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Identification and Chemical Standardization of Licorice Raw Materials and Dietary Supplements Using UHPLC-MS/MS

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Abstract

Defined as the roots and underground stems of principally three *Glycyrrhiza* species, *G. glabra* L., *G. uralensis* Fish. ex DC. and *G. inflata* Batalin, licorice has been used as a medicinal herb for millennia and is marketed as root sticks, powders and extracts. Identity tests described in most pharmacopeial monographs enabled the distinction of *Glycyrrhiza* species. Accordingly, an ultrahigh-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) assay using the method of standard addition was developed to quantify 14 licorice components (liquiritin, isoliquiritin, liquiritin apioside, isoliquiritin apioside, licuraside, liquiritigenin, isoliquiritigenin, glycyrrhizin, glycyrrhetic acid, glabridin, glycy coumarin, licoricidin, licochalcone A, and *p*-hydroxybenzylmalonic acid), representing several natural product classes including chalcones, flavanones, saponins, and isoflavonoids. Using this approach, *G. glabra*, *G. uralensis* and *G. inflata* in a variety of forms including root powders and extracts as well as complex dietary supplements could be differentiated and chemically standardized without concerns due to matrix effects.

Graphical Abstract

Keywords

Botanical dietary supplements; Licorice; Method of standard addition; Tandem mass spectrometry; UHPLC

Introduction

As the global market for botanical dietary supplements grows, ensuring the quality and safety becomes increasingly important.^{1,2} Licorice, used as a medicinal plant since ancient times, consists of three commonly used species, *Glycyrrhiza glabra* L., *Glycyrrhiza uralensis* Fish. ex DC. and *Glycyrrhiza inflata* Batalin. Although there are secondary metabolites in common between licorice species, there are also unique compounds found in the chemical profiles of each.^{3–7} According to most pharmacopeial monographs, the chemical standardization of licorice products is usually carried out by measuring a single compound, glycyrrhizin, a sweet tasting saponin with chemoprevention properties and hypertensive side effects, which is present in all three species.^{8–10}

The U.S. Food and Drug Administration requires the use of current Good Manufacturing Practices in the production of dietary supplements marketed in the United States.¹¹ Although botanical authentication is required, the exact species of licorice, described by its binomial Latin name, is not always accurately disclosed on the label of commercial products. Because each species of licorice has a unique profile of secondary metabolites,^{3–7} its biological activities will also be distinct. For example, the estrogenicity,¹² chemoprevention activity,¹³ and potential for drug-botanical interaction¹⁴ of each species of licorice are different. To enable regulatory compliance, to ensure consumer safety, and to facilitate quality control of licorice dietary supplements, licorice materials used in these products should be identified, authenticated and chemically standardized.²

Plant materials used in botanical dietary supplements are usually identified through macroscopic and microscopic examinations performed on the raw material, followed by chemical methods of identification typically performed on crude extracts. Such chemical methods include thin layer chromatography or high performance liquid chromatography (HPLC) combined with UV or mass spectrometric detection. These identification techniques can be complemented by DNA barcoding methods which are preferably performed on raw plant materials, because extracts and highly processed plant materials usually have low DNA quality unsuitable for accurate DNA identification.^{15,16} Quality control of dietary supplements also requires quantitative measurement of chemical constituents in the starting material and in the finished product. In the case of licorice, a variety of analytical methods such as capillary-zone electrophoresis,¹⁷ HPLC-UV,^{18–22} nuclear magnetic resonance (NMR),⁷ HPLC-mass spectrometry,¹⁰ and HPLC-tandem mass spectrometry (MS/MS)^{23–24} have been used to measure chemical constituents in crude extracts, but rarely in the complex matrices of commercial botanical dietary supplements.

