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Short communication

Determination of active ingredients of Ilex Purpurea Hassk 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 isovanillic acid, gentisic acid, kaempferol, quercetin, caffeic acid and protocatechuic acid in Ilex Purpurea Hassk and its medicinal preparations for the first time. The effects of 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 50 mmol l^{-1} borate buffer (pH 9.0) within 21 min. A 300 μm diameter carbon disk electrode has a good response at +0.95 V (versus SCE) for all analytes. The response was linear over three orders of magnitude with detection limits ($S/N = 3$) ranging from 3×10^{-8} to 2×10^{-7} g ml^{-1} for the analytes. The method has been successfully applied to the analysis of real sample, with satisfactory results.

Keywords: Capillary electrophoresis; Electrochemical detection; Ilex Purpurea Hassk

1. 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]. Chinese traditional medicine Ilex Purpurea Hassk belong to the family of Aquifoliaceae. Ilex Purpurea Hassk is widely distributed in south part of China, especially in Jiangsu, Zhejiang, Anhui Province. The dried leaves of this plant are known in China as 'sijiqing', and have been used ethnically as an effective crude drug in China for hundreds of years [2]. It is used as a remedy (when given internally) to treat bronchitis, pneumonia and ulceration, and as an external treatment for scald, chilblain, etc. [3]. Several bioactive ingredients including isovanillic acid, gentisic acid, kaempferol, quercetin, caffeic acid and protocatechuic acid are found in Ilex Purpurea Hassk [4–6]. The molecular structures of these compounds are shown in Fig. 1. Some related investigations show that kaempferol, quercetin and other flavonoids have a broad range of physiological activities such as anti-inflammatory [7], antiallergic [8] and antioxidant activity for scavenging radicals [9] and inhibition of a variety of enzymes [10]. Modern research has revealed that some phenolic acids [11–12] show anticarcinogenic effects. In order to estimate the quality of Ilex Purpurea Hassk, it is necessary to develop a sensitive, selective, dependable and relative simple assay method to determine the active ingredients in Ilex Purpurea Hassk.

Analysis of the active ingredients in Ilex Purpurea Hassk 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

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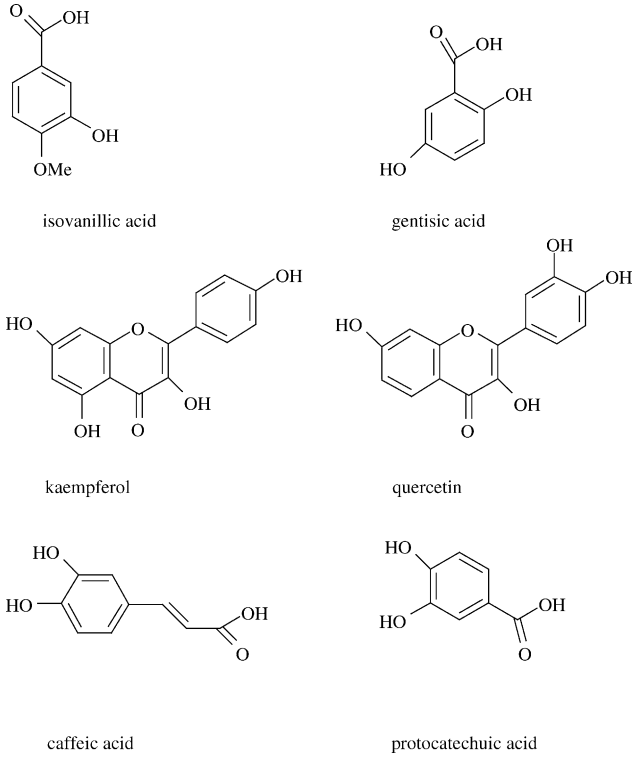

Fig. 1. Molecular structures of isovanillic acid, gentisic acid, kaempferol, quercetin, caffeic acid and protocatechuic acid.

ingredients in medicinal herbs. HPLC, as a prime analytical method, had been applied to analyze the phenolic acids in Ilex Purpurea Hassk [4]. However, HPLC used in the analysis of traditional Chinese medicines has some shortcomings, such as short column lifetime owing to numerous co-existing interfering compounds, some of which can be adsorbed strongly onto the packing materials of HPLC column, resulting in column degradation and higher cost. 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, 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 [13]. In combination with electrochemical detection (ED), CE offers high sensitivity and good selectivity for electroactive species [14]. However, to our knowledge, so far this technique has not been fully explored, and its application to the analysis of Ilex Purpurea Hassk and its medicinal preparations samples has not been conducted. And simultaneous determination of isovanillic acid, gentisic acid, kaempferol, quercetin, caffeic acid and protocatechuic acid has not been reported, either.

