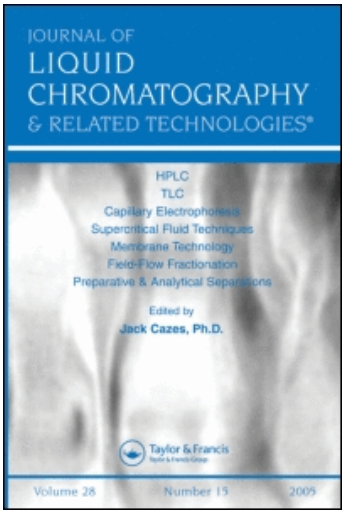

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Determination of Active Ingredients of Spatholobus Suberectus Dunn and Its Medicinal Preparations by Capillary Electrophoresis with Electrochemical Detection

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Abstract: A method based on capillary electrophoresis with electrochemical detection has been developed for the separation and determination of epicatechin, emodin, syringic acid, vanillic acid, rhein, and protocatechuic acid in Spatholobus suberectus dunn and its medicinal preparations for the first time. The effects of a working electrode potential, pH and concentration of running buffer, separation voltage, and injection time on CE-ED were investigated. Under the optimum conditions, the analytes could be separated in a 60 mmol L^{-1} borate buffer (pH 9.0) within 26 min. A 300 μm diameter carbon disk electrode has a good response at +0.95 V (vs. SCE) for all analytes. The response was linear over three orders of magnitude with detection limits (S/N = 3) ranging from 5×10^{-8} g mL^{-1} to 2×10^{-7} g mL^{-1} for the analytes. The method has been successfully applied to the analysis of a real sample, with satisfactory results.

Keywords: Capillary electrophoresis, Electrochemical detection, Spatholobus suberectus dunn

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INTRODUCTION

Traditional Chinese medicines have been extensively used to prevent and cure human disease for over a millennium in oriental countries. Because of its low toxicity and good therapeutical performance, traditional Chinese medicines have attracted considerable attention in many fields.[1] The Chinese traditional medicine, spatholobus suberectus dunn belongs to the family of Leguminosae. Spatholobus suberectus dunn is widely distributed in the south of China, especially in Guangdong, Guangxi, Yunnan Province. The dried rattans of this plant are known in China as "jixueteng", and have been used ethnically as an effective crude drug in China for hundreds of years. It is used as a remedy (when given internally) to treat irregular menstruation, hemopenia, and rheumatism.[2] Several bioactive ingredients including epicatechin, emodin, syringic acid, vanillic acid, rhein, and protocatechuic acid are found in Spatholobus suberectus dunn.[3–5] The molecular structures of these compounds are shown in Figure 1.

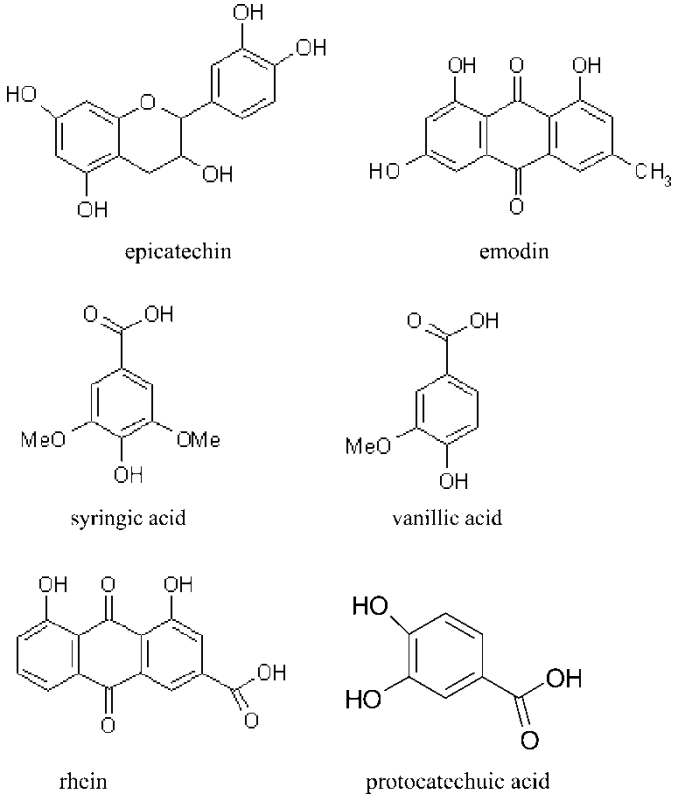

Figure 1. Molecular structures of epicatechin, emodin, syringic acid, vanillic acid, rhein, and protocatechuic acid.