Although more specific than HPLC-UV and more sensitive than NMR, HPLC-MS/MS methods for characterizing and standardizing licorice dietary supplements in the literature have been limited to small numbers of compounds that did not enable the user to distinguish between the three most common species of licorice. For example, the HPLC-MS/MS assay developed by Montoro *et al.*²⁵ was validated for the measurement of only glycyrrhizin. Kong *et al.*²⁶ used HPLC-MS/MS to measure five licorice compounds, and Xie *et al.*²⁴ measured six compounds. Tao *et al.*²³ measured 10 triterpenoid saponins but no flavonoids such as liquiritin, isoliquiritin or their apiosides. Furthermore, these approaches used standard curves

prepared in solvent alone, which did not correct for matrix effects, which Kong *et al.*²⁶ reported included both ion suppression (liquiritin and liquiritigenin) and ion enhancement (liquiritin apioside).

Note that the increased selectivity of HPLC-MS/MS methods enables faster separations than HPLC-UV methods, because not all constituents need to be resolved to baseline. For example, although Wei *et al.*²² measured 14 licorice compounds using HPLC-UV, each separation required 80 minutes. In contrast, we report the development of a 15-min ultrahigh-pressure liquid chromatography (UHPLC)-MS/MS assay using the method of standard addition for the quantitative analysis of 14 licorice compounds. These compounds represent a variety of natural product classes, many with chemoprevention activities such as the chalcones isoliquiritin, isoliquiritin apioside, licuraside, isoliquiritigenin, and licochalcone A, isoflavanoids including licoricidin and glabridin, the flavanones liquiritin, liquiritin apioside and the estrogenic liquiritigenin, the prenyl flavanoid glycycomarin, the triterpene glycyrrhetic acid, and the saponin glycyrrhizin. Our approach simultaneously achieves both botanical identification and chemical standardization of the three pharmacopeial licorice species used in dietary supplements. Meanwhile, the standard addition method eliminates matrix enhancement/suppression issues and provides accurate quantitative result for multiple classes of compounds in a variety of licorice matrices, including commercial dietary supplements.

MATERIALS AND METHODS

Materials and chemicals

HPLC-MS-grade acetonitrile and methanol were purchased from Thermo Fisher (Fair Lawn, NJ). Water was prepared using an Elga Purelab Ultra (Siemens Water Technologies, Woodridge, IL) water purification system. Glycyrrhizin (95.0% w/w), 18 β -glycyrrhetic acid (97.0% w/w) glabridin (87.1% w/w), and licochalcone A (96.1% w/w) were purchased from Sigma-Aldrich (St. Louis, MO). Glycycomarin (92.3% w/w) was obtained from BioBioPha (Kunming Institute of Botany, China). Liquiritigenin (95.5% w/w), liquiritin (95.9% w/w), liquiritin apioside (88.00% w/w), isoliquiritigenin (95.5% w/w), isoliquiritin (89.7% w/w) isoliquiritin apioside/licuraside (74.8/23.8% w/w), and *p*-hydroxybenzylmalonic acid (HBMA, 90.0% w/w) were isolated as previously described.^{20,28,29} Licoricidin (95.2% w/w) was a gift from Dr. Stefan Gafner. The purity of licorice standards was determined by using quantitative NMR as described previously.²⁷

Five commercial licorice dietary supplements and 12 bulk root powders were purchased from a variety of Internet vendors or from Chicago metropolitan area stores. The licorice dietary supplements included dried preparations (such as powdered plant tissue encased in gelatin capsules) and crude extracts. All 12 plant materials were botanically identified through macroscopic/microscopic analyses and comparison with voucher specimens at the Field Museum of Natural History (Chicago, IL), as well as DNA barcoding, as described previously.⁷

Sample preparation

Each licorice sample (2 g) was extracted with 40 mL of ethanol (95%, USP 190 proof) using accelerated solvent extraction (Dionex; Sunnyvale, CA) at 80 °C with 30 min static time. After extraction, the remaining material was washed using 10 mL of the same extraction solvent. The combined extraction solvent was evaporated to dryness under vacuum overnight at 35 °C. Each licorice extract was weighed and dissolved in methanol at 50 µg/mL. A series of 10 standard solutions containing 14 licorice compounds (Figure 1) was prepared by serial dilution in methanol/water (1:1, v/v). Standard solutions or methanol/water (1:1, v/v) were spiked into each extract at a 1:1 (v/v) ratio. Detailed information regarding the standard addition design is shown in Table 1.