In this work, we developed a simple and rapid method to determine isovanillic acid, gentisic acid, kaempferol, quercetin, caffeic acid and protocatechuic acid in Ilex Purpurea Hassk and its medicinal preparations by CE-ED.

2. Experimental

2.1. Apparatus

In this work, a CE-ED system has been constructed and is similar to that described previously [15–16]. 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, TX, 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 distance between the tip of the working electrode and the capillary outlet was as close as possible so that the CE effluent directly impinged upon the electrode surface. 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.

2.2. Reagents

Isovanillic acid and gentisic acid were purchased from Sigma (St. Louis., MO, USA). Kaempferol, quercetin, caffeic acid and protocatechuic acid were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Herba Ilex Purpurea Hassk was purchased from a drug store in Shanghai (Shanghai, China). Compound sijiqing tablets (041201) was obtained from shanghai hanyin Pharmaceutical Corporate (Shanghai, China). Stock solutions of isovanillic acid, gentisic acid, kaempferol, quercetin, caffeic acid 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 (50 mmol l^{-1} borate buffer, pH 9.0). Before use, all solutions were filtered through 0.22 μm nylon filters.

2.3. Sample preparation

One gram of dried Ilex Purpurea Hassk Herbas and 1 g of compound sijiqing tablets were ground into powder in a mortar and accurately weighed. Each weighed sample was extracted with 10 ml anhydrous ethanol (A.R. grade) and water (4:1) for 30 min in an ultrasonic bath. The extract was then filtered through a filter paper and the filter paper was rinsed by the same solution for three times for each sample. Next, a total of extracted solutions were diluted with anhydrous ethanol to 50 ml. In actual sample analysis, 0.5 ml sample solution were again diluted with the running buffer (50 mmol l^{-1} borate buffer, pH 9.0) to 1 ml. After 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.

3. Results and discussion

3.1. 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 Fig. 2, when the applied potential exceeds +0.60 V (versus SCE), all analytes can generate oxidation current at the working electrode. When the applied potential is between +0.60 and +0.95 V (versus SCE), the increase of peak current becomes rapidly. When the applied potential is greater than +0.95 V (versus SCE), both the baseline noise and the background current increase very strongly, resulting in an unstable baseline, which is a disadvantage for sensitive and stable detection. Therefore, the applied potential to the working electrode was

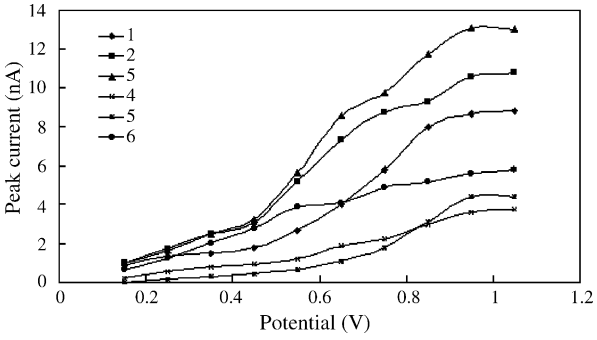

Fig. 2. Hydrodynamic voltammograms (HDVs) for 1 = isovanillic acid, 2 = gentisic acid, 3 = kaempferol, 4 = quercetin, 5 = caffeic acid and 6 = protocatechuic acid in CE. Experimental conditions: fused-silica capillary: 25 μm i.d. × 75 cm; working electrode: 300 μm diameter carbon disk electrode; running buffer: 50 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 isovanillic acid, gentisic acid, kaempferol, quercetin, caffeic acid and protocatechuic acid.

