Chemically Bonded Fullerene C_{60} Capillary Column for the Electrophoretic Separation of Plant Phenols

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A novel mixed-mode stationary phase for open tubular capillary electrochromatography (CEC) was prepared. With 3-aminopropyltrimethoxysilane as the spacer, the chemical reagent in conjunction with the fullerene played an important role in the electrochromatographic separation of the plant phenols. The fullerene C_{60}-based column has the properties of both hydrophilic and hydrophobic interaction sites and provides a positively charged capillary surface responsible for the reversed electroosmotic flow (EOF) in the column during CEC operation. Comparative studies for the fullerene-bonded phase, the intermediate phase with amino group and the bare fused-silica were carried out. Under the conditions of acetate buffer (20 mM, pH 5.0) and applied voltage of -15 kV, the separation of catechin, salicylic acid, myricetin, quercetin, gallic acid, caffeic acid and *p*-coumaric acid could be achieved. The proposed method was also applied to the analysis of plant phenols in grape juice.

INTRODUCTION

Polyphenolic compounds play an important role as natural potent antioxidants, exhibiting various physiological and biological activities, such as inflammatory, anti-allergic and anti-carcinogenic activities, in the human metabolic system.[1] These compounds are found in relatively high concentrations in tea, but only low levels have been identified in other food products such as some fruits and vegetables, wines and buckwheat.[2] High performance liquid chromatography (HPLC) has been the most useful approach for the determination of polyphenols in aqueous and biological samples. Some methods optimize the elution by using the isocratic mode and others by gradient elution, but nearly all workers have adopted reversed phase C_{18} columns.[3-5] Capillary zone electrophoresis (CZE) has been used to analyze polyphenols in tea samples[6,7] but the micellar electrokinetic chromatography (MEKC) methods provide better separation, resolution and quantitation for a larger number of catechins than do the rudimentary CZE methods.[8,9]

Silica has been modified by introducing substituted arenes, phthalocyanins, or tetraphenylporphyrins for the separation of Fullerene C_{60}.[10-12] The opposite approach, namely the use of fullerenes in the modification of HPLC chromatographic supports, has not received comparable attention.[13] Fullerene was reported for the first time as a new stationary phase in capillary gas chromatography for the separation of various organic compounds in 1993.[14] The novel stationary phases of microcolumn liquid chromatography have also been reported for the separation of polycyclic aromatic hydrocarbons[15] and calixarenes.[16]

In view of the above mentioned literature, modification of the capillary column inner wall with buckerminster fullerene C_{60} yielding a neutral surface for the electrophoretic separation sould be an approach of high promise. Since the aromatic portion of the analytes was expected to interact efficiently with the fullerene cage, polyphenols were used as test solutes to investigate the contributions of various migration and separation mechanisms.

EXPERIMENTAL

Apparatus

A high-voltage power supply with a 30 kV capacity (Model 890-CE, Jasco, Tokyo, Japan), a variable-wavelength UV/Vis detector (Jasco 870-CE) and an integrator (Jasco 807-IT) were employed for capillary electrophoresis. The separations were carried out on a fused-silica capillary column with an external coating of polyimide and chemically modified with fullerene. The modified capillaries were of 75 μm i.d. and the total length of the capillary was 80 cm, with a distance of 50 cm between the injection end and the detection window.

Reagents and Chemicals

Fused silica (75 μm i.d.) capillaries were purchased from Resteck (Bellefonte, PA, USA). Most chemicals were of

Dedicated to Professor Sheng-lieh Liu on the occasion of his ninetieth birthday.

an a lyt i cal re agent grade from Merck (Darmstadt, Ger many). Pu rified wa ter (18 MΩ-cm) from a Milli-Q wa ter pu ri fi ca tion sys tem (Millipore, Bed ford, MA, USA) was used to pre pare all so lu tions. 3-Aminopropyltrimethoxysilane was ob tained from Aldrich (Mil wau kee, WI, USA). Ace tic acid, ac e tone, ni tric acid, hy dro chlo ric acid, and ethyl al co hol (Merck), so-dium ac e tate and so dium hy drox ide (Wako, Ja pan), gal lic acid, sal i cylic acid, (+)-catechin, (-)-epicatechin, caffeic acid, quercetin, myricetin, *p*-coumaric acid (Sigma, St. Louis, MO, USA), to lu ene and benzyl al co hol (Acros, Switerland), fullerene from BuckyUSA (Bellaire, USA) were pur chased from the in di cated sources. All liq uid re agents and sol vents used in mois ture-sensitive re ac tions were dis tilled and col-lected over type 4 Å mo lec u lar sieves. All sol vents and so lu-tions for CEC anal y sis were fil tered through a 0.45 μm PTFE mem brane (Millipore) and de gassed prior to use.