UHPLC-MS/MS

All 14 compounds were analyzed in a single run using UHPLC-MS/MS on a Shimadzu (Kyoto, Japan) Nexera UHPLC system and LCMS-8060 triple quadrupole mass spectrometer. The analytes were separated on a Waters (Milford, MA) Acquity UPLC BEH C₁₈ column (2.1 x 100 mm, 1.7 µm) using a 15-min gradient from 12% to 72% acetonitrile in water containing 0.1% formic acid. The flow rate was 0.6 mL/min, and the column oven temperature was 45 °C. Negative and positive ion electrospray were used with polarity switching (15 ms) and selected reaction monitoring (SRM) as indicated in Table 1.

Method validation

The method was validated in terms of selectivity, sensitivity, linearity, accuracy, precision, and stability. Selectivity was evaluated by comparing the retention time and the ratio of SRM MS/MS responses of each analyte (quantifier/qualifier) in licorice samples, standard solutions and spiked licorice samples. Sensitivity and linearity were determined by constructing a solvent calibration curve. Accuracy and inter-day and intra-day precision were evaluated at low, medium and high analyte concentrations (Table 2). The 24-h stabilities of all 14 analytes were measured in three licorice extracts at the UHPLC-MS/MS autosampler temperature of 4 °C.

Statistical analysis

Linear standard curves were fitted using Shimadzu LabSolution software (Kyoto, Japan). Quadratic curve fitting and all other calculations were carried out using Microsoft Excel software (Seattle, WA). Pairwise comparisons were made with R software (version 2.15, R Foundation for Statistical Computing).

RESULTS AND DISCUSSION

UHPLC-MS/MS

Two or three SRM transitions were used for each licorice analyte. The most abundant product ion for each compound was used as the quantifier SRM transition, and the less abundant ions served as qualifiers. All the quantifier SRM transitions, corresponding collision energies (CE) are listed in Table 1. Because licochalcone A (**11**) showed the best MS/MS signal response (lowest limit of detection; Table 2) while at the same time occurring

in some samples at the highest levels of any analyte (Table 3), a sub-optimal CE was used to bring the upper limit of quantitation of licochalcone A (**11**) within the range required for this application. Therefore, all 14 licorice compounds could be measured simultaneously without dilution and reanalysis of those containing high levels of licochalcone A (**11**).

Several of the licorice compounds are isomeric (Figure 1) and share the same SRM transitions (Table 1). These compounds included isomeric liquiritin (**1**) and isoliquiritin (**2**); liquiritin apioside (**3**) isoliquiritin apioside (**4**) and licuraside (**5**); and liquiritigenin (**6**) and isoliquiritigenin (**7**). Therefore, chromatographic separation of these isomers was necessary for accurate quantitative analysis. Using UHPLC, mobile phase parameters were optimized for separation of these isomers as well as the other analytes. Combinations of methanol, acetonitrile, and water with 0.1% formic acid or 5 mM ammonium formate were evaluated. Acetonitrile and 0.1% formic acid in water adequately separated all 14 compounds within 15 min and with good MS/MS sensitivity. UHPLC-MS/MS chromatograms of the 14 analytes in a standard solution of medium concentration and extracts of three botanically authenticated licorice species, *G. glabra*, *G. uralensis* and *G. inflata* are shown in Figure 2.

Although complete chromatographic separation of all analytes is required when using non-selective detectors such as UV absorbance,²² only isomers and other interfering substances need to be resolved when using a highly selective detector such as a tandem mass spectrometer. For example, the mass selectivity of MS/MS detection could measure overlapping compounds of different masses such as liquiritin and liquiritin apioside. As a result, 14 licorice compounds could be measured in 15 min using UHPLC-MS/MS instead of in 80 min using HPLC-UV as reported by Wei, *et al.*²²

Selectivity of the method was confirmed by comparing the retention times of each analyte in standard solution with those of licorice extracts and extracts spiked with standards and by making certain that the ratios of quantifier/qualifier MS/MS SRM transitions were constant for all standards and samples. Extracts of three licorice species, *G. uralensis* (BC629), *G. glabra* (BC694) and *G. inflata* (BC711), were evaluated for stability. All 14 analytes in these extracts were stable for 24 h in the autosampler (4 °C), and the coefficients of variation (CV) for these measurements were within 10.5%.

