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Determination of liquiritigenin and isoliquiritigenin in *Glycyrrhiza uralensis* and its medicinal preparations by capillary electrophoresis with electrochemical detection

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Abstract

A simple, reliable, reproducible and sensitive method, based on capillary electrophoresis with electrochemical detection (ED), for the determination of liquiritigenin and isoliquiritigenin in *Glycyrrhiza uralensis* and its medicinal preparations was described. Operated in a wall-jet configuration, a 300 μm diameter carbon-disk electrode was used as the working electrode, which exhibits good responses at +1000 mV (versus SCE) for the two analytes. Under the optimum conditions, the analytes were base-line separated within 8 min, and excellent linearity was obtained in the concentration range from 5.0×10^{-4} to 1.0×10^{-6} mol/1. The detection limit (S/N = 3) was 4.7×10^{-7} and 2.9×10^{-7} mol/1 for liquiritigenin and isoliquiritigenin, respectively. This work provides a useful method for the analysis of traditional Chinese medicines.
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Keywords: *Glycyrrhiza uralensis*; Pharmaceutical analysis; Liquiritigenin; Isoliquiritigenin; Flavones

1. Introduction

Glycyrrhiza uralensis has been used since ancient Egyptian, Greek, and Roman times in the West and since the Former Han era (the second to third Century B.C.) in ancient China in the East. In traditional Chinese medicine, *G. uralensis* is one of the most frequently used drugs, which exerts antitussive, expectorant and antipyrotic actions and is often used to treat cough, pharyngitis, bronchitis, bronchial asthma [1]. *G. uralensis* and its medical preparations are also used as clinical medicines to treat gastroduodenal ulcers, skin inflammation, contagious hepatitis, etc. [2]. Besides glycyrrhizic acid and glycyrrhetinic acid, chemical constituent investigations show that *G. uralensis* also contains flavones, such as liquiritigenin and isoliquiritigenin [3]; their molecular structures are shown in Fig. 1. Liquiritigenin and isoliquiritigenin from *G. uralensis* have some valuable pharmacological activities. It has been proven that they can be used as naturally occurring xanthine oxidase, monoamine oxidase inhibitor [4,5] and show growth inhibitory effect on cancer cell [6]. It also has the anti-angiogenic effect [7], and antioxidant [8], anti-inflammatory activities [9]. Moreover, isoliquiritigenin from *G. uralensis* has strong inhibitory effect on tyrosinase activity [10], which is known to be a key enzyme in melanin biosynthesis, involved in determining the color of mammalian skin and hair. Various dermatological disorders, such as melasama, age spots, and sites of actinic damage, arise from the accumulation of an excessive level of epidermal pigmentation. The inhibitory effect of isoliquiritigenin on tyrosinase activity shows that extract of *G. uralensis* may serve as a kind of skin-lightening agent. So, it is interesting to study liquiritigenin and isoliquiritigenin contents in *G. uralensis* plants. High-performance liquid chromatography (HPLC) has been developed to determine liquiritigenin and isoliquiritigenin in the traditional Chinese medicinal preparation Zuo et al. [11] and its metabolin [12]. HPLC has also been applied to analyze isoliquiritigenin in several medicinal herbs [13]. It may be due to the contents of liquiritigenin and isoliquiritigen in *G. uralensis* are relatively low, reports on determination of them in this plant are

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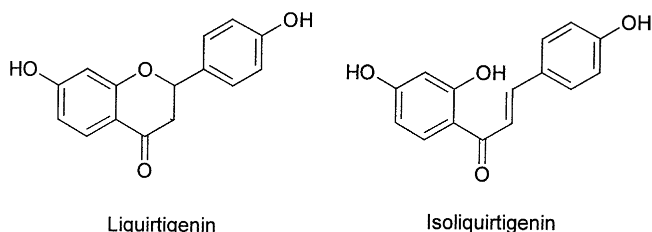

Fig. 1. Molecular structures of liquiritigenin and isoliquiritigenin.

little. Hence it is necessary to establish some simple, economical, accurate and sensitive methods for determination of liquiritigenin and isoliquiritigenin in *G. uralensis*.