Prep a ra tion of Open-tubular Col umns

Fu sed-silica cap il lar ies (80 cm × 75 μm i.d.) were first flushed with 1 M NaOH (30 min), then pure wa ter (15 min), 1 M HCl (30 min) and pure wa ter (15 min). Be fore silanization the cap il lar ies were rinsed with meth a nol (5 min) and then dried in a gas chro ma tog ra phy oven at 110°C for 1 h un der ni-tro gen flow of 2.5 kg cm^{-2}. The pretreated cap il lary col umn was filled with 3-aminopropyltrimethoxysilane (0.5 mL) and fullerene (1.5 mg) in to lu ene (5 mL). Both ends of the cap il-lary were sealed with septa, and the cap il lary was heated un-der the same con di tions over night in a GC oven. Next the col-umn was flushed with to lu ene (10 min) fol lowed with ethyl al co hol (10 min) and re con nected to the GC oven and dried at 150 °C under nitrogen flow. The resulting column was flushed suc ces sively with ac e tone and pure wa ter at am bi ent temperature.

Electrophoresis Conditions

Be fore anal y sis the coated cap il lar ies were pre con di-tioned with the run ning buffer. They were rinsed with buffer be tween runs. Stock so lu tions of the analyte were pre pared in meth a nol. The run ning buffer and the sam ple so lu tions were de gassed in an ul tra sonic bath for 5 min prior to use. In jec tion of the sam ples was done electrokinetically. The analytes were de tected at 215 nm.

RE SULTS AND DIS CUS SION

Characterization of the Fullerene-Coated Capillary Column

The wall-coated fullerene cap il lary col umn was pre-pared by chemical modification of the fused silica with 3-aminopropyltrimethoxysilane, fol lowed by a bond- form-ing step with fullerene as shown in Fig. 1. For char ac ter iza-tion of the pre pared col umn, the di rec tion and quantitation of the EOF were mea sured. In ac e tate buffer (20 mM) with ap-plied volt age of -15 kV, the in ter me di ate, Si-NH_2 and the fi-nal prod uct, Si-NHC_{60} pro vided the an odic EOF (Fig. 2). Since the amino groups in tro duced into the sil ica sur face would be protonated at the pH range stud ied, a dif fer ent EOF di rec tion from that of the bare fused sil ica was to be ex pected. Wall- coated fullerene cre ates a neu tral sur face but there are some unreacted amino groups. Mean while protonation of the sec ond ary amino group in the fullerene-bonded phase would oc cur, al though it is not so easy as protonation of the pri mary amino group. There fore the fi nal prod uct yields a smaller EOF ve loc ity than the in ter me di ate.

Separation of Plant Phenols

For eval u a tion of the pre pared col umn, the fol low ing eight analytes were cho sen: (+)-catechin, (-)-epicatechin, caffeic acid, gal lic acid, myricetin, quercetin, *p*-coumaric acid and sal i cylic acid. Fig. 3. shows the struc tures of the model com pounds.

Ef fect of pH and con cen tra tion of ac e tate buffer

The pK_a val ues of most analytes are be tween 8 and 10 ex cept for phe no lic ac ids. This causes them to be neu tral to par tially an ionic in the tested pH range of 4.0 to 7.0. With ac-e tate buffer (20 mM, pH 4.0) and an ap plied volt age of -15 kV, only four peaks were ob served (Fig. 4), in which myri-