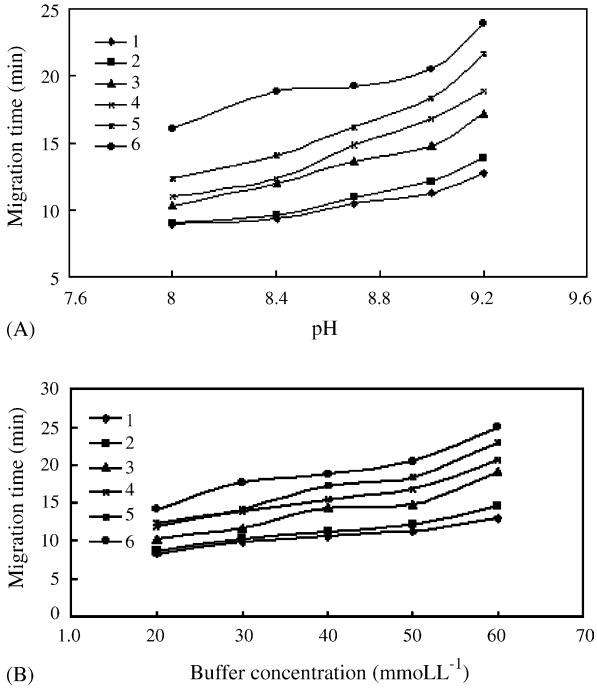

Fig. 3. Effect of buffer pH (A) and concentration (B) on the migration time. Working potential +0.95 V (vs. SCE); other conditions as in Fig. 2.

maintained at +0.95 V (versus 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.

3.2. Effects of the pH value and the buffer concentration

Borate buffer was employed as the running buffer in this work because borate can chelate with the analytes to form more soluble complex anions [17]. The pH dependence of the migration time was investigated in the pH range of 8.0–9.2. As shown in Fig. 3A, 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 pH is lower than 8.7, isovanillic acid cannot be separated from gentisic acid Moreover, higher pH value results in 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 (data showed in Fig. 3B), and the optimum running buffer concentration is 50 mmol l^{-1}.

3.3. 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 that showed in Fig. 4A, higher separation volt-

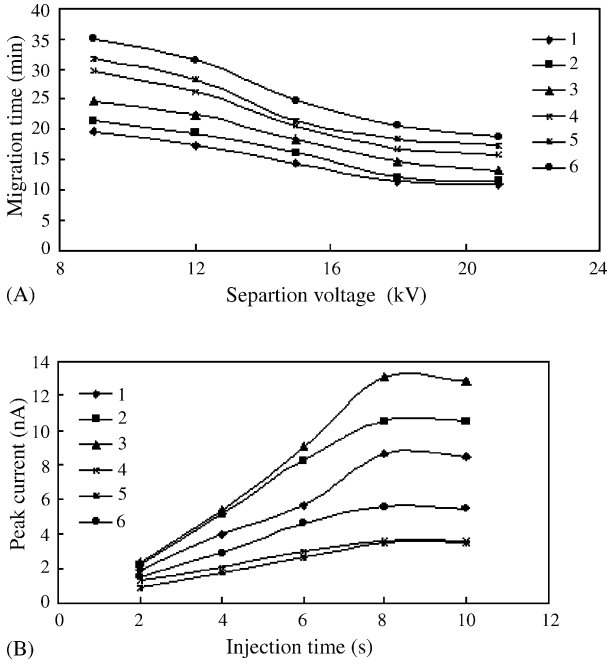

Fig. 4. Effect of separation voltage on the migration time (A) and injection time on the peak current (B). Working potential: +0.95 V (vs. SCE); other conditions as in Fig. 2.

age 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 21 min. The injection time determining the amount of sampling affects both peak current and peak shape. The effect of injection time on peak current was studied by varying injection time from 2 to 10 s at 18 kV. The data in Fig. 4B showed that the peak current increases with 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 experiments above, the optimum conditions for isovanillic acid, gentisic acid, kaempferol, quercetin, caffeic acid and protocatechuic acid have been decided. A 50 mmol l^{-1} borate buffer (pH 9.0) used as the running buffer at a separation voltage of 18 kV. The potential applied to the working electrode was +0.95 V (versus 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 Fig. 5A, and we can see that good separation can be achieved within 21 min.

