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Journal of Chromatography B, 863 (2008) 101–106
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous determination of flavones and phenolic acids in the leaves of *Ricinus communis* Linn. by capillary electrophoresis with amperometric detection

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Received 7 November 2007; accepted 4 January 2008
Available online 16 January 2008

Abstract

Capillary electrophoresis (CE) with amperometric detection (AD) has been developed for the separation and determination of disaccharide glycoside rutin, gentistic acid, quercetin, and gallic acid in the leaves of *Ricinus communis* Linn. for the first time. The effects of the acidity and the concentration of the running buffer, separation voltage, injection time, and detection potential were investigated to acquire the optimum conditions for the determination of the four analytes. The detection electrode was a 300 μm diameter carbon disc electrode at a detection potential of +0.90 V (versus saturated calomel electrode (SCE)). The four analytes could be well separated within 10 min in a 40 cm length fused silica capillary at a separation voltage of 15 kV in a 50 mM borate buffer (pH 9.0). The relation between peak current and analyte concentration was linear over about 3 orders of magnitude with detection limits (S/N = 3) ranging from 0.8 to 2.9 μM for all the analytes. The proposed method has been successfully applied to monitor flavones and phenolic acids in the real plant samples with satisfactory assay results.

Keywords: *Ricinus communis* Linn.; Disaccharide glycoside rutin; Gentistic acid; Quercetin; Gallic acid; Capillary electrophoresis; Amperometric detection

1. Introduction

Ricinus communis Linn. (castor) belongs to Euphorbiaceae family. It is a soft-wooded small tree that is widely distributed in the tropics and the warm temperature regions of the world [1]. Its seeds are squeezed to produce castor oil that has been employed to prepare medicines, paint, lubricating oil, printing ink, etc. Its seeds, leaves and roots have been used as herbal drugs [1,2]. As a commonly used traditional Chinese medicine (TCM), the leaf of *R. communis* L. has the therapeutic functions of reducing swelling, detoxicating, relieving itching, etc. It has been widely used solely or as an important ingredient in some traditional prescriptions for the treatment of eczema, titillation, sores, tumors, etc. A variety of physiologically active compounds (such as disaccharide glycoside rutin, quercetin, gentistic acid, gallic acid, etc.) have been found in the leaves of *R. communis* L. [1]. They have close correlation with the quality of the herbal drug because different functions of the herbal drug come from different bioactive constituents. The disaccharide glycoside rutin has been found widely in plants and usually coexists with its aglycone, quercetin. Some related investigations show that disaccharide glycoside rutin has a broad range of physiological activities such as anti-inflammatory [3], anti-tumor [4] and anti-bacteria [5]. It is thought to improve capillary function by reducing abnormal leakage and has been given to reduce capillary impairment and venous insufficiency of the lower limb [6]. Quercetin has similar effects such as anti-tumor [7], anti-bacteria [8] and inhibition of human platelet aggregation [9]. Gentistic acid (2,5-dihydroxybenzoic acid) has been proved to have the activities of antioxidation [10], enhancing fludioxonil fungicidal activity [11], and anti-bacteria [12]. Gallic acid is widely distributed in fruits and plants [13]. Recent investigations show that it has diverse functions such as antioxidation, anti-tumor, and antifungal [14]. Usually, the higher contents of active constituents indicate the better quality of the herbal drugs. Hence, it is interesting to establish some rapid, simple, and accurate approaches for the determination of the bioactive substances in the leaves of *R. communis* L.