Separation and determination of various constituents in plant drugs is always a complicated and challenge task. HPLC is regarded as a prime separation method in analysis of traditional Chinese medicines. Though the combination of mobile phase and stationary phase makes HPLC high sensitivity and good resolution, numerous coexistent interferences in traditional Chinese medicines lead to pretreatment time-consuming and HPLC column short lifetime. Capillary electrophoresis (CE) is becoming increasingly recognized as an important analytical separation technique for its speed, efficiency, reproducibility, ultra-small sample volume, and minimal consumption of solvent. In addition, electrochemical detection (ED) typically operated in amperometric mode can be coupled with CE to provide high sensitivity and selectivity [14]. As many active constituents in medicinal plants such as flavones, phenols and alkaloids are electroactive, ED coupled with CE should be an alternative and complement technique for determination of constituents in crude drugs. Moreover, because only electroactive substances can be detected, ED also provides higher selectivity, which is important for the analysis of medicinal plants as the constituents in them are complex. Since Kenndler et al. [15] firstly applied CE to analysis arbutin in leaves of *Arctostaphylos uva-ursi* (L.) in 1990, many kinds of traditional Chinese medicines, such as Strychnos pierrian [16], Flos Carthami [17], Atractylodis Rhizoma [18], Pericarpium Citri Reticulatae [19] and so on, have been determined with CE methods. Our research group has applied CE–ED for the determinations of some flavones in pharmaceutical preparations and Chinese herbal drugs, such as *Apocynum venetum*, *Puerariae radix*, *Rhododendron Dauricum L.* and *Ginkgo biloba L.* [20–24].

In this work, liquiritigenin and isoliquiritigenin in *G. uralensis* were determined by CE–ED. As the two analytes are flavones, which have phenolic hydroxyl group that are all electroactive at modest oxidation potential, so ED was employed for sensitive detection. Moreover, two major constituents, glycyrrhizic acid and glycyrrhetinic acid are not electroactive, they obviously can not interfere with the determination of liquiritigenin and isoliquiritigenin, which enhances the selectivity. To our knowledge, so far CE–ED has not been applied to determine liquiritigenin and isoliquiritigenin in *G. uralensis.* In this work, we first developed a simple, efficient and sensitive method to separate and detect liquiritigenin and isoliquiritigenin in *G. uralensis* by CE–ED.

2. Experimental

2.1. Apparatus

The laboratory-built CE–ED system [25] was employed in this work. A 30 kV high-voltage power supply (Shanghai Institute of Nuclear Research, China) provided a voltage between the ends of the capillary. The inlet end of the capillary was held at a positive potential and the outlet end was maintained at ground. A 70 cm length of 25 μm i.d. × 360 μm o.d. fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was used for the separation. Samples were all injected electrokinetically, applying 20 kV for 8 s.

A carbon-disk electrode with 300 μm diameter was employed as the working electrode as described previously. Before use, the surface of the carbon-disk electrode was polished with emery sand paper, sonicated in deionized water, and then positioned carefully opposite the capillary outlet with the aid of a micropositioner (Shanghai Lianyi Instrument Factory). A three-electrode cell system consisting of a carbon-disk working electrode, a platinum auxiliary electrode and a SCE reference electrode was used in combination with a BAS LC-3D amperometric detector (Biochemical Systems, West Lafayette, IN, USA). The electropherograms were recorded using a chart recorder (Shanghai Dahua Instrument factory, China).

