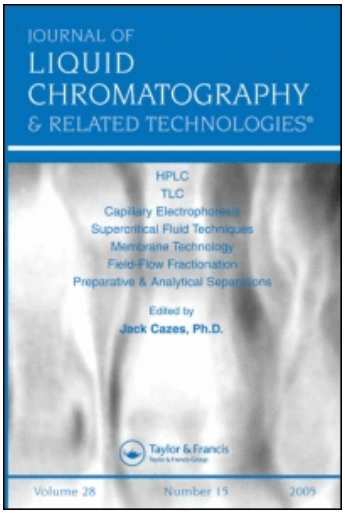

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Shufeng Shen[ab]; Zhidong Chang[ab]; Ji Liu[ab]; Xinghua Sun[ab]; Xin Hu[ab]; Huizhou Liu[ab]
[a] Laboratory of Separation Science and Engineering, State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Beijing, P.R. China [b] Graduate School of the Chinese Academy of Sciences, Beijing, P.R. China

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Shufeng Shen, Zhidong Chang, Ji Liu, Xinghua Sun, Xin Hu, and Huizhou Liu

Laboratory of Separation Science and Engineering, State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Beijing, P.R. China and Graduate School of the Chinese Academy of Sciences, Beijing, P.R. China

Abstract: Simultaneous quantitative determination of glycyrrhizic acid and liquiritin in *Glycyrrhiza uralensis* extract was developed using a high performance liquid chromatography (HPLC) coupled to an evaporative light scattering detector (ELSD). A Luna C_{18} column was used, along with a mobile phase consisting of acetonitrile and 3.0% (v/v) aqueous acetic acid in a gradient elution mode, at a flow rate of 1.0 mL/min. Parameters of ELSD were 105°C for the drift tube temperature and 2.8 L/min for the gas flow-rate. Retention times of liquiritin and glycyrrhizic acid were 4.1 and 8.3 min, respectively. Logarithmic calibration curves were obtained for glycyrrhizic acid from 0.50 to 5.72 μg ($r^2 > 0.999$) with a precision of 0.95% R.S.D and for liquiritin from 0.30 to 6.00 μg ($r^2 > 0.999$) with a precision of 0.59% R.S.D. The limit of quantitation (LOQ) of glycyrrhizic acid and liquiritin were 168.3 ng and 200 ng (S/N = 3), respectively. This method was successfully applied to quality evaluation in licorice raw materials and related commercial formulations.

Keywords: High performance liquid chromatography (HPLC), Evaporative light scattering detector (ELSD), Glycyrrhizic acid, Liquiritin, *Glycyrrhiza uralensis* extract

Address correspondence to Huizhou Liu, Laboratory of Separation Science and Engineering, State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, P. O. Box 353, Beijing 100080, P.R. China. E-mail: hzliu@home.ipe.ac.cn

INTRODUCTION

Licorice is one of the most extensively used medicinal herbs in Eastern and Western medicine. Up to now, above 200 chemical constituents have been characterized by chemical examination and pharmaceutical investigations.[1–4] Triterpenes and flavonoids are considered as important bioactive ingredient. These components have a wide variety of pharmacological activities, including anti-viral, anti-oxidant, anti-inflammatory, immunomodulatory, antiulcer, and have been applied extensively to food, confectionery, tobacco, and pharmaceutical industry.[3]

A qualitative and quantitative analysis of licorice extracts will be very helpful and necessary for an overall viewing of licorice constituents, quality control of raw materials, and production, and further isolation and purification processes. In aqueous licorice extracts, glycyrrhizic acid is usually the most abundant and is used as an indicator to assess the quality of licorice. Moreover, the lowest content of water soluble extractive is stipulated in pharmacopoeial grade licorice roots in several countries, due to the specific bioactivity of some water soluble flavonoids. Triterpenes have a basic skeleton of pentacyclic triterpenoids oleanane and flavonoids are a kind of polyphenolic compound that contains a typical C6-C3-C6 flavone skeleton. Glycyrrhizic acid and liquiritin are the representative compounds, respectively, and their structures are illustrated in Figure 1. Several analytical methods with different techniques have been published, such as polarography,[5] spectrophotometry,[6] thin-layer chromatography (TLC), gas chromatography (GC),[7] high performance liquid chromatography (HPLC)[1,8–11] coupled to mass spectrometry (MS),[4,12] capillary zone electrophoresis (CZE),[7,13–15] and micellar electrokinetic chromatography (MEKC).[16] Among these methods, HPLC with UV detection is proposed for the determination of licorice extracts. However, some problems have been widely recognized. Though many chemical constituents of licorice have been identified, some real