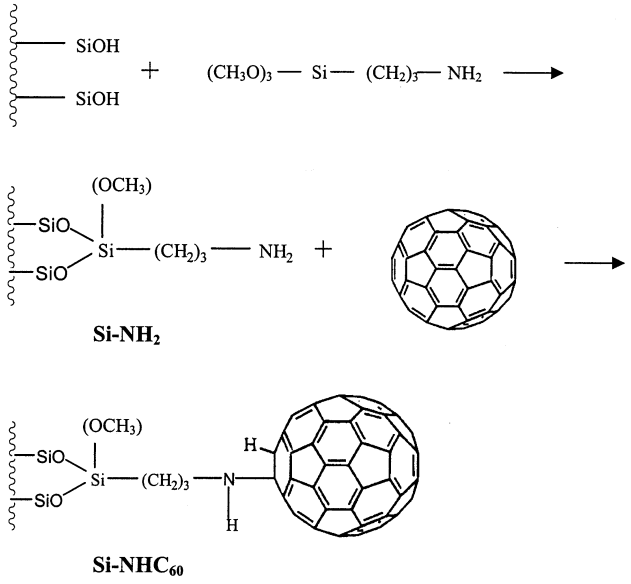

Fig. 1. The pro ce dures for the prep a ra tion of chem i-cally bonded fullerene C_{60} cap il lary col umn.

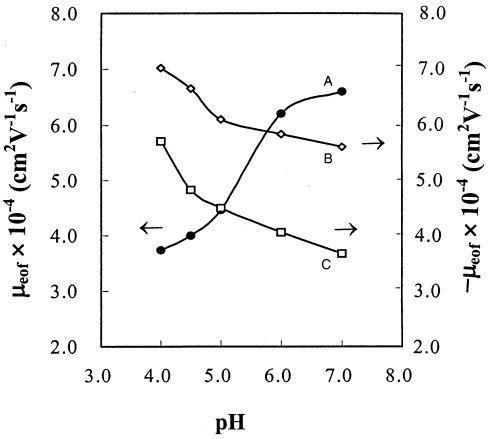

Fig. 2. In flu ence of pH on electroosmotic flow mo bility in var i ous cap illary ma te ri als. Cap illary column: 80 cm × 75 mm i.d. (50 cm to de tec tor). Curve A: bare fused sil ica. Curve B: amino-bonded phase. Curve C: fullerene-bonded phase. Electrokinetic in jec tion: A. 5 kV, 10 s; B and C. -5 kV, 10 s. Back ground elec tro lyte: ac e-tate buffer (20 mM). De tec tion: UV at 215 nm. Ap plied volt age: A. 15 kV. B and C. -15 kV.

cetin and quercetin were coeluted. Gal lic acid, caffeic acid and *p*-coumaric acid were nonbaseline re solved. At pH 5.0 on the other hand, seven re solved peaks and faster mi gra tion for most of the analyte were dem on strated. A greater dis so ci a-tion of the phe no lic ac ids may ex plain this phe nom ena. In the above cases, catechin mi grated faster than myricetin and quercetin. An ex tra ketone group pres ent in the myricetin and quercetin and more hydrogen bond formation with the bonded phase than catechin may ex plain this be hav ior. As can be seen in Fig. 3, myricetin pos sesses one more -OH group than quercetin. More hydrophilic behavior might therefore result in a faster migration for myricetin than quercetin. When the buffer pH was in creased to 6.0 and 7.0, the re duc tion of the EOF due to lower protonation of the amino group in the bonded phase led to a poorer res o lu tion for the pairs of myricetin and quercetin as well as caffeic acid and *p*-coumaric acid. Based on the above-mentioned re sults, the use of ac e tate buffer at pH 5 is rec om mended.

Conventionally, as the buffer concentration is in-creased, the elec tro pho retic and electroosmotic mo bil i ties are both re duced. The net mo bil ity is, there fore, ex pected to

