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Quality Analysis of Raw and Honey-Processed Licorice of *Glycyrrhiza uralensis* Fisch. and *G. glabra* L. by Simultaneous Determination of Five Bioactive Components Using RP-HPLC/DAD Method

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ABSTRACT

A reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed for simultaneous determination of five bioactive components, liquiritin apioside, liquiritin, licuraside, isoliquiritin and glycyrrhizin in licorice with diode-array detection (DAD). The method was performed with a mobile phase consisting of acetonitrile and 0.1% (v/v) phosphoric acid under gradient elution. All calibration curves of the five bioactive components showed excellent linearity ($r^2 \geq 0.9997$) within the ranges of 91.2 - 1824 μg/mL, 54.4 - 2720 μg/mL, 6.4 - 512 μg/mL, 6.4 - 512 μg/mL and 20.4 - 2040 μg/mL, respectively. The average recoveries ranged from 95.86% to 103.11%. This analytical method was also validated with respect to precision, repeatability and accuracy, and proved to be sensitive and accurate to simultaneously determine the five components in licorice. The developed method was further applied to quantify the contents of five components in forty two batches of either raw or honey-processed licorice samples of *Glycyrrhiza uralensis* Fisch. and *G. glabra* L. The results revealed that the contents of five components had descended *in toto* after the honey process. The content ratio of glycyrrhizin to liquiritin was more than 5.0 in licorice of *G. glabra* whereas it was less than 3.0 in that of *G. uralensis* in both raw and honey-processed licorice materials. This content ratio difference may be used to distinguish among licorice materials.

Key words: licorice, honey-procedure, flavonoids, glycyrrhizin, RP-HPLC/DAD, correlation

INTRODUCTION

Licorice is derived from dried roots and rhizomes of *Glycyrrhiza* species (Leguminosae Family) plants which are widely distributed in Europe and Asia[1]. It has been used medically to treat diseases of respiratory tract, gastrointestinal and cardiovascular system for a long time[2]. In China, only *Glycyrrhiza uralensis* Fisch., *G. glabra* L. and *G. inflata* Bat. are officially used and called Gancao in the Chinese Pharmacopeia[3]. Besides Gancao, Zhi-Gancao, a honey-mix-and-bake processed licorice is also a commonly prescribed licorice in traditional Chinese medicine (TCM)[4]. According to the TCM theory, Zhi-Gancao does better at tonifying and replenishing, which is considered as immunoregulation in pharmacology research[5], while Gancao has a greater anti-inflammatory effect[6].

More than 400 compounds have been isolated from *Glycyrrhiza* species[7]. Flavonoids and triterpene saponins are the major components, which are believed to be responsible for their pharmacological activities, including anti-inflammatory, antimicrobial and antiviral, antioxidant, antispasmodic, hepatoprotective, expectorant and memory enhancing[6]. Specifically, glycyrrhizin, a triterpene saponin, is a major component in licorice exhibiting anti-inflammatory, antiulcer and immunological activity[6]. Liquiritin apioside, liquiritin, licuraside and isoliquiritin in licorice belong to the flavonoids which exhibit antioxidant activity in many *in vitro* and *in vivo* studies[8]. According to the Chinese Pharmacopoeia, liquiritin and glycyrrhizin are recommended as quality control markers of raw licorice, however, they are monitored by two separate HPLC methods[3]. Even though, many HPLC methods have been developed to quantify the contents of flavonoids or/and triterpene saponins in licorice, most of which were able to determine only one or two components[9-13]. Due to the co-existence of multiple bioactive components in TCM products, many researchers have developed sophisticated methods to simultaneously quantify as many components as possible in their studies. In Li's work, liquiritin, isoliquiritin, liquiritigenin, isoliquiritigenin, glycyrrhizin and glycyrrhetinic acid in licorice and

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related Chinese herbal preparations were determined[14]. In Wang's report, liquiritin, liquiritigenin, isoliquiritigenin, glycyrrhizin, 18α-glycyrrhetinic acid, 18β-glycyrrhetinic acid and 18β-glycyrrhetinic acid methyl ester were quantified in twenty raw licorice samples[15]. Simultaneous determination of liquiritin, isoliquiritin, liquiritigenin, isoliquiritigenin, glycyrrhizin, licorice-saponin G_2 and uralsaponin B in eleven batches of licorice were reported recently by Li *et al.* as well[16]. In these studies, separation of several compounds was achieved and the content of each analyte in different raw licorice samples or related preparations were successfully quantified. However, analytical studies on honey-processed licorice samples were rarely reported. In our previous preliminary study, liquiritin apioside, liquiritin, licuraside, isoliquiritin and glycyrrhizin were analyzed by RP-HPLC/UV method and contents of all five components in raw, baked, honey-baked and honey-soaked licorice samples were compared[17].