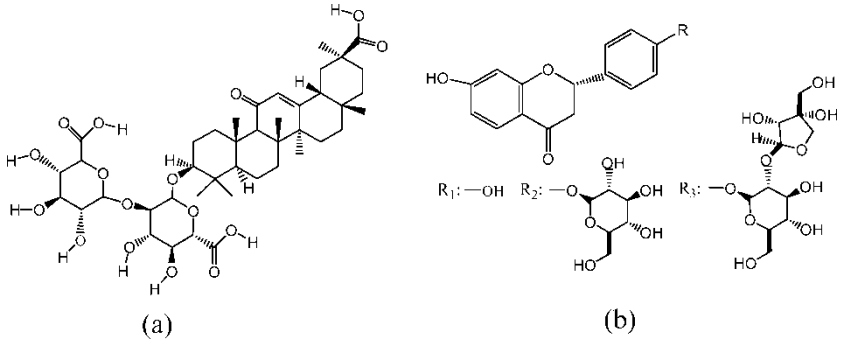

Figure 1. Chemical structures of compounds. (a) glycyrrhizic acid; (b) R_1: liquiritigenin; R_2: liquiritin; R_3: liquiritin apioside.

reference standard materials are still difficult to obtain, and different compounds usually have different response factors. Moreover, mobile phase gradient elutions may produce unstable baselines and some non-chromophoric components cannot be detected either. These characteristics make quantitative analysis of some constituents not easy or feasible.

An evaporative light scattering detector (ELSD) is being used increasingly in HPLC as a universal detection method in recent years, as long as any sample is less volatile than the mobile phase.[17–19] ELSD is gradient compatible and can maintain a stable baseline, because the mobile phase is evaporated before detection. Due to the response signal of ELSD based on mass concentrations of the analyte and the amount of light scattered, molecules with a similar structure formula and approximate molecular mass can show nearly equivalent response factors. These characteristics can also offer the possibility of quantitative evaluation of some substances with a lack of standard material. The quantitative analysis of water soluble constituents in licorice using HPLC coupled with ELSD was not reported until now.

In this work, a new HPLC-ELSD method was developed for simultaneous quantitative determination for glycyrrhizic acid and liquiritin in *Glycyrrhiza uralensis* licorice. Effective separation conditions were obtained by optimizing the compositions of mobile phase and the ELSD parameters. Logarithmic calibration curves of glycyrrhizic acid and liquiritin were used for quantitative analysis of components in licorice raw materials and their formulations.

EXPERIMENTAL

Plant Materials and Chemicals

Sample slices (thickness about 3–5 mm, diennial cultivated) and roots (triennial cultivated) of *Glycyrrhiza uralensis* were provided by Inner Mongolia Yili Science and Technology Industry Co. Ltd. (China). Glycyrrhizae and compound licorice tablets were purchased from a local market (Beijing, China). Glycyrrhizic acid monoammonium salt trihydrate ($C_{42}H_{65}O_{16}3H_2O$, MW 894.03, 98%) was purchased from the Acros Organics (USA). Liquiritin ($C_{21}H_{22}O_9$, MW 418.39, 98%) was purchased from National Pharmaceutical Engineering Center for Solid Preparation in Chinese Herbal Medicine (China). Acetonitrile, acetic acid, methanol, and water were of HPLC grade. Other chemicals were of analytical reagent grade.

Sample Preparation from Licorice Extract

Dried slices or roots of *Glycyrrhiza uralensis* (20.0 g) were extracted twice, each with 150 mL of aqueous ammonia solution (0.5% v/v) for 45 min by ultrasonic treatment. The residue was filtrated and washed with 25 mL of

distilled water. The combined extracts were used for the stock solution and the samples were diluted ten times for analysis.

Equipment and Chromatographic Conditions

Chromatographic separations were carried out on HP1100 liquid chromatography (Agilent Technologies, USA) equipped with an automatic injector and a Luna C_{18} (2) column (250 × 4.6 mm, 5.0 μm, Phenomenex, Torrance, CA, USA) was used. An Alltech ELSD2000ES (Alltech, Deerfield, IL, USA) and Agilent 35900E signal transmitter (Agilent Technologies, USA) were also used.