Liquid chromatography (LC) was the most commonly used method for the determination of the bioactive constituents in

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doi:10.1016/j.jchromb.2008.01.002

R. communis L. [14–16]. Given the increasing interest in phenolic antioxidants in plant products, a variety of methods based on gas chromatography [17–19], high-performance liquid chromatography [17,20,21], and capillary electrophoresis (CE) [22] have been developed for the determination of flavonoids and phenolic acids occurred in both free and conjugated forms in plant products. Separation and determination of various constituents in plant drugs is always a complicated and challenging task. Nowadays, the application of CE for the separation of various active constituents in medicinal plants has become increasingly widespread because of its minimal sample volume requirement, short analysis time and high separation efficiency [23,24]. After CE separation, ultraviolet (UV) detector has been commonly employed for the detection of the constituents extracted from TCMs [25–27]. Because the absorbance path length of the capillary (the inner diameter, 25–100 μm i.d.) is very short, the low sensitivity of the UV detector used results in poor detection limit (typically 10 μM). Usually, the content of the constituents in the TCMs is very low. High sensitive detection methods are highly demanded. To meet the requirement, electrospray ionization mass spectrometry was coupled with CE for the efficient determination of constituents in TCMs without derivatization [19–21]. Recently, electrochemical detection (ED) has been coupled with CE for the sensitive detection of TCMs [24]. ED usually operated in the amperometric mode can be coupled with CE to provide high sensitivity and selectivity for the determination of electroactive substances [28–30]. ED can also provide higher selectivity as only electroactive substances can be detected so that the electropherograms are simplified, which is important for the analysis of medicinal plants because the constituents in them are usually complex.

In this study, CE with amperometric detection (AD) was employed for the determination of disaccharide glycoside rutin, gentistic acid, quercetin, and gallic acid (their molecular structures are shown in Fig. 1) in the dried leaves of *R. communis* L. Because all four constituents in the crude drugs contain phenolic hydroxyl groups that are electroactive at modest oxidation potential on the carbon electrode, AD was employed for their sensitive and selective detection in this work. To our best knowledge, there are no earlier reports published on the determination of the distribution of the active constituents in *R. communis* L. by CE. The optimization, detailed characterization, and advantages of the CE-AD approach are reported in the following sections in connection with the measurement of the four active constituents in the crude drugs.

2. Experimental

2.1. Reagent and solutions

Disaccharide glycoside rutin, gentistic acid, quercetin, and gallic acid were all supplied by Shanghai Chemical Reagent Company (SinoPharm, Shanghai, China). All aqueous solutions were made up in doubly distilled water. Other chemicals were of analytical grade.

Stock solutions of all analytes (10 mM) were prepared in water and were kept in a 4 °C refrigerator. They were stable for at least 1 month. The electrophoretic separation medium was 50 mM borate buffer (pH 9.0) unless mentioned otherwise. The stock solutions were diluted to the desired concentration with the separation medium just prior to use.

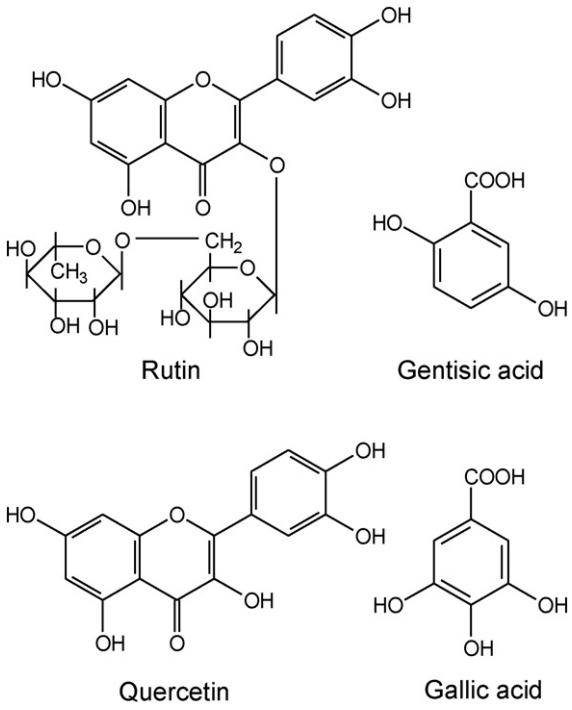

Fig. 1. Molecular structure of disaccharide glycoside rutin, gentistic acid, quercetin, and gallic acid.

