm.

^bHistidine-bonded phase (100 cm \times 75 μm I.D.).

be due to the hydrophobic interaction of the aromatic ring and conjugated double bond of the polyphenols. Therefore, a methanol-water mixture (1:9, v/v), followed by acetate buffer, was needed as the washing solution between runs. If extensive strong adsorption occurs, 0.1 mM hydrochloric acid may be necessary to improve the cleaning.

The prepared column could be used for a long period of time, up to 1 month, without noticeable change in the separation properties. The relative standard deviation (RSD) of the migration times for five injections within-day was better than $\pm 1.0\%$ for most of the analytes (Table 2). The day-to-day RSD of migration times obtained at interval of 10 tests among 50 injections ($n = 5$) was better than $\pm 2.0\%$ for most analytes, except the two early eluting analytes, caffeic acid and salicylic acid (RSD = 3.5%).

Analytical Application

The fundamental aspects of a new stationary phase are of utmost importance in the further development of the column for the analysis of complex matrix samples. Red wines may have very complex phenolic composition that changes over their shelf-life (24). The occurrence of these substances in wines is not only a consequence of their extraction from grapes during winemaking. Once grapes are crushed, before the beginning of alcoholic fermentation, several condensation reactions which involve some of those molecules, especially anthocyanins, catechins, and procyanidins take place, resulting in the formation of new polymeric pigments (25).

The method developed above was used to determine polyphenols present in wines. Both red and white wine were 10-fold diluted with pure water, then filtered

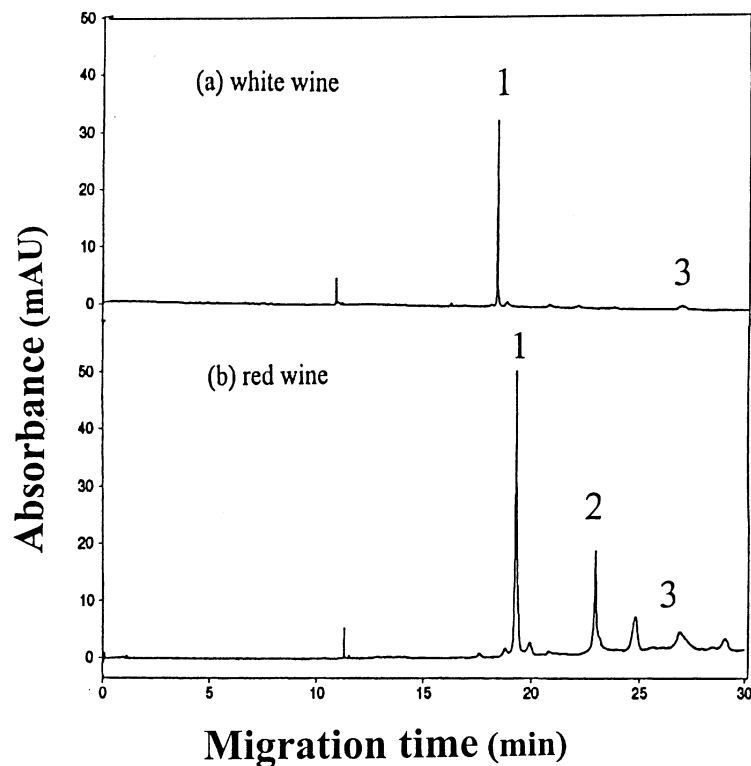


Figure 6. Electropherogram for the determination of catechin in wine. Column: histidine-bonded phase, 100 cm (80 cm to detector) \times 100 μ m I.D.; Background electrolyte: acetate buffer (10 mM, pH 5.0); Sample injection: electrokinetic (-5 kV, 10 s); Applied voltage: -20 kV; Detection: UV at 280 nm. Sample injected after ten-fold dilution with pure water (a) white wine (b) red wine. Peak identification: 1. (+)-catechin.

through a membrane (cellulose, 0.45 μ m) and directly injected (-5 kV, 10 s) into the system. Figure 6 shows the electropherogram of the wine polyphenols. The peaks were identified using the standard addition method. Using multiple standard addition ($n = 5$), (+)-catechin was found more abundant in red wine ($11.9 \pm 0.7 \mu$ M) than that in the white wine ($3.8 \pm 0.3 \mu$ M). The linear equation for the catechin determination in red wine was $y = 1.65x + 19.67$ ($r^2 = 0.9972$) and that in white wine was $y = 2.88x + 10.85$ ($r^2 = 0.9983$), where y is the peak area (μ V) and x is the spiked concentration (μ M). Some additional peaks in red wine were also observed, but were not identified. According to the literature (23), (+)-catechin and (–)-epicatechin are flavonoids that are widely distributed in plant-derived foods, including red wine, green tea, chocolate, and many fruits. The result seems rational.

CONCLUSIONS

With this new bonded phase, under the experimental condition of pH 3 ~ 6, the EOF was reversed. Highly efficient separation of seven polyphenols, including the optical isomers, (+)-catechin and (-)-epicatechin, was achieved within 40 min. Only 17 min was needed if the smaller diameter tube (75 mm I.D.) was used. It could be employed for the determination of plant phenols in many plant-derived foods. These coatings appear attractive; strong adsorption was seen due to the hydrophobic interaction of the aromatic ring and the conjugated double bond of the analytes. Moreover, methanol/water mixture (1:9, v/v), followed by pure water as the washing solution, could be used to solve the problem.

ACKNOWLEDGMENT

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