Some related investigations show that quercetin and other flavonoids have a broad range of physiological activities such as anti-inflammatory,[6] antiallergic,[7] and antioxidant activity for scavenging radicals,[8] and inhibition of a variety of enzymes.[9] Modern research has revealed that some phenolic acids[10,11] show anti-carcinogenic effects. In order to estimate the quality of Spatholobus suberectus dunn, it is necessary to develop a sensitive, selective, dependable, and relatively simple assay method to determine the active ingredients in Spatholobus suberectus dunn.

Analysis of the active ingredients in Spatholobus suberectus dunn is a challenging task because of the composition diversity, the significant concentration difference of active ingredients, as well as effects of many factors such as climates, regions of growth, and seasons of harvest, on the contents of active ingredients in medicinal herbs. HPLC, as a prime analytical method, had been applied to analyze Spatholobus suberectus dunn.[5] However, HPLC used in the analysis of traditional Chinese medicines has some shortcomings, such as short column lifetime owing to numerous coexisting interfering compounds, some of which can be adsorbed strongly onto the packing materials of HPLC columns, resulting in column degradation and higher costs. Besides, the theoretical plate number of a HPLC column is often much lower than that of a capillary tube with the same length. Capillary electrophoresis (CE) is becoming increasingly recognized as an important analytical separation technique due to its speed, efficiency, reproducibility, ultra-small sample volume, and ease of clearing up the contaminants. In 2000, the U.S. Food and Drug Administration (FDA) published a draft of Guidance for Industry Botanical Drug Products. Before a plant drug becomes logically marketed, its spectroscopic or chromatographic fingerprints and chemical assay of characteristic markers are required. CE should find more applications in this area.[12] In combination with electrochemical detection (ED), CE offers high sensitivity and good selectivity for electroactive species.[13–16] However, to our knowledge, this technique has not been fully explored so far, and its application to the analysis of Spatholobus suberectus dunn and its medicinal preparations of samples has not been conducted. Also, the simultaneous determination of epicatechin, emodin, syringic acid, vanillic acid, rhein, and protocatechuic acid has not as of yet been reported.

In this work, we developed a simple and rapid method to determine epicatechin, emodin, syringic acid, vanillic acid, rhein, and protocatechuic acid in Spatholobus suberectus dunn and its medicinal preparations by CE–ED.

EXPERIMENTAL

Apparatus

In this work, a CE-ED system has been constructed and is similar to that described previously.[17,18] A high voltage (± 30 kV) power supply (Shanghai

Institute of Nuclear Research, Shanghai, China) provided a voltage between the ends of the capillary. The separation was undertaken in a 75 cm length, 25 μm i.d., and 360 μm o.d. fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA).

A three electrode electrochemical cell consists of a 300 μm diameter carbon disc working electrode, a platinum auxiliary electrode, and a saturated calomel electrode (SCE) as the reference electrode. An electrochemical Analyzer CHI 830B (CH Instruments, Austin, Texas, USA) was used as the amperometric detector, which was connected to a high performance PC with the Windows XP operating system installed. Before use, the carbon disc electrode was polished with emery paper and sonicated in doubly distilled water, and finally carefully positioned opposite the outlet of the capillary. The capillary was rinsed with 0.1 mol L^{-1} NaOH 30 min before use. The injector electrode was kept at high positive voltage, and the electrochemical cell for detection was kept at ground. A Plexiglas box with an interlock on the access door was used to enclose the high voltage output and to protect the operators from accidental electric shock.