In this study, a sensitive and accurate RP-HPLC/DAD method was developed for the simultaneous determination of five major bioactive components in raw and honey-processed licorice samples collected from different regions of China. Based on the determination of various batches of samples, changes in content, correlations of the five individual components in raw and honey-processed licorice and differences between licorice of *Glycyrrhiza uralensis* and *G. glabra* were compared and discussed. We believe these findings should be considered as quality control markers to be implemented into the Pharmacopeia.

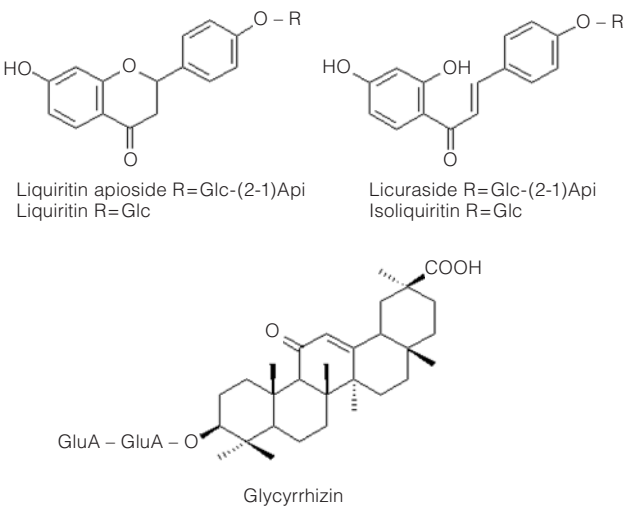

Figure 1. Chemical structures of the five major components in licorice.

MATERIALS AND METHODS

I. *Chemicals and Reagents*

Reference standards, liquiritin apioside, liquiritin, licuraside, isoliquiritin and glycyrrhizin were isolated and purified from licorice (roots of *G. uralensis*) by the authors. The chemical structures of all five components were confirmed by ^{1}H-NMR, ^{13}C-NMR and HPLC/MS analyses and are showed in Figure 1. The relative purities were all more than 95% by HPLC-UV analysis. HPLC grade acetonitrile was purchased from Shanghai Xingke Biochemistry Co., Ltd (Shanghai, China). Analytical grade methanol and phosphoric acid were from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Water was purified by Milli-Q system (Millipore, Bedford, MA, USA). Honey was supplied by Shanghai Jing-an Pharmaceutical Co., Ltd (Shanghai, China).

II. *Apparatus and Chromatographic Conditions*

An Agilent 1200 series HPLC system (Agilent, Waldbronn, Germany) was employed for the analysis, which consisted of quaternary pump, on-line degasser, autosampler, thermostatic column compartment and DAD. An Agilent Zorbax Elipse XDB-C_{18} column (5 μm, 250 × 4.6 mm) connected with an Alltech Brava BDS-C_{18} guard column (5 μm, 7.5 × 4.6 mm) was used for chromatographic separation at a temperature of 35°C. The mobile phase consisted of (A) acetonitrile and (B) 0.1% aqueous phosphoric acid (v/v) using gradient elution. The gradient program for quantitative analysis was: 0 - 20 min, 13% - 19% A, 0.6 mL/min, 276 nm; 20 - 37 min, 19% - 35% A, 0.8 mL/min, 360 nm; 37 - 55 min, 35% - 55% A, 0.8 mL/min, 254 nm.

III. *Plant Materials*

Fifteen batches of raw licorice and twelve batches of honey-processed licorice were collected from local pharmacies in Xinjing Autonomous Region, Inner Mongolia Autonomous Region, Guangxi Province, Jiangsu Province, Beijing and Shanghai. A portion of each batch of raw materials of licorice was then prepared in house by the honey process (honey-mix-and-bake process, described below) under identical conditions. A total of forty two batches of licorice, fifteen raw, fifteen in-house honey-processed, and twelve purchased honey-processed licorice samples were used in this study.

IV. *Preparation of In-House Honey-Processed Licorice Samples*

According to the general guidance for raw materials preparation in the Chinese Pharmacopoeia, 100 g of raw materials require 25 g of refined honey to process. In this study, the raw licorice was mixed with refined honey in the same ratio as recommended by the Chinese Pharmacopoeia and baked to dry at 150°C in 1 h.