2.2. Reagents

As liquiritigenin standard sample was not available commercially, liquiritigenin and isoliquiritigenin were synthesized by Professor Jianxin Wang (College of Chemical and Material Engineering, Southern Yangze University, Wuxi, China), and have been identified by melting point, $[\alpha]_D$, ^{1}HNMR, IR, UV and MS. All the data coincide well with the previous reports [26,27]. *G. uralensiss* were purchased from Shanhe drugstore in Wuxi (China). *G. uralensis* medical preparations, compound Liquorice tablets and compound Liquorice mixture, were obtained from Nanjing Second Pharmaceutical Factory (Nanjing, China) and Shanghai Meiyou Pharmaceutical Co. (Shanghai, China), respectively. Stock solutions of two analytes(1.00×10^{-3} mol/1, each) were prepared in methanol and were diluted to the desired concentration with the running buffer (50 mmol/l borax buffer with pH value from 7.8 to 9.2). Before use, all solutions were filtered through 0.22 μm nylon filters.

2.3. Sample preparation

Two grams of dried *G. uralensis* herbs and 1 g of compound Liquorice tablets (about 15 tablets) were ground into powder and accurately weighed. Each weighed sample was

extracted with 15 ml methanol for 15 min in an ultrasonic bath. The extract was then filtered through a filter paper. The extraction procedure was repeated three times. Next, a total of extracted solutions were diluted with methanol to 50 ml. In actual sample analysis, 0.4 ml sample solution was again diluted with 50 mmol/l running buffer to 3 ml. After filtered through 0.22 μm nylon filter, all solution can be directly injected electrokinetically.

0.1 ml liquid sample of compound Liquorice complex was diluted with 50 mmol/l running buffer to 3 ml. After filtered through 0.22 μm nylon filter, it was directly injected to capillary for separation and determination.

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 electrochemical response of the analytes. In order to obtain best detection results, optimum potential applied to the working electrode should be selected; therefore hydrodynamic voltammetry experiment was conducted to find this optimum potential. As shown in Fig. 2, when the applied potential exceeds +0.70 V (versus SCE), oxidation currents of liquiritigenin and isoliquiritigenin increase rapidly; when the applied potential passes +1.00 V (versus SCE), however, the peak currents of two analytes increase slower. Therefore, the applied potential of +1.00 V (versus SCE) was selected, where the background current is not too high and the S/N ratio is the highest.

3.2. Effects of the pH value on migration time and peak currents

Since Liquiritigenin and isoliquiritigenin have phenolic hydroxyl groups, they could be partly ionized to form electronegative analytes in the alkalescent medium. Moreover, borate can chelate with the analytes to form more soluble complex anions. So, the borate buffer was employed as the running buffer in this experiment. As expected, the pH value of the running buffer directly affects the migration time and the peak current of the analytes. The pH dependence of the migration time was investigated in the pH range of 7.8–9.2. As is well know that the velocity of electroosmotic flow increases with increasing pH value, but the electrophoretic velocity of anionic analytes increases correspondingly. Because electroosmosis and electrophoresis of the analytes move in opposite direction, and the increase of the velocity of electrophoresis is faster than that of electroosmosis in the pH range of 7.8–9.2, apparent migration velocity of the analytes decreases with increasing pH value. Therefore, the migration time of the analytes increases with increasing pH value, which was testified as shown in Fig. 3A. Although Liquiritigenin and isoliquiritigenin are isomeric compounds, their molecular structures are remarkably different. As shown in Fig. 1, liquiritigenin have two phenolic hydroxyl groups, whereas isoliquiritigenin have

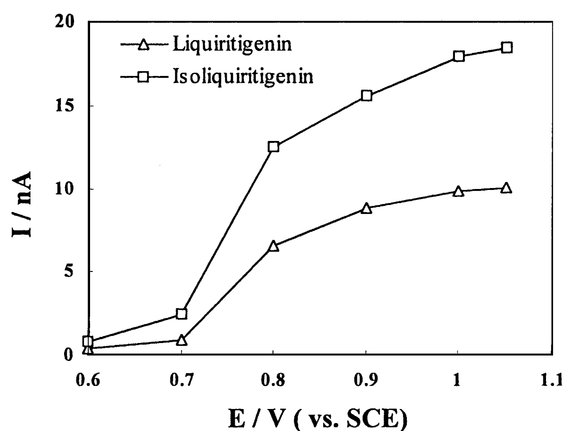

Fig. 2. Hydrodynamic voltammograms (HDVs) of liquiritigenin and isoliquiritigenin. Fused-silica capillary: 70 cm × 25 μm i.d.; concentrations of two analytes: 1.0×10^{-4} mol/l each. Working electrode: 300 μm diameter carbon disk electrode; running buffer: 50 mmol/l borate (pH 8.7); separation voltage: 20 kV; injection time: 20 kV/8 s.