Reagents

Syringic acid was purchased from Sigma (St. Louis. Mo, USA). Epicatechin, emodin, vanillic acid, rhein, and protocatechuic acid were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Herba Spatholobus suberectus dunn was purchased from a drug store in Shanghai (Shanghai, China). Spatholobus suberectus dunn capsules (050211) was obtained from Guangxii Lingfeng Pharmaceutical Corporate (Guangxi, China). Stock solutions of epicatechin, emodin, syringic acid, vanillic acid, rhein, and protocatechuic acid (2.0×10^{-3} g · mL^{-1}, each) were prepared in anhydrous ethanol (A.R. grade), stored in the dark at 4°C, and were diluted to the desired concentrations with the running buffer (60 mmol L^{-1} borate buffer, pH = 9.0). Before use, all solutions were filtered through 0.22 μm nylon filters.

Sample Preparation

Four grams of dried Spatholobus suberectus dunn Herbas and 4 g of Spatholobus suberectus dunn capsules were ground into powder in a mortar and accurately weighed. Each weighed sample was refluxed with anhydrous ethanol (A.R. grade) and water (4:1) 30 mL for 30 min in a water bath at 70°C. After cooling, the mixture was filtered through filter paper and the residues were washed with the same solution (2 × 10 mL). The extract and washings were combined and concentrated to approximately 45 mL under vacuum, and then diluted to 50 mL with the same solution in a volumetric flask. In the actual

sample analysis, 0.5 mL sample solutions were again diluted with the running buffer to 1 mL. After being filtered through a 0.22 μm syringe filter, all solutions can be injected directly to the CE-ED system for analysis. Before use, all sample solutions were stored in the dark at 4°C.

RESULTS AND DISCUSSION

Effect of the Potential Applied to the Working Electrode

The potential applied to the working electrode directly affects the sensitivity, detection limit, and stability of this method. Therefore, hydrodynamic voltammetry was investigated to obtain optimum detection results. As shown in Figure 2, when the applied potential exceeds +0.50 V (vs. SCE), all analytes can generate an oxidation current at the working electrode. When the applied potential is between +0.60 V and +0.95 V (vs. SCE), the increase of peak current becomes rapid. When the applied potential is greater than +0.95 V (vs. SCE), both the baseline noise and the background current very strongly increased, resulting in an unstable baseline, which is a disadvantage for sensitive and stable detection. Therefore, the applied potential to the working electrode was maintained at +0.95 V (vs. SCE), where the background current was not too high and the S/N ratio was the highest. Moreover, the working electrode showed good stability and high reproducibility at this optimum potential.

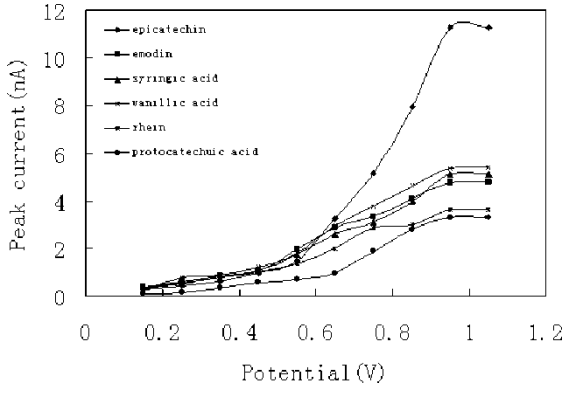

Figure 2. Hydrodynamic voltammograms (HDVs) for 1 = epicatechin, 2 = emodin, 3 = syringic acid, 4 = vanillic acid, 5 = rhein, and 6 = protocatechuic acid in CE. Experimental conditions: Fused-silica capillary: 25 μm i.d. × 75 cm; working electrode: 300 μm diameter carbon disk electrode; running buffer: 60 mmol L^{-1} borate buffer (pH 9.0); separation voltage: 18 kV; electrokinetic injection: 8 s (18 kV); concentration: 2.0×10^{-5}g mL^{-1} for epicatechin, emodin, syringic acid, vanillic acid, rhein and protocatechuic acid.