V. *Preparation of Licorice Sample Solutions*

0.5 g of licorice powder (passed through 50-mesh screen and dried at 60°C for 8 h before weighing) was mixed with 25 mL of 60% (v/v) methanol aqueous solution in a

conical flask with plunger, and subject to ultrasonic extraction for 15 min. The sample solutions were passed through a 0.22-μm filter and analyzed by HPLC.

VI. *Preparation of Standard Solutions*

The stock solutions of standards were prepared by accurately weighing 5.7 mg of liquiritin apioside, 6.8 mg of liquiritin, 1.6 mg of licuraside, 1.6 mg of isoliquiritin and 5.1 mg of glycyrrhizin standards and dissolving in 25 mL of methanol, individually, and kept at -20°C. The working solutions were prepared by diluting the respective stock solutions with methanol and stored at 4°C.

RESULTS AND DISCUSSION

I. *Optimization of Chromatographic Conditions*

Acetonitrile-aqua system was adapted to separate the five components due to its good resolution. In the pre-test, peak of glycyrrhizin was too blunt to identify without any acid in the aqueous phase. Hence, acid was used to optimize the peak shape. The base line was not stable when the aqueous phase contained more than 1.5% (v/v) of acetic acid. Subsequently, phosphoric acid was considered, and good peak shape of the five components and steady base line were obtained from the aqueous phase consisted of 0.1% (v/v) phosphoric acid (pH 2.2).

It was observed that baseline of liquiritin apioside and liquiritin were not baseline separated at the flow rate of 0.8 mL/min. Better separation (R > 1.5) between the two components was obtained when the flow rate was reduced to 0.6 mL/min. The maximum absorption of all five components were measured by the DAD and the detection wavelengths were thus set at 276 nm for both liquiritin apioside and liquiritin , 360 nm for both licuraside and isoliquiritin and 254 nm for glycyrrhizin.

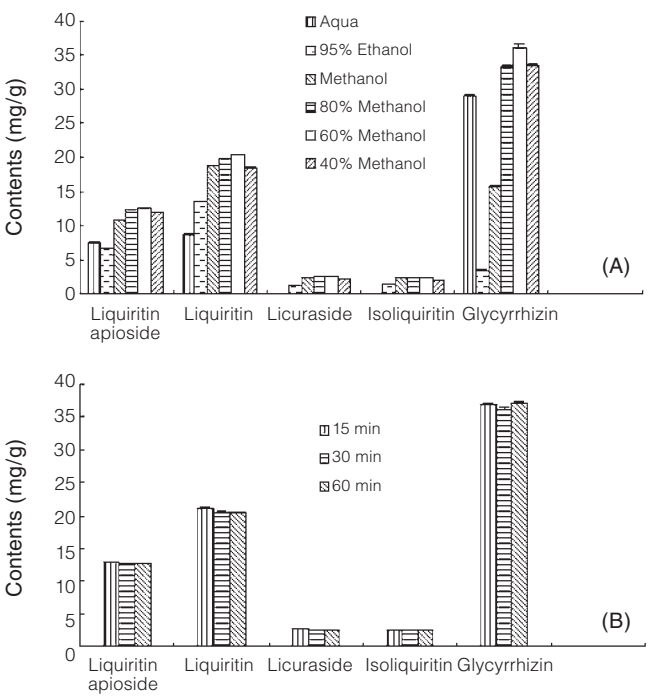

Figure 2. Extraction efficiencies of the five major components by (A) different extraction solutions and (B) different extraction times.

II. *Optimization of Sample Preparation*

Ultrasonic extraction was considered as a simple, effective method and widely used in TCM. Extraction solvent of water, 95% ethanol aqueous solvent and methanol/water solutions (100, 80, 60, and 40%, v/v) were studied . Extraction time of 15, 30 and 60 min were compared. The extraction efficiencies under different conditions are summarized in Figure 2. The results showed that 60% methanol aqueous solvent system offered an overall best extraction efficiency. It is noticed that the extraction time had no significant effect on the extraction efficiency after 15 min of extraction, probably due to high solvent volume to licorice sample ratio (25 mL to 0.5 g, 50 times). Therefore, the extraction time was determined as 15 min in this study.