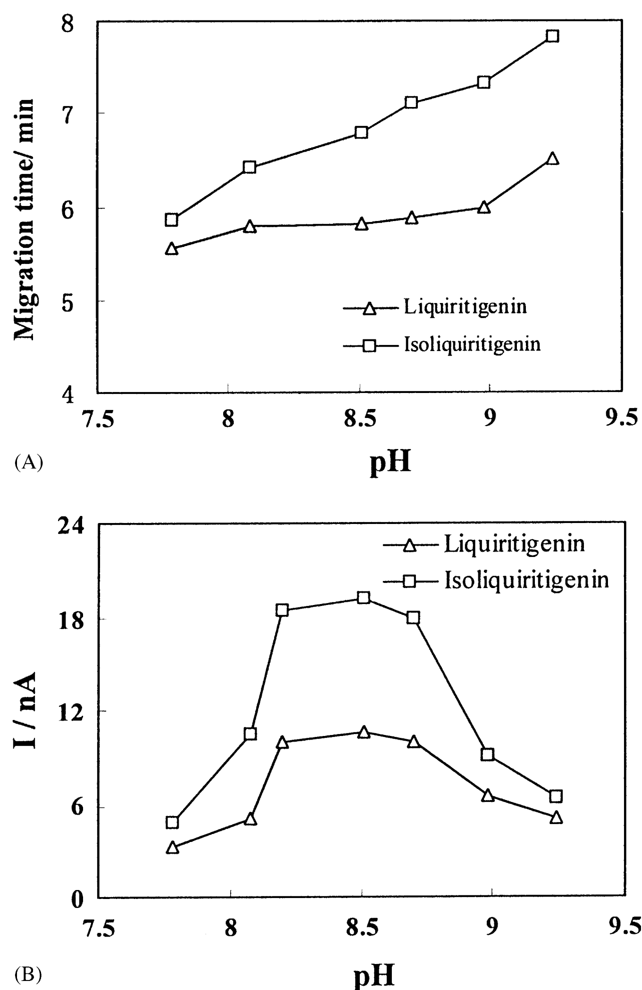

Fig. 3. Effects of acidity on migration time (A) and on peak current (B). Working electrode potential is 1.00 V (vs. SCE); other conditions are the same as in Fig. 2.

three. So, isoliquiritigenin has more negative charge than liquiritigenin, which results in larger electrophoretic mobility in basic medium, in reverse smaller apparent migration velocity. Therefore, baseline separation of liquiritigenin and isoliquiritigenin can be achieved from pH 8.0 to 9.2. Unfortunately, the two analytes were not baseline resolved with other compounds existing in the sample matrix in the pH value of 8.0–8.5. The effect of pH value on peak currents was also studied. Fig. 3B shows that from pH 8.2 to 8.7, peak currents of analytes are higher. Therefore, in considering the resolution, stability, analysis time and sensitivity, pH 8.7 was selected as the optimum pH value for running buffer.

The concentration of the running buffer is also important. The effect of the running buffer concentration on migration time was also studies. From 25 to 100 mmol/1, liquiritigenin and isoliquiritigenin can be baseline separated. Considering the coexistent compounds interference and analysis time, 50 mmol/1 borate buffer was chosen as the appropriate running buffer.

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 charged analytes, which in turn determine the migration time of the analytes. In this experiment, the influence of the separation voltage on the migration time of the analytes is studied. As expected, higher separation voltage gives shorter migration time for the analytes. However when the separation voltage exceeds above 20 kV, baseline noise becomes much larger. Therefore the optimum separation voltage selected is 20 kV, at which good separation can be obtained for the analytes within 8 min.