Effects of the pH Value and the Buffer Concentration

Two buffers, namely phosphate and borate were tested. The experimental results showed that the six analytes could be baseline separated only in borate running buffer under the same conditions. So borate buffer was employed as the running buffer in this work. The pH dependence of the migration time was investigated in the pH range of 8.0–9.2. As shown in Figure 3, the migration time of all analytes increases with increasing pH value, separation of the analytes can be achieved from pH 8.7 to 9.2. When the pH is lower than 8.7, syringic acid can not be separated from vanillic acid. Moreover, a higher pH value results in a long analysis time and easy oxidation of the analytes. Therefore, pH 9.0 was selected as the optimum pH value. Besides the pH value, the running buffer concentration, which effects peak height and theoretical plate number is also an important parameter. The effect of the running buffer concentration on migration time was studied, and the optimum running buffer concentration is 60 mmol L^{-1}.

Effects of Separation Voltage and Injection Time

For a given capillary length, the separation voltage determines the electric field strength, which affects both the velocity of electroosmotic flow and the migration velocity of the analytes, which, in turn, determines the migration time of the analytes. As expected, higher separation voltage gives shorter migration time for all analytes. However, when the separation voltage exceeds 18 kV, baseline noise becomes larger. Therefore the optimum separation voltage selected is 18 kV, at which good separation can be obtained for all analytes within 26 min. The injection time determining the amount of sampling affects both peak current and peak shape. The effect of injection time on peak current

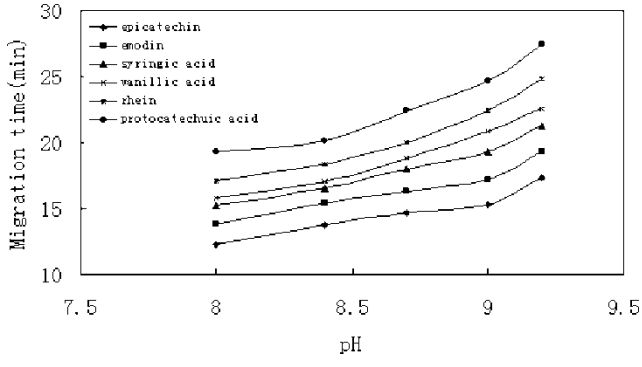

Figure 3. Effect of buffer pH on the migration time. Working potential + 0.95 V(vs.SCE); other conditions as in Figure 2.

was studied by varying injection time from 2 s to 10 s at 18 kV. It was found that the peak current increases with the increasing sampling time. When the injection time is longer than 8 s, the peak current nearly levels off and peak broadening becomes more severe. In this experiment, 8 s (18 kV) is selected as the optimum injection time.

Through the above experiments, the optimum conditions for epicatechin, emodin, syringic acid, vanillic acid, rhein, and protocatechuic acid have been decided. A 60 mmol L^{-1} borate buffer (pH 9.0) was used as the running buffer at a separation voltage of 18 kV. The potential applied to the working electrode was +0.95 V (vs. SCE). Samples were injected electrokinetically at 18 kV for 8 s.

The typical electropherogram for a standard solution of the six analytes is shown in Figure 4(A), and we can see that good separation can be achieved within 26 min.