III. *Validation of the Analytical Method*

(I) *Specificity*

The specificity validation was conducted on a mixture of the five standards solution, licorice sample solution, and mobile phase or 60% (v/v) methanol aqueous solution (as a blank). HPLC chromatograms, displayed in Figure 3, indicated that the blank solutions did not interfere with the separation and good separation for the five components in licorice was obtained under this chromatographic condition.

(II) *Linearity, LOD and LOQ*

Calibration curves were established from seven different concentrations of the standard solutions and analyzed using

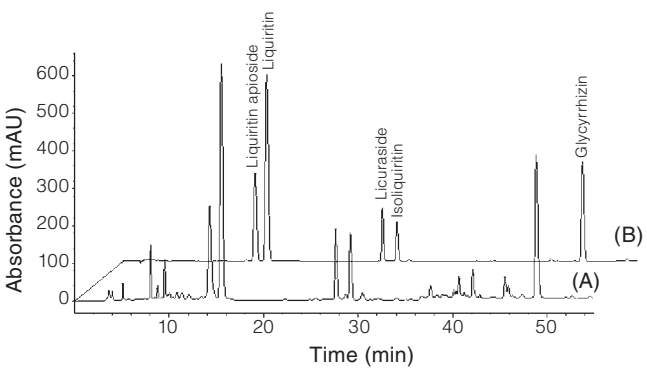

Figure 3. Specificity validation for the HPLC analytical method for liquiritin apioside, liquiritin, licuraside, isoliquiritin and glycyrrhizin in licorice: (A) raw licorice samples solution and (B) standard solution of liquiritin apioside, liquiritin, licuraside, isoliquiritin and glycyrrhizin at the concentration of 11.4, 18.13, 2.56, 1.83 and 29.14 μg/mL, respectively.

the linear least-square regression equation based on the peak areas as a function of concentrations of each component. LOD and LOQ were separately determined at a signal-to-noise ratio (S/N) of three and ten, respectively.

Linear regressions, LOD and LOQ values were estimated with the external standard method and are reported in Table 1. Liner regression analysis of each component had wide linear concentration range and correlation coefficient was greater than 0.9998. The LOD and LOQ values suggested that the developed HPLC method was sufficiently sensitive for the determination of the five components in licorice.

(III) *Precision*

The instrument precision was validated by performing the intra- and inter-day assays on the mixed standard solutions. The intra-day precision assay was carried out with six replicate injections in a single day, while the inter-day precision was analyzed everyday in duplicate for three consecutive days.

Table 1. Calibration curves of the five components in licorice (n = 3, mean value)

Component	Regression equation[a]	*r*	Linear range (μg/mL)	Limit of detection (LOD, μg/mL)	Limit of quantification (LOQ, μg/mL)
Liquiritin apioside	y = 2109.03x + 146.36	0.9998	91.2 - 1824	9.12×10^{-2}	5.02×10^{-1}
Liquiritin	y = 2926.23x + 120.39	0.9999	54.4 - 2720	8.70×10^{-2}	3.04×10^{-1}
Licuraside	y = 5380.81x – 30.40	0.9999	6.4 - 512	3.84×10^{-2}	1.92×10^{-1}
Isoliquiritin	y = 5811.35x – 15.71	0.9999	6.4 - 512	3.84×10^{-2}	1.41×10^{-1}
Glycyrrhizin	y = 1000.94x + 4.61	0.9999	20.4 - 2040	6.20×10^{-2}	2.04×10^{-1}

[a] y is the peak area and x is the concentration (μg/mL) of the component.

Table 2. Intra- and inter-day precision data for concentrations of the five components in licorice

Component	Intra-day precision (n = 6, mean ± SD)						Inter-day precision (n = 18, mean ± SD)	
	Day 1		Day 2		Day 3			
	Concentration (μg/mL)	RSD (%)	Concentration (μg/mL)	RSD (%)	Concentration (μg/mL)	RSD (%)	Concentration (μg/mL)	RSD (%)
Liquiritin apioside	229.03 ± 1.05	0.46	229.58 ± 2.20	0.96	231.82 ± 1.51	0.65	230.14 ± 1.47	0.64
Liquiritin	2774.92 ± 13.96	0.51	2792.27 ± 14.63	0.53	2802.58 ± 1.19	0.43	2789.92 ± 13.98	0.51
Licuraside	51.10 ± 0.22	0.43	51.22 ± 0.23	0.45	51.46 ± 0.19	0.37	51.53 ± 0.35	0.68
Isoliquiritin	48.53 ± 0.22	0.45	48.72 ± 0.23	0.48	48.92 ± 0.20	0.41	48.73 ± 0.20	0.41
Glycyrrhizin	116.75 ± 0.55	0.47	116.77 ± 0.74	0.64	117.40 ± 0.57	0.49	116.97 ± 0.37	0.32