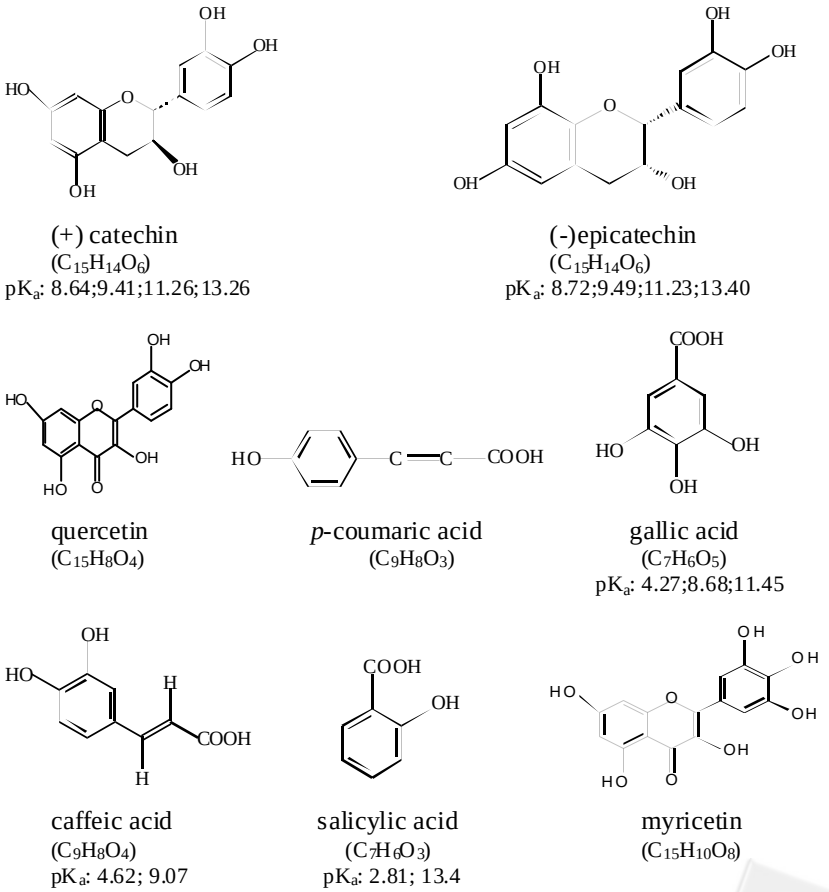

Fig. 3. Struc tures of the model-compounds.

fol low the same trend. More over in this work, in creas ing the buffer con cen tra tion from 20 to 80 mM, the mi gra tion of the analytes de creased steadily and no better res o lu tion was in di-cated. The rea son might be due to the fact that most analytes are neu tral to par tially an ionic in the tested pH range. There-fore the buffer con cen tra tion used through out this work was 20 mM.

Ef fect of ap plied volt age

Vari a tion of the ap plied volt age from -5 kV to -25 kV un der ac e tate buffer (20 mM, pH 5.0) has been tested. It does not mod ify sig nif i cantly the ap par ent mo bil i ties and the res o-lu tion. There fore we adopted the in ter me di ate volt age of -15 kV for fur ther ex per i ments.

Ef fect of type of buffer

Be sides buffer pH and con cen tra tion, buffer type might also in flu ence re ac tion rate and sep a ra tion mech a nism. It has al ready been dem on strated that ac e tate buffer (20 mM, pH 5.0) with an ap plied volt age of -15 kV al lows good sep a ra tion of plant phe nols, ex cept for the iso mer of catechins, (+)-catechin and (-)-epicatechin (Fig. 3b). Thus phos phate buffer was also tested as the back ground elec tro lyte. A faster mi gra-tion for the cor re spond ing analytes un der sim i lar con di tions as those of ac e tate buffer were dem on strated. But the pairs of myricetin and quercetin as well as gal lic acid and caffeic acid were coeluted.

Separation Mechanism

In or der to elu ci date the role of fullerene, the mi gra tion be hav ior was also com pared with those of the bare fused sil-ica and the in ter me di ate amino bonded phase (Fig. 5). The re-sults can be ex plained as fol lows. Since the analytes have very sim i lar elec tro pho retic mo bil ity, bare fused sil ica cap il-lary can not be used for the sep a ra tion un der the tested con di-tion. When the amino bonded phase was employed, only some re solved peaks were in di cated. Mean while sal i cylic acid was eluted last among the analytes, and a faster mi gra-tion for the analytes was ob served due to a greater EOF than