4. 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 solu-

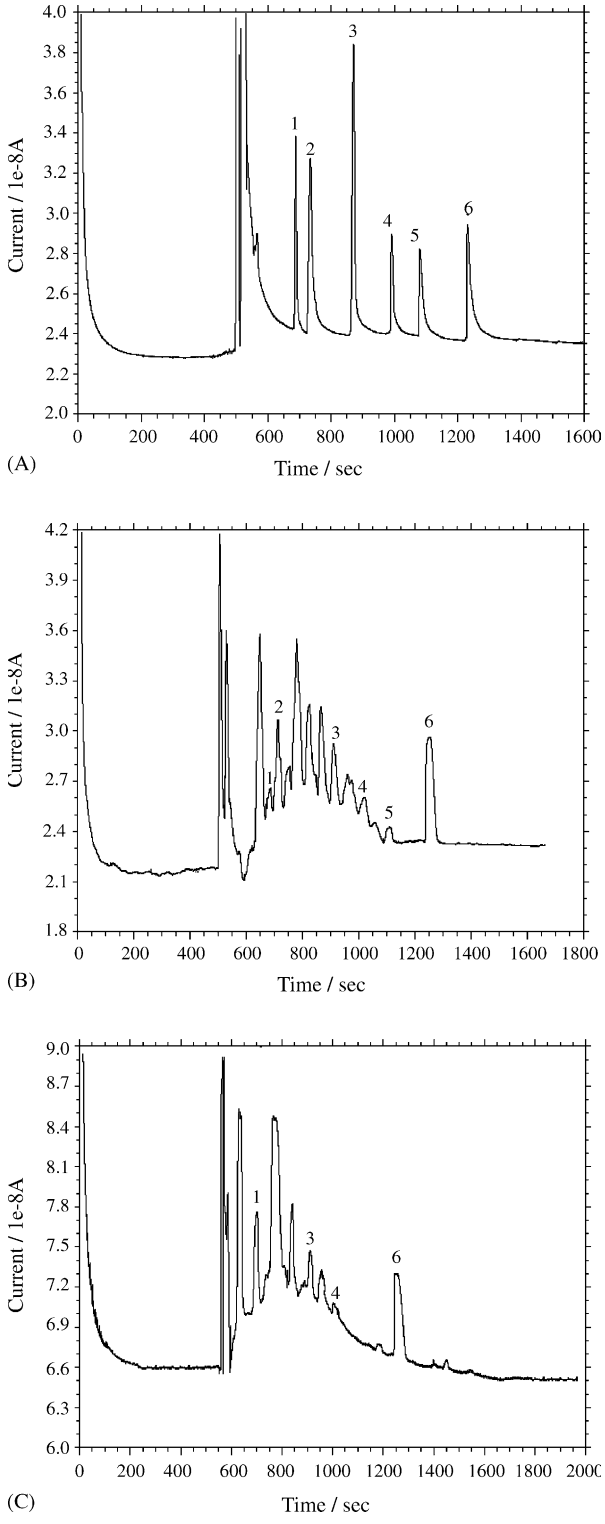

Fig. 5. The electropherogram of a standard mixture solution (2.0×10^{-5} g ml^{-1} for isovanillic acid, gentisic acid, kaempferol, quercetin, caffeic acid and protocatechuic acid) (A), and the typical electropherograms of herba Ilex Purpurea Hassk (B) and its medicinal preparations compound sijiqing tablets (C). Working potential: +0.95 V (vs. SCE); other conditions as in Fig. 2. Peak identification: 1 = isovanillic acid, 2 = gentisic acid, 3 = kaempferol, 4 = quercetin, 5 = caffeic acid and 6 = protocatechuic acid.

tions, linearity, reproducibility, detection and quantification limits.

4.1. 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 1 day. The results showed that the peak area and migration time of each analyte were almost unchanged (R.S.D.% <3.7) and that no significant degradation is observed within the given period, indicating the solutions are stable for at least 24 h.

4.2. Linearity

To determine the linearity of the peak area response on concentration for isovanillic acid, gentisic acid, kaempferol, quercetin, caffeic acid and protocatechuic acid, a series of mixed standard solutions from 0.1 to 200 $\mu g\,ml^{-1}$ were tested. The results of regression analysis on calibration curves and detection limits are presented in Table 1.

4.3. Reproducibility

The reproducibility of the peak area and migration time was estimated by making repetitive injections of a standard mixture solution (20 $\mu g\,ml^{-1}$ for each analyte) under the optimum conditions. The relative standard derivations (R.S.D.s) of the peak area and migration time were 2.7 and 2.3% for isovanillic acid, 2.4 and 1.9% for gentisic acid, 3.1 and 2.5% for kaempferol, 2.2 and 1.9%for quercetin, and 3.1 and 1.4% for caffeic acid and 2.9 and 1.5% for protocatechuic acid, respectively ($n=7$).