The optimized conditions were obtained by a linear gradient elution. The mobile phase was composed of acetonitrile and 3.0% aqueous acetic acid at a flow rate of 1.0 mL/min in a gradient elution, and the complete gradient conditions are summarized in Table 1. The drift tube temperature of ELSD was set at 105°C and the gas flow rate was 2.8 L/min.

Method Validation

Calibration Curves and Linearity

Stock solutions of Glycyrrhizic acid monoammonium salt and liquiritin were separately prepared. Samples were accurately weighed and dissolved into the volumetric flasks with HPLC grade methanol to make stock solutions of 1.0 mg/mL. Solutions were sonicated for 5 min to ensure complete dissolution. Calibration standards were prepared by diluting the stock solution with methanol in appropriate concentrations, and the calibration curves were generated by plotting the logarithm of peak areas versus logarithm of the mass of each analyte. The limit of quantitation (LOQ) was defined as the lowest mass of analyte able to be clearly detected at a signal-to-noise ratio (S/N) of 3.

Precision and Accuracy

The precision was expressed as the percentage relative standard deviations (RSDs) of the data for each analyte of five injections. The assays to

Table 1. Gradient elution profile of HPLC separation

Time (min)	0	8	12	15	18
A (%)	30	70	70	90	90
B (%)	70	30	30	10	10

A: acetonitrile; B: 3.0% (v/v) aqueous acetic acid.

evaluate accuracy and precision were preformed at different mass levels in replicate injections three times. The mean deviations were counted by the following equation:

$$\text{Mean Deviation (\%)} = \frac{\text{Calc. Mean Mass} - \text{Actual Mass} \times 100\%}{\text{Actual Mass}} \quad (1)$$

All the percent mean deviations were calculated to evaluate the accuracy by the difference between the calculated mean mass from calibration curves and their actual mass. The deviations lower than 5% was to be acceptable.

Intra- and inter-day variations were chosen to determine the precision of the developed method. Approximately 5.0 g of dried slices samples of *Glycyrrhiza uralensis* licorice were weighed, extracted, and analyzed, as described in the aforementioned Sample Preparation Section. For the intra-day variability test, the samples were analyzed in triplicate, three times (at 2.0 h intervals) within one day, while for inter-day evaluation, the samples were examined in triplicate for three consecutive days. Variations were expressed by the relative standard deviations (RSDs).

Application of Method

This developed HPLC-ELSD method was applied to determine the contents of some components in different licorice raw materials and related formulations. Two kinds of licorice extracts from diennial and triennial cultivated licorice were prepared according to the Sample Preparation Section. Samples of extractum glycyrrhizae or compound licorice tablets (200 mg) were dissolved into 100 mL of methanol solution (50%, v/v), and the top clarified solution was to be analyzed. The contents of components were calculated according to their corresponding calibration curves.

RESULTS AND DISCUSSION

Optimization of the HPLC-ELSD System

The compositions and gradient of mobile phase, the temperature of the drift tube, and the gas flow rate were very important parameters to determine whether major constituents in licorice extract could achieve good separation and response signals. The critical first step was that the different constituents should be eluted gradually from the analytical column by the mobile phase. Results showed that the use of a low gradient slope was proven to be an advantage resulting in high resolution, but greatly lengthens the elution time. Optimum separation and resolution could be achieved by the mobile phase composed of acetonitrile and 3.0% aqueous acetic acid in gradient elution,

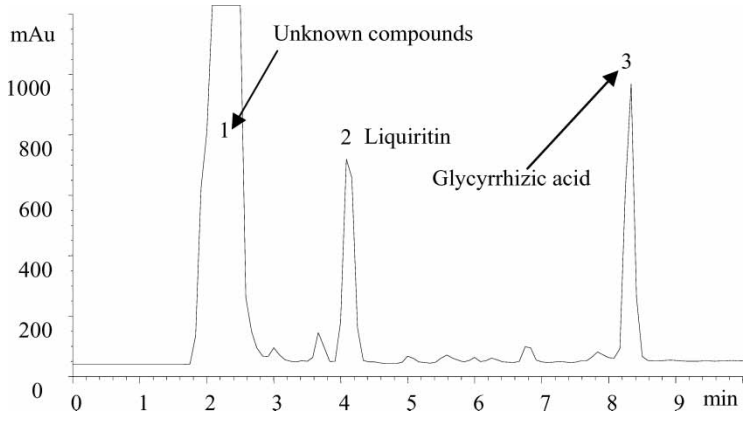

Figure 2. Representative HPLC-ELSD chromatographic profile of licorice extract. The gradient conditions are described in Table 1. ELSD parameters: drift tube temperature 105°C, gas flow-rate 2.8 L/min. Chromatographic peaks: 1, Retention time (Rt) = 2.5; 2, Rt = 4.1; 3, Rt = 8.3.