2.2. Apparatus

The CE-AD system used has been described previously [30,31]. A ±30 kV high-voltage dc power supply (Shanghai Institute of Nuclear Research, China) provided a separation voltage between the ends of the capillary. The inlet of the capillary was held at a positive potential and the outlet of capillary was maintained at ground. The separations were carried out in a 40 cm length of 25 μm i.d. and 360 μm o.d. fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA). In order to improve the reproducibility of the peak current and migration time, the whole CE system was assembled in a laboratory that was air-conditioned at 25 °C to reduce temperature fluctuation.

A three-electrode electrochemical cell consisting of a laboratory-made 300 μm diameter carbon disc detection electrode, a platinum auxiliary electrode, and a saturated calomel electrode (SCE) as the reference electrode, was used in combination with a BAS LC-4C amperometric detector (Bioanalytical Systems Inc., West Lafayette, IN, USA). The filter of the detector was set at 0.1 Hz. The detection electrode was positioned carefully opposite the outlet of the capillary with the aid of a micromanipulator (CORRECT, Tokyo, Japan) and arranged in a wall-jet configuration. The distance between the tip of the detection electrode and the capillary outlet was adjusted to ~25 μm by comparison with the bore (25 μm) in the capillary while being viewed under a microscope. The electropherograms were

recorded using a LKB REC 1 chart recorder (Pharmacia, Sweden). A YS 38-1000 220 V alternate constant-voltage power supply (Shanghai Instrumental Transformer Factory, Shanghai, China) was employed to suppress the voltage fluctuation of the power line.

2.3. Sample preparation

Three samples of the leaves of *R. communis* L. were collected from the east campus of Medical Center of Fudan University (Shanghai, China) and air dried. All samples were identified by Prof. S. Ding (Medical Center of Fudan University, Shanghai, China). They were all dried at 60 °C for 2 h and then pulverized. About 2.0 g of the powder was weighed accurately, dispersed in 50 ml of methanol, and was kept in a 60 °C water bath for 3 h. After cooling, it was sonicated for 30 min and filtered through a filter paper. Additional methanol was added to the extract in a volumetric flask to adjust the final volume to be 50 ml. The extract was diluted using 50 mM borate buffer (pH 9.0) at a ratio of 25 (1–25) just prior to CE analysis.

2.4. Procedures

Before use, the carbon disc electrode was successively polished with emery paper and alumina powder, and sonicated in doubly distilled water. CE was performed at a separation voltage of 15 kV, unless otherwise indicated. The potential applied to the detection electrode was +0.90 V (versus SCE). The fused-silica capillary used in CE was rinsed with a 0.1 M NaOH aqueous solution for at least 15 min to clean the inner wall before use, and then it was flushed with doubly distilled water and the running buffer successively. Samples were injected electrokinetically at 15 kV for 6 s. Before injection, both the anode end of the capillary and the platinum-wire anode were moved from the anode solution to the sample solution. After an injection voltage of 15 kV was applied between the two ends of the capillary for 6 s, several nanolitres of the sample solution were introduced into the capillary. The anode end of the capillary together with the anode was then quickly returned to the anode solution. A voltage of 15 kV was subsequently applied in the constant-voltage mode for CE separation. The amperometric detector was on during the injection procedures. Note that the cathode solution in the electrochemical detection cell, the anode solution, and the sample solution were all at the same level. Moreover, sample solutions, standard solutions, and the separation medium were all filtered through a polypropylene filter (0.22 μm, Shanghai Bandao Industry Co., Ltd., Shanghai, China) prior to their use.