The injection time determining the amount of sampling affects both peak current and peak shape. The effect of injection time on sampling was studied by varying injection time from 2 to 14 s at 20 kV. With increasing injection time, peak current increases, and the peak width increases simultaneously. When the injection time is longer than 12 s, peak current levels off and peak broadening becomes severer. In order to separate the two analytes from the co-existent interferences in sample matrix, the small injection time is helpful for separation efficiency. Balancing the resolution and sensitivity, 8 s (20 kV) is selected as the optimum injection time in this experiment.

Through the experiments above, the optimum conditions for the determination of liquiritigenin and isoliquiritigenin were decided. The typical electropherogram for a standard solution of the analytes is shown in Fig. 4, as we can see baseline separation can be achieved within 8 min.

3.4. Reproducibility, linearity, detection limit of the two analytes

The reproducibility of the peak current is estimated by making repetitive injections of a standard mixture solution

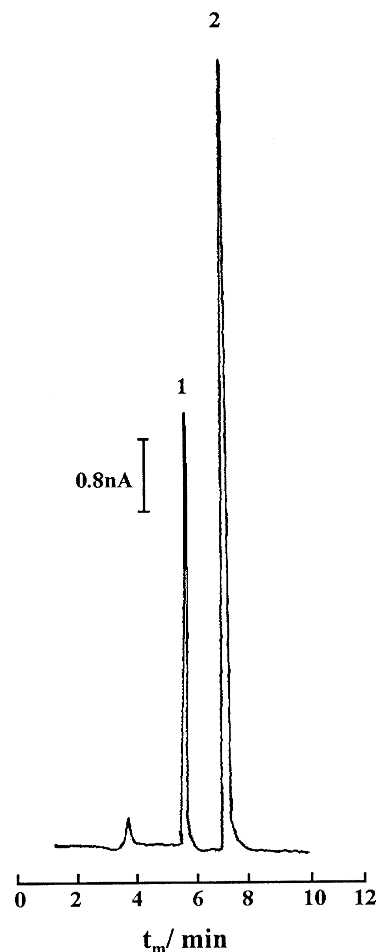

Fig. 4. The electropherogram of standard solution containing liquiritigenin and isoliquiritigenin. Peak identification: (1) liquiritigenin; (2) isoliquiritigenin. Working potential is 1.00 V (vs. SCE). The concentration of two analytes: 5.0×10^{-5} mol/1, each. Other conditions as in Fig. 2.

(5.0×10^{-5} mol/1 for each analyte) under the selected optimum conditions. The relative standard derivations (R.S.D.s) of the migration time and the peak current are 0.63, 0.78 and 2.6, 2.9% for liquiritigenin and isoliquiritigenin, respectively ($n = 7$).

To determine the linearity of liquiritigenin and isoliquiritigenin, a series of standard mixture solutions containing 2.0×10^{-7} to 5.0×10^{-4} mol/1 of each analyte were tested. The detection limit is evaluated on the basis a signal-to-noise ratio of 3. The results of regression analysis on calibration curves and detection limits are summarized in Table 1.

3.5. Sample analysis and recovery

Liquiritigenin and isoliquiritigenin in *G. uralensis* herb and its commercial medicinal preparations were determined by CE–ED under the optimum conditions. Typical electropherograms of *G. uralensis* herbs, compound Liquorice tablets and compound Liquorice mixture are shown in Fig. 5A, Fig. 5B and Fig. 5C, respectively. By adding

Table 1
The regression equations and detection limits[a]

Compound	Regression equation[b]	Correlation coefficient	Linear range (mol/1)	Detection limit (10^{-7}mol/l)
Liquiritigenin	$y = 9.89 \times 10^4 x + 0.02$	0.9999	1×10^{-6}–5×10^{-4}	4.7
Isoliquiritigenin	$y = 1.79 \times 10^5 x - 0.15$	0.9989	1×10^{-6}–5×10^{-4}	2.9

[a] CE–ED conditions as in Fig. 4.
[b] In the regression equation, the x value is the concentration of analytes (mol/1), the y value is the peak current (nA).