Table 3. Recovery of the five components in licorice (n = 3, mean value)

Component	Original (mg)	Spiked (mg)	Determined (mg)	Recovery (%)	RSD (%)
Liquiritin apioside	3.26	1.57	4.81	98.72	0.80
		3.34	6.76	101.96	0.33
		4.90	8.31	103.11	1.97
Liquiritin	5.26	2.54	7.70	95.86	1.38
		5.55	10.69	97.79	0.24
		7.92	13.05	98.23	1.43
Licuraside	0.65	0.27	0.92	96.51	0.79
		0.60	1.24	99.16	1.08
		0.85	1.51	100.88	1.54
Isoliquiritin	0.62	0.32	0.95	101.56	1.39
		0.64	1.28	101.56	0.58
		0.96	1.58	98.96	2.05
Glycyrrhizin	9.38	4.90	14.21	98.67	1.92
		9.80	19.27	100.97	2.54
		13.72	23.31	101.57	0.83

Table 4. Contents of the five components in fifteen batches of raw and their in-house honey-processed licorice samples

No.	Region collected	Date collected	Original plant		Content of each component (mg/g, n = 3, mean ± SD, dried matter)					Content ratio of glycyrrhizin to liquiritin
					Liquiritin apioside	Liquiritin	Licuraside	Isoliquiritin	Glycyrrhizin	
1	Urumqi, Xinjiang	2008.12	*G. glabra*	Raw	10.71 ± 0.22	9.79 ± 0.10	2.73 ± 0.02	1.45 ± 0.01	63.93 ± 0.17	6.53
				Honey-processed	6.88 ± 0.08	4.32 ± 0.04	1.17 ± 0.02	1.01 ± 0.03	28.80 ± 0.32	6.67
2	Urumqi, Xinjiang	2009.05	*G. glabra*	Raw	12.54 ± 0.10	8.72 ± 0.04	3.22 ± 0.02	1.32 ± 0.01	73.04 ± 0.38	8.38
				Honey-processed	6.26 ± 0.05	4.05 ± 0.02	1.44 ± 0.30	1.03 ± 0.02	34.83 ± 0.32	8.60
3	Aksu, Xinjiang	2009.03	*G. glabra*	Raw	9.51 ± 0.14	10.58 ± 0.13	2.40 ± 0.04	1.43 ± 0.02	57.98 ± 0.89	5.48
				Honey-processed	4.63 ± 0.06	4.47 ± 0.05	1.02 ± 0.02	1.11 ± 0.02	26.03 ± 0.40	5.82
4	Kashi, Xinjiang	2009.01	*G. glabra*	Raw	15.07 ± 0.20	10.44 ± 0.14	3.27 ± 0.04	1.41 ± 0.02	59.92 ± 0.79	5.74
				Honey-processed	7.87 ± 0.07	4.09 ± 0.02	1.25 ± 0.02	0.92 ± 0.02	24.49 ± 0.18	5.99
5	Turfan, Xinjiang	2009.01	*G. glabra*	Raw	15.91 ± 0.14	8.37 ± 0.09	3.78 ± 0.04	1.20 ± 0.02	67.09 ± 0.61	8.02
				Honey-processed	7.97 ± 0.05	3.85 ± 0.05	1.55 ± 0.03	0.98 ± 0.02	31.09 ± 0.20	8.08