3. Results and discussion

3.1. Hydrodynamic voltammograms (HDVs)

Because disaccharide glycoside rutin, gentistic acid, quercetin, and gallic acid all contain phenolic hydroxyl groups, they are electroactive on a carbon electrode. In this work, they were amperometrically detected at a carbon disc electrode after CE separation. The potential applied to the detection electrode directly affects the sensitivity and the detection limits of this method, and it is necessary to determine the hydrodynamic voltammograms for the analytes to obtain the optimum potential. Fig. 2 illustrates the HDVs of disaccharide glycoside rutin, gentistic acid, quercetin, and gallic acid. It is obvious that peak current begins to increase rapidly when the applied potential exceeds +0.4 V (versus SCE) for all the four analytes. However, the current response for all compounds increases much more slowly at potentials above +0.9 V (versus SCE). Although an applied potential greater than +0.90 V (versus SCE) results in higher peak currents, both the baseline noise and the background current increase substantially. The high background current led to an unstable baseline, which is a disadvantage for sensitive and stable detection. Considering the sensitivity and background current, subsequent amperometric detection work employed a detection potential of +0.90 V (versus SCE), offering the most favorable signal-to-noise characteristics. The stability of the detection electrode was good and the reproducibility was high at the optimum potential.

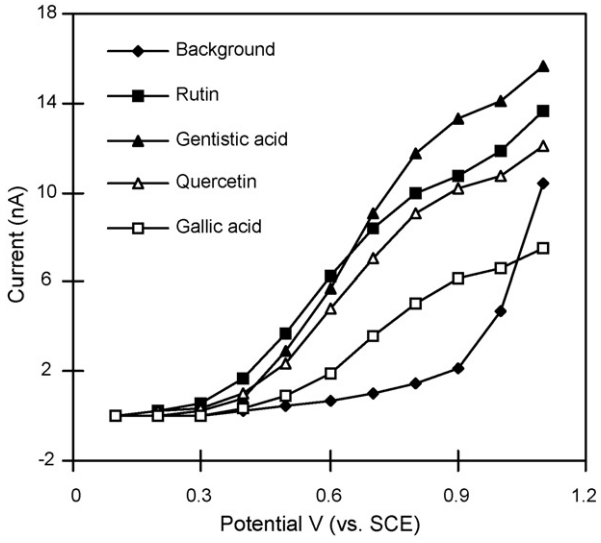

Fig. 2. Hydrodynamic voltammograms (HDVs) for 0.1 mM disaccharide glycoside rutin, 0.2 mM gentistic acid, 0.1 mM quercetin, and 0.2 mM gallic acid in CE analysis. Fused-silica capillary: 25 μm i.d. × 40 cm; detection electrode: 300 μm diameter carbon disc electrode; running buffer: 50 mM borate (pH 9.0); separation voltage: 15 kV; injection: 6 s (at 15 kV).

3.2. Effects of the acidity and concentration of the running buffer

In order to improve the resolution and solubility of analytes, alkaline borate buffer was employed in this study. Disaccharide glycoside rutin, gentistic acid, quercetin, and gallic acid are partially negatively charged in an alkaline borate buffer because their phenolic hydroxy and carboxylic groups can dissociate to form anions. In addition, the nearby hydroxyl groups in disaccharide glycoside rutin, quercetin, and gallic acid can form negatively charged complexes with boric acid in alkaline solutions [32].

The acidity of the running buffer affects the zeta-potential (ζ), the electro-osmotic flow (EOF) as well as the overall charge of