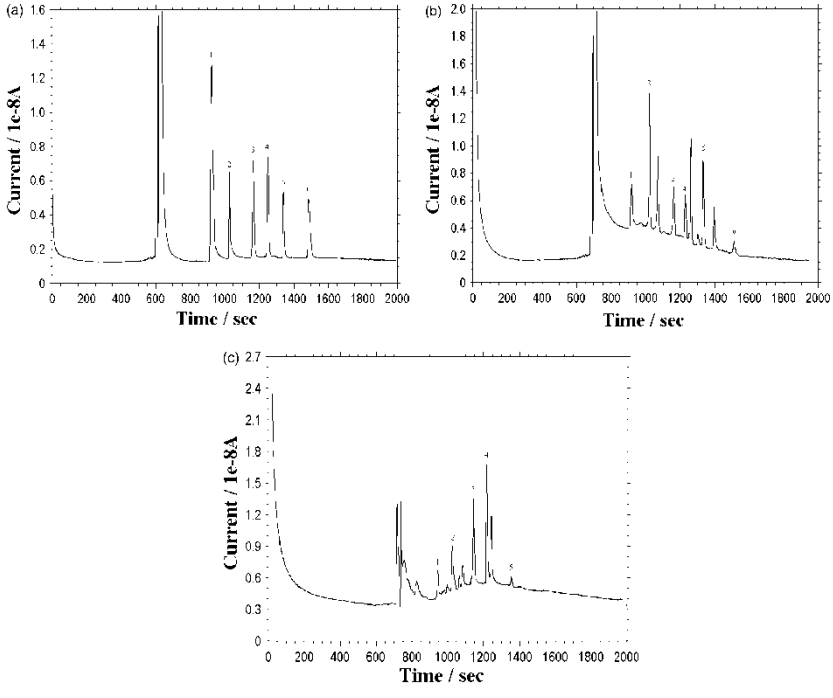

Figure 4. The electropherogram of a standard mixture solution (2.0×10^{-5} g mL^{-1} for epicatechin, emodin, syringic acid, vanillic acid, rhein, and protocatechuic acid) (A), and the typical electropherograms of herba Spatholobus suberectus dunn. (B) and its medicinal preparations Spatholobus suberectus dunn capsules (C). Working potential: +0.95 V (vs. SCE); other conditions as in Figure 2. Peak identification: 1 = epicatechin, 2 = emodin, 3 = syringic acid, 4 = vanillic acid, 5 = rhein, and 6 = protocatechuic acid.

Method Validation

Appropriate method validation information concerning new analytical techniques for analyzing pharmaceuticals is required by regulatory authorities. Validation of such methods include assessment of the stability of the solutions, linearity, reproducibility, detection, and quantification limits.

Stability of the Solutions

The stability of standard and sample solutions was determined by monitoring the peak area of standard mixture solutions and sample solutions over a period of one day. The results showed that the peak area and migration time of each analyte were almost unchanged (R.S.D. % <3.6) and that no significant degradation is observed within the given period, indicating that the solutions are stable for at least 24 hours.

Linearity

To determine the linearity of the peak area response on concentration for epicatechin, emodin, syringic acid, vanillic acid, rhein, and protocatechuic acid, a series of mixed standard solutions from 0.1 $\mu g \cdot mL^{-1}$ to 200 $\mu g \cdot mL^{-1}$ were tested. The results of regression analysis on calibration curves and detection limits are presented in Table 1.

Reproducibility

The reproducibility of the peak area and migration time was estimated by making repetitive injections of a standard mixture solution (20 $\mu g \cdot mL^{-1}$ for each analyte) under the optimum conditions. The relative standard deviations (RSDs) of the peak area and migration time were 3.1% and 2.9% for epicatechin, 2.3% and 1.6% for emodin, 3.4% and 2.2% for syringic acid, 3.0% and 1.7% for vanillic acid, 3.7% and 2.6% for rhein, and 3.5% and 1.9% for protocatechuic acid, respectively ($n = 7$).

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The detection limits are evaluated on the basis of a signal to noise ratio of 3. The calibration curves exhibit excellent linear behavior over the concentration range of about three orders of magnitude, with the detection limits ranging from 0.05 to 0.20 $\mu g \cdot mL^{-1}$ for all the analytes.

The LOQ is defined as the level at, or above, which the measurement precision is satisfactory for quantitative analysis. In our case, LOQ was evaluated on the basis of a signal to noise ratio of 10. The LOQ was 0.31,

Table 1. Results of regression analysis on calibration and the detection limits[a]

Compound	Regression equation $Y = a + bx$[b]	Correlation coefficient	Linear range ($\mu g \cdot mL^{-1}$)	Detection Limit[c] ($\mu g \cdot mL^{-1}$)
Epicatechin	$Y = 9897.2X - 0.5583$	0.9996	0.3–100	0.10
Emodin	$Y = 1496X + 1.0574$	0.9994	0.1–150	0.05
Syringic acid	$Y = 2742X + 0.7453$	0.9992	0.5–200	0.20
Vanillic acid	$Y = 2896.4X - 0.0103$	0.9993	0.4–150	0.15
Rhein	$Y = 1370.8X - 0.058$	0.9991	0.6–200	0.20
Protocatechuic acid	$Y = 2504.1X + 1.6365$	0.9993	0.3–200	0.10

[a]Working potential is +0.95 V(vs. SCE). Other conditions as in Figure 2.
[b]Where the y and x are the peak area (nQ) and concentration ($mg \cdot mL^{-1}$) of the analytes, respectively.
[c]The detection limits corresponding to concentrations giving signal to noise ratio of 3.