6	Wusu, Xinjiang	2009.03	*G. glabra*	Raw	8.47 ± 0.09	5.16 ± 0.09	2.23 ± 0.03	0.89 ± 0.02	59.24 ± 0.11	11.48
				Honey-processed	3.84 ± 0.05	2.19 ± 0.02	0.87 ± 0.02	0.63 ± 0.02	27.68 ± 0.51	12.64
7	Qitai, Xinjiang	2009.01	*G. glabra*	Raw	22.88 ± 0.23	6.26 ± 0.07	5.83 ± 0.06	1.03 ± 0.01	69.74 ± 0.31	11.14
				Honey-processed	10.40 ± 0.14	2.92 ± 0.02	2.15 ± 0.02	0.77 ± 0.01	30.00 ± 0.52	10.27
8	Shihezi, Xinjiang	2009.02	*G. uralensis*	Raw	5.02 ± 0.02	18.55 ± 0.01	0.94 ± 0.01	1.21 ± 0.01	30.86 ± 0.06	1.66
				Honey-processed	3.92 ± 0.07	10.48 ± 0.16	0.49 ± 0.01	1.75 ± 0.03	11.12 ± 0.11	1.06
9	Hetian, Xinjiang	2009.02	*G. uralensis*	Raw	6.04 ± 0.03	30.87 ± 0.11	1.04 ± 0.01	2.57 ± 0.02	36.86 ± 0.29	1.19
				Honey-processed	2.53 ± 0.02	6.80 ± 0.05	0.48 ± 0.01	1.41 ± 0.02	11.74 ± 0.14	1.73
10	Beijing	2009.03	*G. uralensis*	Raw	7.19 ± 0.05	23.93 ± 0.18	1.41 ± 0.01	2.09 ± 0.01	39.52 ± 0.28	1.65
				Honey-processed	5.26 ± 0.09	10.09 ± 0.13	0.84 ± 0.02	2.20 ± 0.05	17.13 ± 0.12	1.70
11	Shanghai	2009.04	*G. uralensis*	Raw	6.28 ± 0.08	25.49 ± 0.14	1.53 ± 0.02	3.56 ± 0.05	38.20 ± 0.46	1.50
				Honey-processed	3.68 ± 0.08	9.94 ± 0.14	0.77 ± 0.01	2.63 ± 0.06	14.62 ± 0.20	1.47
12	Changshu, Jiangsu	2009.03	*G. uralensis*	Raw	7.81 ± 0.14	21.35 ± 0.31	1.49 ± 0.03	2.76 ± 0.05	38.21 ± 0.50	1.79
				Honey-processed	5.05 ± 0.02	8.06 ± 0.07	0.72 ± 1.21	1.20 ± 0.04	14.22 ± 0.14	1.76
13	Guilin, Guangxi	2009.04	*G. uralensis*	Raw	11.71 ± 0.25	19.72 ± 0.19	2.16 ± 0.02	1.41 ± 0.01	42.76 ± 0.51	2.17
				Honey-processed	5.68 ± 0.12	7.35 ± 0.11	0.90 ± 0.02	1.20 ± 0.03	8.95 ± 0.24	1.22
14	Beihai, Guangxi	2009.03	*G. uralensis*	Raw	6.32 ± 0.05	27.24 ± 0.25	1.44 ± 0.01	2.56 ± 0.03	43.10 ± 0.38	1.58
				Honey-processed	2.99 ± 0.04	8.40 ± 0.50	0.50 ± 0.01	1.64 ± 0.04	12.30 ± 0.14	1.46
15	Hohhot, Inner Mongolia	2007.02	*G. uralensis*	Raw[a]	12.97 ± 0.14	21.00 ± 1.76	2.60 ± 0.02	2.53 ± 0.01	37.47 ± 0.42	1.78
				Honey-processed	11.72 ± 0.13	16.64 ± 0.10	2.33 ± 0.05	4.08 ± 0.04	32.61 ± 0.33	1.96