and the optimized gradient conditions are described in Table 1. Then, the temperature for determination of two reference compounds was optimized from 90°C to 115°C and the gas flow rate from 2.0 L/min to 3.0 L/min, according to the ELSD reference. The drift tube temperature of 105°C and the gas flow rate of 2.8 L/min were the optimized parameters to obtain a good chromatographic response. The typical HPLC-ELSD chromatograms of licorice extract as shown in Figure 2. Three major chromatographic peaks (1, 2, and 3) were found and their retention times (Rt) were 2.5, 4.1, and 8.3 min, respectively.

Two major chromatographic peaks (Rt = 4.1 and 8.3) could be assigned to glycyrrhizic acid and liquiritin according to their standards. The chromatographic peak 1 (Rt = 2.5) had a wide and strong response signal for ELSD but almost invisible UV-vis absorption. Moreover, primary results by HPLC combined with mass spectroscopy showed that it might probably be a mixture of saccharides, amino acids, and flavanones, and need to be further analyzed in future work.

Table 2. Calibration curves for glycyrrhizic acid and liquiritin

Analytes	Calibration curves[a]	Correlation coefficient (r^2)	Linear range (μg)	LOQ (ng)
Glycyrrhizic acid	y = 1.7861 x −2.4582	0.9999	0.50–5.72	168.3
Liquiritin	y = 1.7834 x −2.5714	0.9994	0.30–6.00	200.0

[a]y: the logarithm of peak areas; x: logarithm of the mass of each analyte (ng).

Table 3. Validation of precision and accuracy of method

	Actual mass (μg)	Calculated mean mass (μg)	Mean deviation[a] (%)	Variation within replicates (%, n = 3)
Glycyrrhizic acid	1.44	1.41	2.08	0.46
	2.40	2.37	1.25	1.60
	4.80	4.73	1.46	0.21
Liquiritin	1.00	1.02	2.00	0.86
	4.00	4.13	3.25	0.46

[a]Mean deviation (%)= (calculated mean mass − actual mass)/actual mass × 100%.

Method Validation

Calibration curves of liquiritin and glycyrrhizic acid monoammonium salt were investigated over the mass range of 0.2–7.0 μg. Under the optimized HPLC-ELSD conditions, all calibration curves showed good linearity ($r^2 > 0.999$) within the corresponding ranges, as shown in Table 2. The LOQ of this method for glycyrrhizic acid and liquiritin was 168.3 ng and 200 ng, respectively. Moreover, it could be, surprisingly, found that glycyrrhizic acid and liquiritin had nearly equivalent response factors under the analytical conditions, even though they had obviously different structures and molecular weights, which would also offer a great convenience for a rough quantitative estimate by one of two compounds.

Table 4. Determination of intra- and inter-day precision of method

		Intra-day variability			Inter-day variability		
Analyte		Mass (μg)	Mean	RSD (%)	Mass (μg)	Mean	RSD (%)
Glycyrrhizic acid	1	2.124	2.113	0.58	2.109	2.104	0.32
		2.107			2.104		
		2.109			2.098		
	2	3.206	3.205	0.09	3.206	3.197	0.94
		3.202			3.217		
		3.206			3.168		
Liquiritin	1	2.004	2.006	0.46	2.000	1.978	2.46
		2.014			2.005		
		2.000			1.930		
	2	3.001	2.997	0.43	3.005	3.001	1.63
		2.986			3.040		
		3.005			2.959		

Validation of Precision and Accuracy

The RSDs between replicate injections for glycyrrhizic acid and liquiritin were 0.95% and 0.59% (n = 5), respectively. The mean deviations were evaluated by injecting a set of known validation samples at low and high mass concentrations. The results are summarized in Table 3. It was observed that the analytical method for the two compounds had good accuracies within the deviation ranges of 1.2–3.5% and variation within replicates were within the ranges of 0.2–1.6%. Intra- and inter-day evaluation of the method is shown in Table 4. The intra-and inter-day variations were less than 1.5% for two analytes, indicating that this developed method was accurate and precise enough for their quantitative evaluation.