0.16, 0.62, 0.47, 0.66, 0.32 $\mu g \cdot mL^{-1}$ for epicatechin, emodin, syringic acid, vanillic acid, rhein, and protocatechuic acid, respectively.

Sample Analysis and Recovery

Under optimum conditions, the determination of epicatechin, emodin, syringic acid, vanillic acid, rhein, and protocatechuic acid in Spatholobus suberectus dunn and its medicinal preparation Spatholobus suberectus dunn capsules were carried out according to the procedures described in the earlier sample preparation section. Typical electropherograms obtained from herba Spatholobus suberectus dunn. (B) and Spatholobus suberectus dunn capsules (C) are shown in Figure 4. By adding the standard samples of epicatechin, emodin, syringic acid, vanillic acid, rhein, and protocatechuic acid into the respective actual samples, the active ingredients, namely epicatechin (peak 1), emodin (peak 2), syringic acid (peak 3), vanillic acid (peak 4), rhein (peak 5), and protocatechuic acid (peak 6) can be qualitatively determined. The amounts of each compound were calculated through the calibration equations and the results were displayed in Table 2. Six active ingredients were all detected in Herba Spatholobus suberectus dunn, but protocatechuic acid was not found in the capsules, which revealed that the amounts of the analyte in these preparations were below the determination limit.

Under the optimum conditions, the recovery and reproducibility experiments were also conducted to evaluate the precision and accuracy of the method. Recovery was determined by the standard addition method. The average recoveries and RSDs for the analytes are listed in Table 3 ($n = 3$).

Table 2. Assay results for herba spatholobus suberectus dunn and its medicinal preparations spatholobus suberectus dunn capsules ($n = 3$)

Sample	Ingredient	Found ($mg \cdot g^{-1}$)	RSD (%)
Herba spatholobus suberectus dunn	Epicatechin	0.08	2.9
	Emodin	0.86	3.3
	Syringic acid	0.24	2.7
	Vanillic acid	0.22	3.1
	Rhein	0.75	2.5
	Protocatechuic acid	0.05	3.4
Spatholobus suberectus dunn capsules	Epicatechin	0.04	2.6
	Emodin	0.72	2.9
	Syringic acid	0.64	3.0
	Vanillic acid	0.74	3.4
	Rhein	0.10	2.8
	Protocatechuic acid	Not found	—

Working potential is +0.95 V(vs. SCE) other conditions as in Figure 2.

Table 3. The determination results of recovery for this method with herba spatholobus suberectus dunn sample ($n = 3$)

Sample	Ingredient	Original amount ($\mu g \cdot mL^{-1}$)	Added amount ($\mu g \cdot mL^{-1}$)	Found amount ($\mu g \cdot mL^{-1}$)	Recovery (%)	RSD (%)
Herba	Epicatechin	3.09	10	12.91	98.2	2.8
spatholobus	Emodin	34.47	10	44.86	103.9	3.7
suberectus	Syringic acid	9.45	10	19.91	104.6	3.5
dunn	Vanillic acid	8.95	10	18.67	97.2	2.9
	Rhein	29.90	10	39.58	96.8	2.5
	Protocatechuic acid	1.84	10	11.58	97.4	3.1

Working potential is +0.95 V (vs. SCE) other conditions as in Figure 2.

CONCLUSIONS

This work presents the first application of CE-ED for the qualitative and quantitative assay of epicatechin, emodin, syringic acid, vanillic acid, rhein, and protocatechuic acid in Spatholobus suberectus dunn and its medicinal preparation Spatholobus suberectus dunn capsules. The above assay results indicate that this method is accurate, sensitive, and reproducible.

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