[a] The raw licorice collected from Hohhot, Inner Mongolia was applied to the validation of the analytical method.

The results are summarized in Table 2. The RSD values at each concentration were less than 1.0% for both intra- and inter-day precision assays, indicating the high precision of the chromatographic system.

(IV) *Reproducibility and Sample Stability*

The reproducibility was examined by analyzing six sample solutions from the same batch under identical preparation conditions. The room-temperature sample stability was confirmed by analyzing one sample solution at 0, 3, 6, 9, 12 and 24 h, respectively.

The RSD values from the reproducibility experiments were less than 2.10%, which revealed this analytical method was repeatable. In the stability validation, neither appreciable changes of the five components nor degradation products were detected in the chromatograms. The RSD values of concentrations were below 2.10%. The licorice sample solution was stable within 24 h at room temperature after preparation.

(V) *Accuracy*

For accuracy validation, standard solutions at low, medium and high levels (50%, 100% and 150% of the original contents) were spiked individually to licorice sample with known contents of the five components. The samples with the spiked standard solutions were then extracted and analyzed according to the procedure developed in this study.

The results are reported in Table 3. The average recoveries ranged from 95.86% to 103.11%, with the RSD values raging from 0.24% to 2.54%, indicating that the method was accurate.

IV. *Quantification of Licorice Samples*

The validated HPLC method was applied to simultaneously determine liquiritin apioside, liquiritin, licuraside, isoliquiritin and glycyrrhizin in fifteen batches of raw and their in-house honey-processed licorice and twelve batches of purchased honey-processed licorice. The contents are summarized in Tables 4 and 5.

In all licorice samples, glycyrrhizin was the most abundant (8.95 ± 0.24 to 73.04 ± 0.38 mg/g), followed in descending order by liquiritin (2.19 ± 0.02 to 30.87 ± 0.11 mg/g), liquiritin apioside (2.53 ± 0.02 to 23.62 ± 0.24 mg/g), licuraside (0.48 ± 0.01 to 5.83 ± 0.06 mg/g) and isoliquiritin (0.63 ± 0.02 to 4.14 ± 0.04 mg/g). The contents of liquiritin and glycyrrhizin in most raw licorice were more than 10 and 20 mg/g, respectively, which are the minimum contents required by the Chinese Pharmacopoeia. In the USP 28-NF 23[18] and the JP XIV[19], only glycyrrhizin content in licorice at higher than 25 mg/g is required of dried licorice. The contents of glycyrrhizin in raw samples ranged from 37.47 ± 0.42 to 73.04 ± 0.38 mg/g, all of which were far better than the requirements. The contents of the five components in all fifteen batches of raw samples had descended *in toto* after the

Table 5. Contents of the five components in twelve batches of purchased honey-processed licorice samples

No.	Region collected	Date collected	Original plant	Content of each component (mg/g, n = 3, mean ± SD, dried matter)					Content ratio of glycyrrhizin to liquiritin
				Liquiritin apioside	Liquiritin	Licuraside	Isoliquiritin	Glycyrrhizin	
1	Urumqi, Xinjiang	2009.05	*G. glabra*	21.37 ± 0.13	10.73 ± 0.10	4.50 ± 2.70	1.45 ± 0.02	65.68 ± 1.20	6.12
2	Kashi, Xinjiang	2009.01	*G. glabra*	19.63 ± 0.13	12.10 ± 0.09	4.59 ± 0.04	1.56 ± 0.01	70.03 ± 1.22	5.79
3	Turfan, Xinjiang	2009.01	*G. glabra*	23.62 ± 0.24	11.69 ± 0.10	4.81 ± 0.06	1.53 ± 0.01	71.49 ± 2.05	6.12
4	Aksu, Xinjiang	2009.03	*G. uralensis*	3.57 ± 0.03	19.26 ± 0.11	0.87 ± 0.01	3.08 ± 0.02	27.77 ± 0.61	1.44
5	Wusu, Xinjiang	2009.03	*G. uralensis*	3.96 ± 0.04	19.68 ± 0.18	0.93 ± 0.01	2.97 ± 0.03	29.39 ± 0.35	1.49
6	Qitai, Xinjiang	2009.01	*G. uralensis*	4.39 ± 0.04	27.75 ± 0.19	1.01 ± 0.01	4.14 ± 0.04	38.83 ± 0.34	1.40
7	Shihezi, Xinjiang	2009.02	*G. uralensis*	4.06 ± 0.03	21.27 ± 0.19	0.72 ± 0.01	1.74 ± 0.02	29.96 ± 0.33	1.41
8	Beijing	2009.03	*G. uralensis*	6.78 ± 0.05	16.06 ± 0.14	1.68 ± 0.02	2.10 ± 0.02	35.02 ± 0.53	2.18
9	Shanghai	2009.04	*G. uralensis*	4.84 ± 0.05	25.44 ± 0.26	1.01 ± 0.01	2.78 ± 0.04	32.55 ± 0.43	1.28
10	Changshu, Jiangsu	2009.03	*G. uralensis*	5.01 ± 0.03	28.93 ± 0.24	0.97 ± 0.01	2.64 ± 0.03	32.45 ± 0.85	1.12
11	Guilin, Guangxi	2009.04	*G. uralensis*	9.63 ± 0.08	13.74 ± 0.01	1.61 ± 0.02	1.42 ± 0.02	32.26 ± 0.47	2.35
12	Beihai, Guangxi	2009.03	*G. uralensis*	4.70 ± 0.02	23.44 ± 0.07	1.00 ± 0.01	2.55 ± 0.02	33.60 ± 0.33	1.43

Table 6. Content correlation tests (two-tailed) between liquiritin apioside and liquiritin, licuraside and isoliquiritin in raw and honey-processed licorice samples