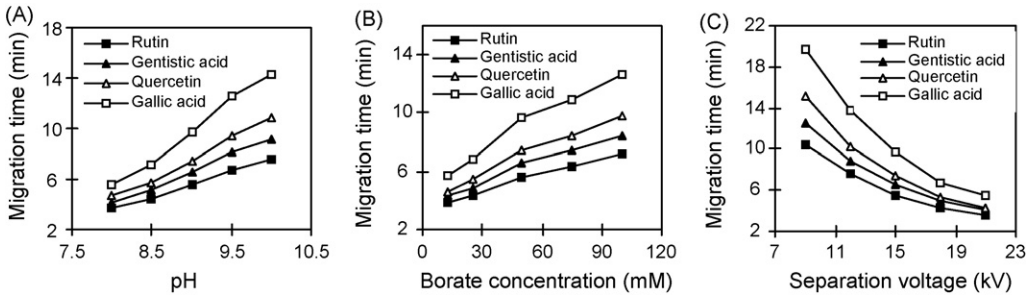

Fig. 3. Effect of the acidity (A) and the concentration (B) of running buffer, and separation voltage (C) on the migration time of the analytes. Detection potential: +0.90 V (versus SCE); other conditions as in Fig. 2.

the analytes, which determines the migration time, peak height, and the separation of the analytes. The effect of the running buffer pH on the migration time of the analytes is shown in Fig. 3A. The running buffers were 50 mM borate buffers at five different pH values (8.0, 8.5, 9.0, 9.5 and 10.0). When the running buffer pH increases from 8.0 to 10.0, the migration time increases with the resolution improved due to the dissociation of the analytes. Meanwhile, the peak current was low and the peak shape becomes poor at pH values above 9.0. At pH 9.0, the four analytes could be well separated within a relatively short time. In this experiment, 50 mM borate buffer at pH of 9.0 was chosen as the running buffer in considering the peak current, resolution, the analytical time, and the stability of the running buffer.

Because the buffer concentration influences the viscosity coefficient of the solution, the diffusion coefficient of analytes and the zeta-potential (ζ) of the inner surface of capillary tube, it affects not only the resolution and migration time of the analytes but also the peak current. The effect of the concentration of the