Quantitative Analysis of Major Constituents

The HPLC-ELSD method was applied for content determination of constituents in two different plant samples of *Glycyrrhiza uralensis* from Inner Mongolia (China) and their formulations. The representative chromatograms and contents of main components are shown in Figure 3 and Table 5, respectively. It was found that there were remarkable differences of constituents

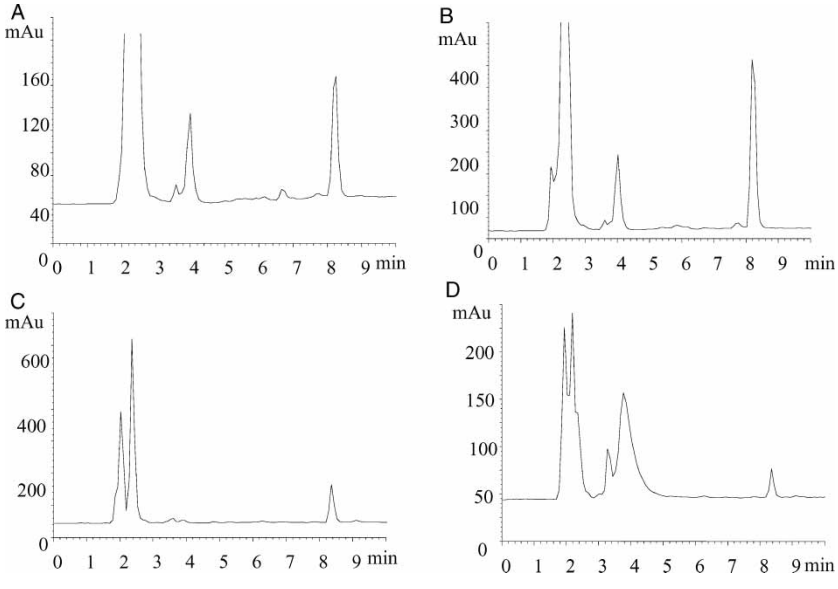

Figure 3. Representative HPLC-ELSD chromatograms of four samples. The gradient conditions as described in Table 1. ELSD parameters: drift tube temperature 105°C, gas flow rate 2.8 L/min. A: licorice slices extract; B: licorice roots extract; C: extractum glycyrrhizae; D: compound licorice tablets.

Table 5. Contents of constituents in different plant sources of licorice or formulations

	Content, % (g/g), (R.S.D.%)[a]	
Samples	Glycyrrhizic acid	Liquiritin
Licorice slices[b]	2.18 (1.21)	2.06 (0.89)
Licorice roots[c]	4.35 (1.55)	3.06 (0.73)
Extractum glycyrrhizae	6.79	–
Compound licorice tablets	4.15	–

[a]R.S.D. % of contents of constituents calculated from four batch extract samples of the same source of licorice.

[b]Diennial cultivated licorice samples from Inner Mongolia (China).

[c]Triennial cultivated licorice samples from Inner Mongolia (China).

– Undetectable.

and their contents between plant samples in different cultivated stages (diennial and triennial licorice). For the triennial licorice, the mean contents of glycyrrihizic acid and liquiritin were 4.35% and 3.06%, respectively, which met with the requirement of some pharmacopoeias (not less than 4% glycyrrihizic acid). Moreover, the RSDs between four batch extract samples of the same source of licorice were less than 3.0% (Table 5). In the two pharmaceutical formulations investigated, glycyrrihizic acid, one of the important active ingredients, was analyzed quantitatively to inspect and control the quality of their formulations. Moreover, this method may be of great help to qualitatively distinguish different origins of licorice according to their HPLC-ELSD chromatographic profiles.

CONCLUSIONS

A new HPLC-ELSD method has been developed for quantitative determination of glycyrrhizic acid and liquiritin. All calibration curves showed good linearity ($r^2 > 0.999$) and good reproducibility (RSDs < 2.0 %). The LOQ of glycyrrhizic acid and liquiritin was 168.3 ng and 200 ng, respectively. This described method could be useful in the quality analysis of different kinds of licorice raw materials and their formulations, and it has potential applications for simultaneous content evaluation of related compounds with their similar structures.

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