Liquiritin apioside		Liquiritin		Licuraside		Isoliquiritin	
		Pearson Correlation	Significant			Pearson Correlation	Significant
Raw	*G. glabra*	-0.219	0.636	Raw	*G. glabra*	-0.307	0.503
	G. uralensis	-0.433	0.284		*G. uralensis*	0.062	0.884
In-house	*G. glabra*	0.040	0.931	In-house	*G. glabra*	-0.088	0.851
Honey-processed	*G. uralensis*	0.854	0.007**	Honey-processed	*G. uralensis*	0.872	0.005**
Purchased	*G. glabra*	-0.217	0.861	Purchased	*G. glabra*	0.528	0.646
Honey-processed	*G. uralensis*	-0.587	0.097	Honey-processed	*G. uralensis*	-0.427	0.252

** significant at the 0.01 level.

honey process, which was probably caused by heating with honey.

In all licorice samples of *G. glabra*, the abundance of glycyrrhizin was much higher than liquiritin. The content ratio of glycyrrhizin to liquiritin was more than 5.0 (from 5.48 to 12.62) in licorice of *G. glabra* whereas it was less than 3.0 in that of *G. uralensis* in both raw and honey-processed licorice materials. Compared to it in raw samples, the ratio did not change significantly after the honey process. It could be considered as a stable marker to distinguish licorice of *G. glabra* from that of *G. uralensis*. It also implied that the descent ranges of glycyrrhizin and liquiritin after the honey process were consistent.

In Sung's study, it was concluded that baking licorice under controlled baking temperature and duration resulted in the conversion of glycyrrhizin to 18-β glycyrrhetinic acid, which provided some scientific evidence to understand the differences in therapeutic values of raw and baked licorice[20]. Comparing the chemical structures of the four flavonoid glycosides, liquiritin and isoliquiritin are the products of liquiritin apioside and licuraside, respectively, by hydrolyzing a terminal apiose. It was assumed that the honey process would effect hydrolyzing the sugar chain. Therefore, content correlation of the four flavonoid glycosides in both raw and honey-processed licorice samples were tested (two-tailed) by SPSS. The results are summarized in Table 6. Liquiritin apioside and liquiritin, licuraside and isoliquiritin content in raw materials of *G. uralensis* were highly correlated after the in-house honey process (the raw licorice was mixed with 25% refined honey and baked to dry at 150°C in 1 h) which indicated that the honey process did influence liquiritin apioside and licuraside sugar chain hydrolyzation in licorice of *G. uralensis* more significantly than that of *G. glabra*. However, no correlation was exhibited between liquiritin apioside and liquiritin, licuraside and isoliquiritin in purchased honey-processed samples. It is possible that the sugar chain hydrolyzation would not reach the significant level if the baking temperature was not high enough since no definite requirement to the baking temperature in the honey process is established in the Chinese Pharmacopoeia. The study on influences on content changes under different baking temperatures are now carrying out by our team.

The content disparities of the same component in different raw samples could be mainly caused by diversities of species and growth environments. Accumulation of components can be influenced by years of growing as well[15, 21]. And enzymes or entophytes existing in plants may play an important role on sugar chains hydrolyzation[22]. Because roots and rhizomes of most herbs must be cut to thin pieces prior to their usage or process, dried roots and rhizomes are intenerated with water before cutting[22]. Loss of water soluble ingredients like glycosides are inevitable during water intenerating.

CONCLUSIONS

An RP-HPLC/DAD analytical method has been developed for simultaneous determination of liquiritin apioside, liquiritin, licuraside, isoliquiritin and glycyrrhizin in licorice and applied to monitor forty two batches of raw and honey-processed licorice. The complete validation results showed that the developed method could be considered as a reliable and sensitive quality control for licorice materials. Due to its high abundance and bioactivity, liqiritin apioside content is suggested as a reasonable measure of quality. The content ratio of glycyrrhizin to liquiritin can be considered as a stable marker to distinguish licorice of *G. glabra* (more than 5.0) from that of *G. uralensi* (less than 3.0). All contents of the five components would descend *in toto* after the honey process. The contents of liquiritin apioside and liquiritin, licuraside and isoliquiritin in licorice of *G. uralensis* are significantly correlative after the in-house honey process, evidently due to the hydrolyzation of liquiritin apioside and licuraside to liquiritin and isoliquiritin during the honey-mix-and-bake process. This change could be a significant indication of the therapeutic difference between *G. glabra* and *G. uralensis* in clinical application.

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