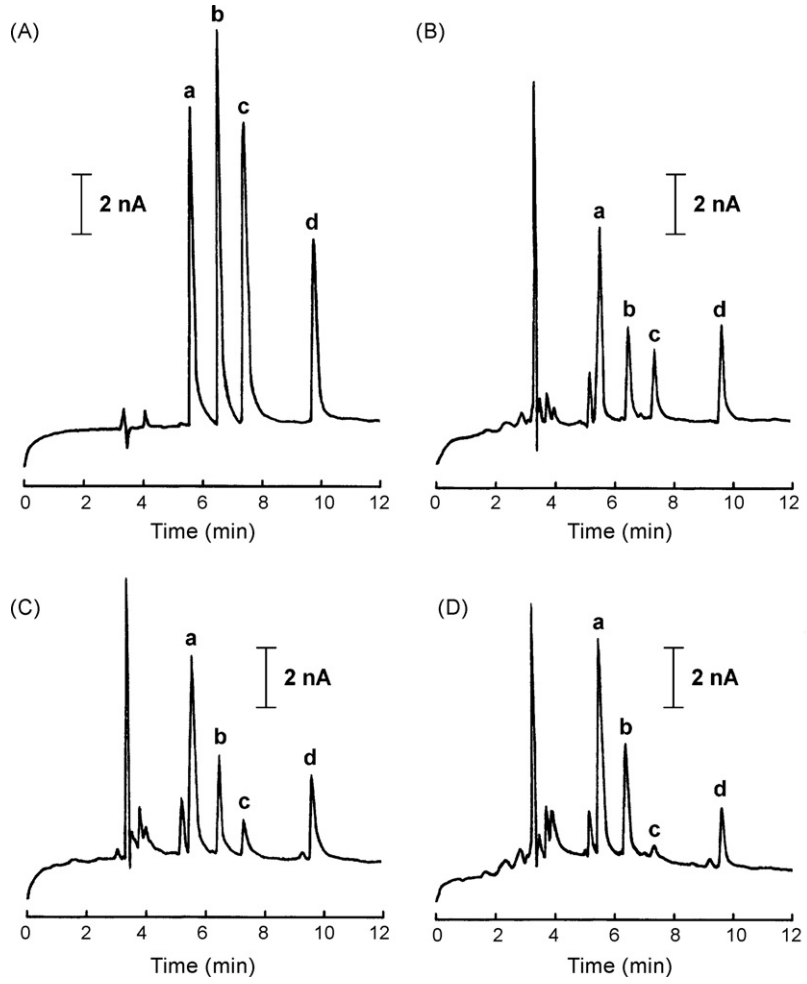

Fig. 4. (A) Electropherogram of a standard mixture solution containing 0.1 mM disaccharide glycoside rutin, 0.2 mM gentistic acid, 0.1 mM quercetin, and 0.2 mM gallic acid and (B–D) the typical electropherograms of the diluted extracts from the dried leaves of *Ricinus communis* L. (sample 1–3) under the optimum conditions. Detection potential: +0.90 V (versus SCE); other conditions as in Fig. 2.

Table 1
The results of regression analysis on calibration curves and the detection limits

Compound	Regression equation $Y = a + bX$[a]	Correlation coefficient	Linear range (mM)	Detection limit (μM)[b]
Disaccharide glycoside rutin	$Y = 0.1241 + 106.89X$	0.9992	0.001–1.0	0.8
Gentistic acid	$Y = 0.1407 + 66.438X$	0.9991	0.002–1.0	1.4
Quercetin	$Y = 0.1305 + 101.32X$	0.9994	0.001–1.0	0.9
Gallic acid	$Y = 0.0932 + 30.667X$	0.9988	0.005–1.0	2.9

Detection potential is +0.90 V (versus SCE). Other conditions are the same as in Fig. 2.
[a] Where the Y and X are the peak current (nA) and concentration of the analytes (mM), respectively.
[b] The detection limits correspond to concentrations giving a signal-to-noise ratio of 3.

running buffer (pH 9.0) on CE separation was investigated by changing the borate concentration from 25 to 100 mM. Fig. 3B indicates that the migration time and the resolution increase with increasing buffer concentration. Upon raising the borate concentration above 50 mM, the peak current was low and the peak shape became poor because the electric current in the capillary also increased, resulting in Joule heating and peak broadening.

3.3. Effect of separation voltage and injection time

Fig. 3C illustrates the influence of separation voltage on the migration time of the four analytes. Increasing the separation voltage 9–21 kV in steps of 3 kV not only dramatically decreases the migration time of all analytes, but also increases the baseline noise, resulting in poorer detection limits. It was found that higher separation voltages were not beneficial to the resolution of the four compounds. Moreover, higher separation voltages resulted in higher Joule heating that directly affected the separation efficiency of this method. However, too low separation voltages would increase the analysis time considerably, which in turn would cause peak broadening. Based on experiments, 15 kV was chosen as a good compromise.

In this study, samples were introduced into the capillary electrokinetically. The injection time directly affected the amount of samples, which affected the peak height and the peak shape. The effect of the injection time on CE separation was investigated by changing the injection time from 2 to 10 s in increments of 2 s at an injection voltage of 15 kV. It has been found that both the peak current and the peak width increased with increasing the sampling time. When the injection time exceeded 6 s, the peak current leveled off, and peak broadening became more severe (not shown). In this experiment, 6 s (at 15 kV) was selected as the optimum injection time, considering the separation and sensitivity.

Table 2
Assay results of the analytes in the plant samples ($n = 3$, mg/g)

Sample	Disaccharide glycoside rutin	Gentistic acid	Quercetin	Gallic acid
1	25.04 (2.7)[a]	4.510 (3.1)	4.308 (4.2)	11.48 (2.9)
2	25.91 (3.6)	4.768 (3.7)	2.278 (4.0)	9.627 (3.3)
3	28.50 (2.8)	5.412(3.0)	0.7456 (4.8)	6.778 (4.1)

Detection potential is +0.90 V (versus SCE). Other conditions are the same as in Fig. 2.
[a] The data in the brackets are the R.S.D.s (%).