4.4. 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 0.03 to 0.20 $\mu g\,ml^{-1}$ 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 were 0.20, 0.69, 0.67, 0.21, 0.26, 0.13 $\mu g\,ml^{-1}$ for isovanillic acid, gentisic acid, kaempferol, quercetin, caffeic acid and protocatechuic acid, respectively.

Table 2
Assay results for herba Ilex Purpurea Hassk and its medicinal preparations compound sijiqing tablets ($n=3$)

Sample	Ingredient	Found $mg\,g^{-1}$	R.S.D. (%)
Herba Ilex Purpurea Hassk	Isovanillic acid	0.76	2.1
	Gentisic acid	1.54	2.5
	Kaempferol	1.22	1.9
	Quercetin	1.41	3.8
	Caffeic acid	0.74	2.9
	Protocatechuic acid	5.87	3.3
Compound sijiqing tablets	Isovanillic acid	4.55	2.5
	Gentisic acid	Not found	–
	Kaempferol	0.66	2.1
	Quercetin	0.23	3.7
	Caffeic acid	Not found	–
	Protocatechuic acid	6.53	3.5

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

4.5. Sample analysis and recovery

Under optimum conditions, the determination of isovanillic acid, gentisic acid, kaempferol, quercetin, caffeic acid and protocatechuic acid in Ilex Purpurea Hassk and its medicinal preparations compound sijiqing tablets were carried out according to the procedures described earlier. Typical electropherogram obtained from herba Ilex Purpurea Hassk. (B) and compound sijiqing tablets (C) are shown in Fig. 5. By adding the standard samples of isovanillic acid, gentisic acid, kaempferol, quercetin, caffeic acid and protocatechuic acid into the actual samples, respectively, the active ingredients namely isovanillic acid (peak 1), gentisic acid (peak 2), kaempferol (peak 3), quercetin (peak 4), caffeic acid (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 Ilex Purpurea Hassk, but gentisic acid and caffeic acid were not found in the tablets, which revealed that the amounts of the two analytes in these preparations were below the determination limit.

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

Compound	Regression equation $Y=a+bX$[b]	Correlation coefficient	Linear range ($\mu g\,ml^{-1}$)	Detection limit[c] ($\mu g\,ml^{-1}$)
Isovanillic acid	$Y=2512.3X+2.9069$	0.9994	0.2–200	0.06
Gentisic acid	$Y=5191.7X-0.4407$	0.9997	0.5–100	0.20
Kaempferol	$Y=5738.7X-0.0421$	0.9995	0.5–200	0.20
Quercetin	$Y=1652X+2.9845$	0.9996	0.2–200	0.06
Caffeic acid	$Y=2138.9X+1.188$	0.9993	0.2–200	0.07
Protocatechuic acid	$Y=2638.6X+5.9087$	0.9995	0.1–200	0.03

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

Table 3
The determination results of recovery for this method with Ilex Purpurea Hassk sample ($n = 3$)

Sample	Ingredient	Original amount ($\mu g\,ml^{-1}$)	Added amount ($\mu g\,ml^{-1}$)	Found amount ($\mu g\,ml^{-1}$)	Recovery (%)	R.S.D. (%)
Herba Ilex Purpurea Hassk	Isovanillic acid	7.60	10	17.29	96.9	1.5
	Gentisic acid	15.36	10	25.47	101.1	2.6
	Kaempferol	12.21	10	22.08	98.7	2.9
	Quercetin	14.08	10	23.63	95.5	3.9
	Caffeic acid	7.411	10	17.26	98.5	3.4
	Protocatechuic acid	58.70	10	69.13	104.3	2.7

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

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 standard addition method. The average recoveries and R.S.D.s for the analytes were listed in Table 3 ($n = 3$).

5. Conclusions

The work presents the first application of CE-ED for qualitative and quantitative assay of isovanillic acid, gentisic acid, kaempferol, quercetin, caffeic acid and protocatechuic acid in Ilex Purpurea Hassk and its medicinal preparations compound sijiqing tablets. The above assay results indicate that this method is accurate, sensitive and reproducible.

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