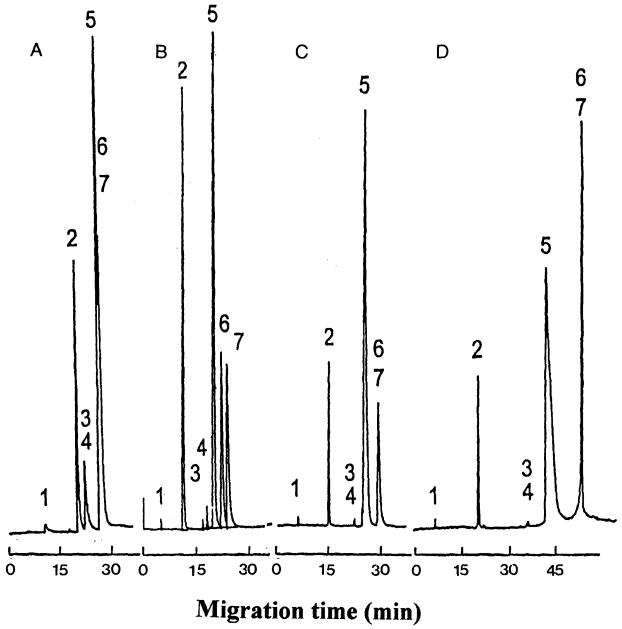

Fig. 4. Ef fect of pH on the sep a ra tion of plant phe nols. Cap il lary col umn: fullerene-bonded phase, 80 cm × 75 mm i.d. (50 cm to de tec tor). Electro-kinetic in jec tion: -5 kV, 10 s. Ap plied volt age: -15 kV. Analyte concentration: 0.1 mM for each. De tec tion: UV at 215 nm. Back ground elec tro lyte: ac e tate buffer (20 mM), A. pH 4.0. B. pH 5.0. C. pH 6.0. D. pH 7.0. Peak iden ti fi ca-tion: 1. catechin. 2. sal i cylic acid. 3. myricetin. 4. quercetin. 5. gal lic acid. 6. caffeic acid. 7. *p*-coumaric acid.

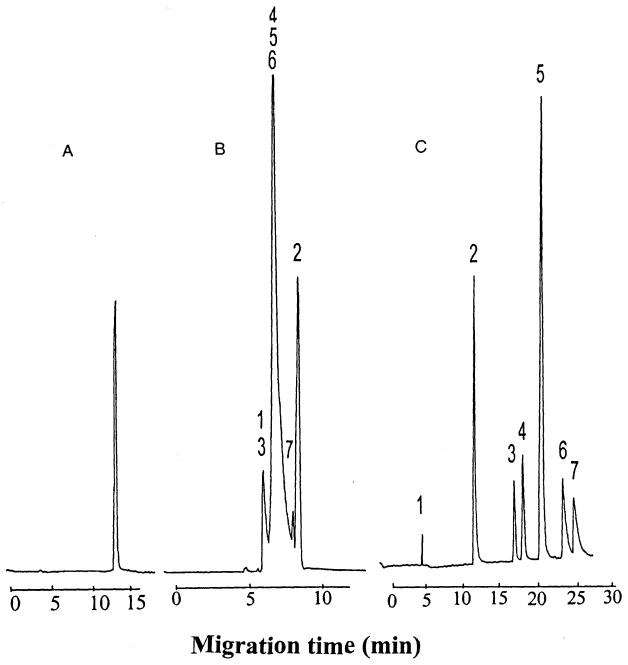

Fig. 5. Electropherograms of plant phe nols with dif fer-ent cap il lary col umns. Cap il lary col umn: 80 cm × 75 mm i.d. (50 cm to de tec tor). A. bare fused-silica. B. amino-bonded phase. C. fullerene-bonded phase. Electrokinetic in jec tion: A. 5 kV, 10 s; B and C. -5 kV, 10 s. Ap plied volt age: A. 15 kV; B and C. -15 kV. Analyte con cen tra-tion: 0.1 mM for peak 1, 2, 5, 6 and 7. 1.5 mM for 3 and 4. De tec tion: UV at 215 nm. Back-ground elec tro lyte: ac e tate buffer (20 mM, pH 5.0). Peak iden ti fi ca tion: 1. catechin. 2. sal i-cylic acid. 3. myricetin. 4. quercetin. 5. gal lic acid. 6. caffeic acid. 7. *p*-coumaric acid.

Table 1. Stability and Separation Efficiency of the Fullerene-bonded Phase[a]

Analyte	Migration time (min)[b]	Theoretical plate/m^{-1}	Plate height (μm)	Rs[c]
Catechin	5.70 ± 0.16%	89100	5.61	---
Salicylic acid	12.42 ± 0.22%	19600	25.57	24.53
Myricetin	18.30 ± 0.17%	17600	28.47	15.23
Quercetin	19.47 ± 0.07%	16600	30.06	2.95
Gallic acid	21.24 ± 0.07%	20500	24.42	3.97
Caffeic acid	23.62 ± 0.11%	17600	28.46	5.38
p-Coumaric acid	25.23 ± 0.08%	22600	22.13	3.28
Benzyl alcohol[d]	5.08 ± 0.62%[e]			
	5.11 ± 2.46%[f]			