Through the experiments above, the optimum conditions for determining disaccharide glycoside rutin, gentistic acid, quercetin, and gallic acid were acquired. The typical electropherogram for a mixture containing 0.1 mM disaccharide glycoside rutin, 0.2 mM gentistic acid, 0.1 mM quercetin, and 0.2 mM gallic acid is shown in Fig. 4A. Baseline separation for all the four analytes can be achieved within 10 min.

3.4. Reproducibility, linearity and detection limits

The precision was examined from a series of 7 repetitive injections of a sample mixture containing 0.1 mM disaccharide glycoside rutin, 0.2 mM gentistic acid, 0.1 mM quercetin, and 0.2 mM gallic acid under the optimum conditions. The time for each run is about 12 min. Reproducible signals were obtained with an R.S.D. of 3.2% (disaccharide glycoside rutin), 2.9% (gentistic acid), 3.5% (quercetin), 3.7% (gallic acid) for the peak currents. Such good repeatability reflects the reduced surface fouling of the carbon detection electrode and indicates that this approach is suitable for the analyses of real samples.

A series of the standard mixture solutions of the four analytes with concentration ranging from 1 μM to 1.0 mM were tested to determine the linearity at the carbon disc electrode in this method. The carbon electrode detector offered a well-defined concentration dependence. The results of regression analysis on calibration curves and detection limits are presented in Table 1. The determination limits are evaluated on the basis of a signal-to-noise ratio of 3. The calibration curves exhibit satisfactory linear behavior over the concentration range of about 3 orders of magnitude with the detection limits ranging from 0.8 to 2.9 μM for all the analytes.

3.5. Sample analysis and recovery

Under the optimum conditions, CE-AD was applied for the determination of disaccharide glycoside rutin, gentistic acid, quercetin, and gallic acid in the dried leaves of *R. communis* L. The typical electropherograms for the diluted extracts from three plant samples are shown in Fig. 4B–D. Peak identification was performed by a standard-addition method [33]. The standard solution of disaccharide glycoside rutin, gentistic acid, quercetin, or gallic acid was added to the diluted extract of the plant sample. The electropherogram of each standard-added sample solution was measured and compared with the electropherogram of the plant sample without standard added.

The raised peak was assigned to the added compound. The assay results are summarized in Table 2.

Recovery experiments were performed by adding accurate amounts of disaccharide glycoside rutin, gentistic acid, quercetin, and gallic acid to the diluted extract of the dried leaves of *R. communis* L. in the running buffer. Subsequently, the standard-spiked sample solution was analyzed under the optimum conditions. The average recoveries and the corresponding R.S.D.s were 97.5 and 3.2% for disaccharide glycoside rutin, 98.1 and 2.9% for gentistic acid, 96.3 and 3.6% for quercetin, and 99.3 and 4.0% for gallic acid, respectively ($n = 3$). The results indicated that this method had both high recovery and good precision for the four analytes.

4. Conclusions

In this study, CE-AD has been employed for the determination of disaccharide glycoside rutin, gentistic acid, quercetin, and gallic acid in the dried leaves of *R. communis* L. It is characterized by its higher performance and sensitivity, lower expense of operation, and minimal solvent/reagent consumption. The main advantage of CE as an analytical technique for the analysis of plant samples is that the capillary can be cleaned much easier by flushing. Because disaccharide glycoside rutin, gentistic acid, quercetin, and gallic acid are directly detected on the carbon electrode, samples do not need derivatization before determination. Because only electroactive compounds can be detected, the interferences of some electroinactive coexistent substances were minimized so that the electropherograms were simplified. In conclusion, CE-AD is an efficient approach for the constituent and fingerprint study of plant drugs due to its special attributes.

Acknowledgments

The authors are grateful for the financial supports provided by the National Nature Science Foundation of China (Grant Nos.: 20675017 and 20405002) and the 863 program of China (2007AA04Z309).

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