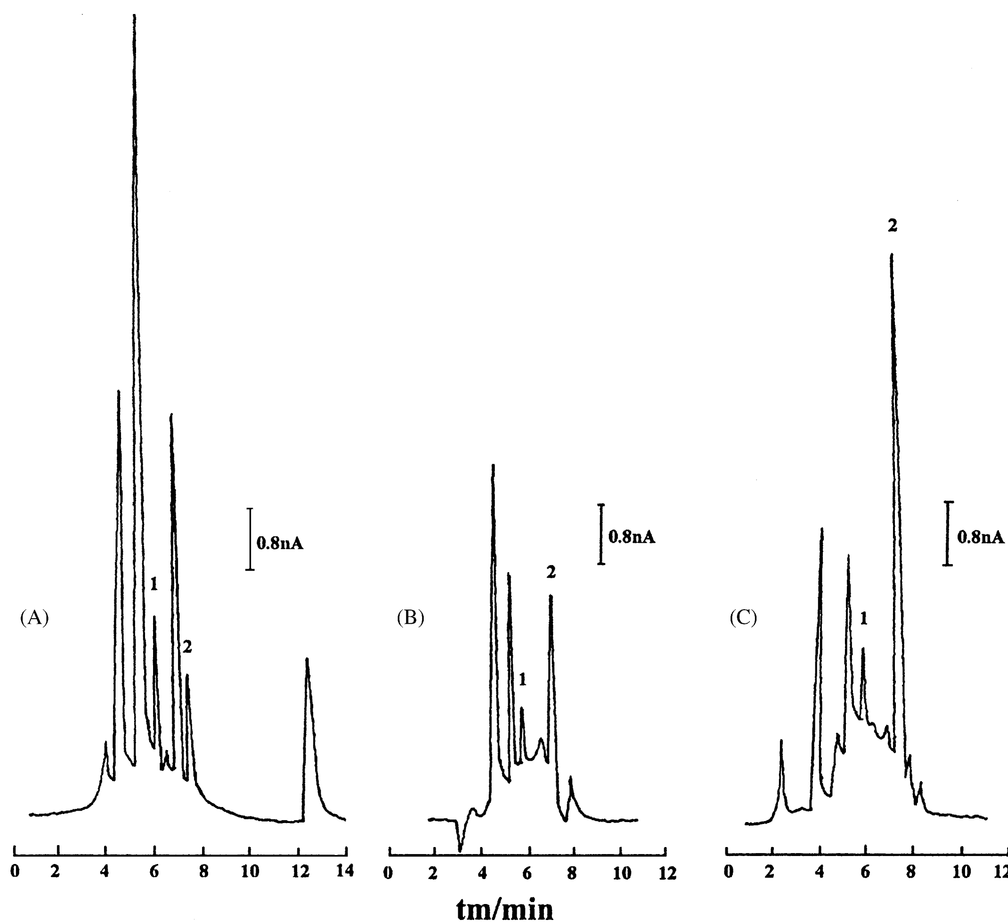

Fig. 5. The electropherograms of actual samples: *G. uralensis* herbs (A), compound Liquorice tablets (B) and compound Liquorice mixture (C). Peak identification: (1) liquiritigenin; (2) isoliquiritigenin. Conditions in Fig. 4.