[a] Column dimension, 80 cm (effective length, 50 cm) × 75 μm i.d.; electrokinetic injection (-5 kV, 10 s), acetate buffer (20 mM, pH 5.0); applied potential, -15 kV; detection at 215 nm. [b] Average of five measurements. [c] $Rs = \Delta t/W_{av} = 2\,(t_2-t_1)/(w_1+w_2)$. [d] Neutral marker. [e] Average of seven measurements for the neutral marker within column. [f] Average of seven measurements for the neutral marker between columns.

that of the fullerene-bonded phase. The strong interaction of the amino group in the bonded phase with the carboxylate group of the salicylic acid could explain this phenomenon. Thus, it is obvious that the separation of plant phenols is essentially controlled by the π-π interaction of the fullerene bonded phase and the analytes in addition to the van der Waals force or hydrogen bonding with the amino group of the spacer.

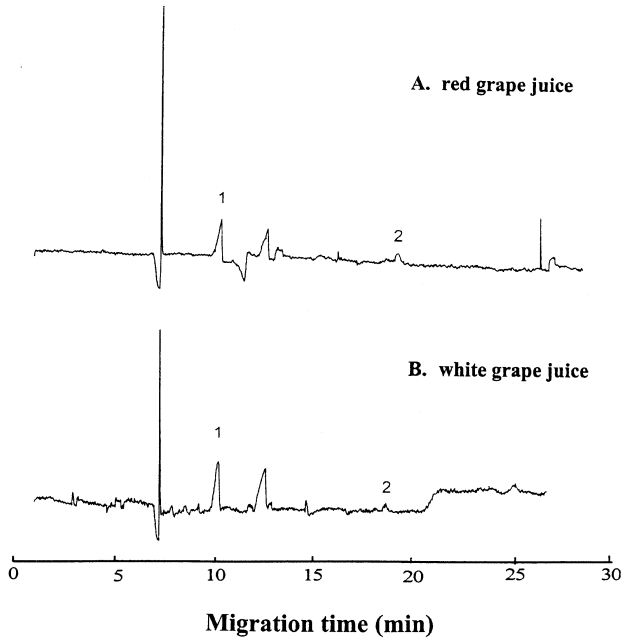

Fig. 6. Electrophoretic determination of plant phenols in grape juice. Capillary column: fullerene-bonded phase, 80 cm × 75 mm i.d. (50 cm to detector). Electrokinetic injection: -5 kV, 10 s. Applied voltage: -15 kV. Background electrolyte: acetate buffer (20 mM, pH 5.0). Detection: UV at 215 nm. Peak identification: 1. catechin. 2. salicylic acid. A. red grape juice. B. white grape juice.

Stability of the Coatings

Under electrophoretic conditions, the columns discussed here were usable for a long period of time (up to several weeks) without noticeable change in the separation properties. The run-to-run reproducibility of migration time for the column is shown in Table 1. The RSD values of migration times for the neutral marker, benzyl alcohol by seven measurements were 0.62%, indicating the good repeatability of this method. Column to column reproducibility has also been evaluated with benzyl alcohol. The RSD values of migration times for seven different columns were less than 2.5%.

Analytical Application

Grapes contain a large amount of different phenolic compounds in the skins, pulp and seeds that are partially extracted during wine making. Several studies have pointed out that many of them may show biological properties of interest, related to their antioxidant properties.[17] Fig. 6 shows the electropherogram recorded for grape juice in which the different peaks were identified by the standard addition method. Qualitative data showed that a slightly greater amount of catechin was present in the white grape juice, while a lower amount of salicylic acid was in it by comparison with the result for red grape juice.

CONCLUSIONS

Reversed-phase HPLC and capillary electrophoresis

are the most cited techniques for the separation of polyphenols. Dalluge et al. reported that separation could be improved by the use of deactivated monomeric C_{18} LC columns and gradient elution systems utilizing acid-containing buffers.[18] Therefore the present study was designed in order to determine the feasibility of the use of a capillary column containing fullerene in the electrophoretic separation of plant phenols. The mix-mode stationary phase combines the protonated amino group and the neutral surface of fullerene. It has two major advantages over a fused silica capillary in the CZE applications. A reversal of the EOF could be obtained with the introduction of an amino silane. And second, the fullerene-bonded phase could provide the π-π interaction with the aromatic ring of the plant phenols. Thus neutral compounds and anion mixtures could be separated in a single run with the established system. These results indicate that the proposed method can be useful for the analysis of plant phenols in many application areas.

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Key Words

Fullerene-bonded phase; Open tubular; Capillary electrochromatography; Plant phenols.

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