Table 2
Assay results for *G. uralensis* herbs, compound liquorice tablets and compound liquorice mixture ($n = 3$)

Sample	Ingredients	Found	R.S.D. (%)
G. uralensis herbs	Liquiritigenin	0.81 mg/g	3.8
	Isoliquiritigenin	0.45 mg/g	4.0
Compound Liquorice tablets	Liquiritigenin	0.64 mg/g	2.9
	Isoliquiritigenin	1.49 mg/g	3.5
Compound Liquorice mixture	Liquiritigenin	0.101 mg/ml	4.5
	Isoliquiritigenin	0.299 mg/ml	1.6

CE–ED condition as Fig. 4.

the standard samples of liquiritigenin and isoliquiritigenin into the actual samples respectively, the active ingredients namely liquiritigenin (peak 1) and isoliquiritigenin (peak 2) in samples can be identified. Because the peaks of the analytes can not baseline separated from the adjacent peak the contents of the analytes in the actual sample were determined with standard addition method. The calibration curves with standard addition method for determination of liquiritigenin and isoliquiritigenin in *G. uralensis* herbs, compound Liquorice tablets and compound Liquorice mixture are shown in Fig. 6A–C, respectively. The assay results are listed in Table 2. The recovery and reproducibility experiments under the optimum conditions were also conducted to evaluate the precision and accuracy of the method. Recovery was determined by standard addition method,

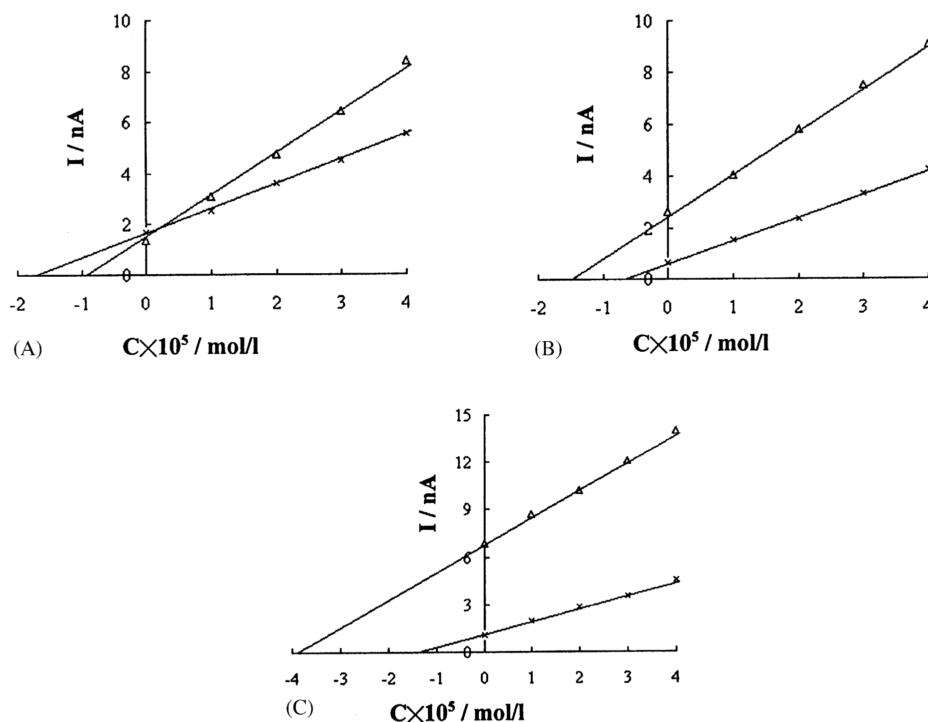

Fig. 6. The calibration curves with standard addition method for determination of actual samples: *G. uralensis* herbs (A), compound Liquorice tablets (B) and compound Liquorice mixture (C) symbol (X) represents liquiritigenin, and (Δ)stands for isoliquiritigenin.

Table 3
Results of recovery of this method with compound liquorice mixture sample ($n = 3$)

Ingredient	Original amount (mol/L)	Added amount (mol/L)	Found (mol/L)	Recovery (%)	R.S.D. (%)
Liquiritigenin	1.31×10^{-5}	1.00×10^{-5}	2.25×10^{-5}	97.5	3.5
Isoliquiritigenin	3.89×10^{-5}	1.00×10^{-5}	4.80×10^{-5}	98.1	1.8

and the results are listed in Table 3. The above assay results indicate that this method is accurate, sensitive and reproducible, providing a useful quantitative method for the analysis of traditional Chinese medicines.

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