The limit of detection (defined as a signal-to-noise of 3:1), linear range, accuracy, and precision were determined for each analyte and are summarized in Table 2. Most compounds had a wide linear range that encompassed the concentrations of analytes present in all samples. For example, the standard curve for glycycomarin (**10**) was linear from 0.12–3880 ng/mL (R^2 0.9999). An exception was licochalcone A (**11**) which produced a linear calibration curve only from 0.11–215 ng/mL (R^2 = 0.9987) but was fit to a quadratic equation for the concentration range of 86–4300 ng/mL (R^2 = 0.9994). The lower limit of quantitation listed in Table 2 was the lower boundary of the linear range.

Standard addition method

The method of standard addition was used to compensate for matrix effects such as ion suppression or enhancement that can interfere with quantitative measurements using electrospray mass spectrometry. The typical standard addition calculation was based on the

linear relationship of the MS/MS response and analyte concentration as shown in Figure 3. In the case of licochalcone A (**11**), the standard addition data were fit to a quadratic equation and then analyzed using inverse nonlinear regression (Figure 4) as described by Meija *et al.*³⁰ Accurate quantitative analysis of all 14 compounds was obtained during a single analysis while avoiding the need for dilution and time-consuming reanalysis.

The method of standard addition was used to overcome matrix effects which can decrease the accuracy of mass spectrometry-based assays, and in particular, the minor matrix effects (approximately 2–3%) reported by Kong *et al.*²⁶ for liquiritin, liquiritin apioside, and liquiritigenin. An alternate approach to minimize these matrix effects and improve accuracy is the use of labeled internal standards (also known as surrogate standards), which co-elute with the analytes during UHPLC-MS/MS and correct for minor ion suppression or enhancement. However, surrogate standards of the 14 licorice compounds used in this investigation were not available. Other approaches to overcome matrix effects include optimizing sample preparation to remove interfering substances prior to UHPLC-MS/MS or modification of the chromatographic method to resolve all interfering substances from the analytes.

Phytochemical identification of licorice samples

Another objective of this study was to develop an assay that could be used to identify licorice plant material used in dietary supplements and to distinguish the three *Glycyrrhiza* species based on their chemical profiles. To validate the method, the chemical profiles of all 14 analytes were determined in extracts of 12 DNA-authenticated licorice root powders.⁷ From the quantitative results summarized in Table 3, distinct differences were observed in the chemical compositions among the three licorice species.

Glabridin (**12**) was detected in all the *G. glabra* samples but not in any of the *G. uralensis* or *G. inflata* root preparations. Only *G. uralensis* root preparations contained licoricidin (**13**) and substantial levels of glycycomarin (**10**). Licochalcone A (**11**) was abundant only in *G. inflata*. These results are consistent with those reported previously by Kondo *et al.* in 2007.¹⁸ In addition to these species-specific compounds, *G. uralensis* root preparations contained more liquiritin (**1**) and isoliquiritin (**2**) than did *G. glabra* (p-value < 0.001), and *G. glabra* contained more apiosides (**3–5**) than did *G. uralensis* (p-value < 0.05). Since only one authenticated *G. inflata* root powder was obtained and tested, which was a limitation of this study, no additional comparisons could be made with statistical significance involving this species of licorice.

To visualize species distinguishing information, percentages of liquiritin+isoliquiritin (**1,2**), apiosides (**3–5**), and liquiritigenin+isoliquiritigenin (**6,7**) were computed with respect to their total amounts (Table 4). *G. glabra* root preparations contained $94.1 \pm 2.8\%$ of apiosides, while *G. uralensis* had $40.9 \pm 13.6\%$. The level of apiosides in the *G. inflata* root preparation fell in between the other two species at 82.8%. Meanwhile, the amount of liquiritin +isoliquirtin was abundant only in *G. uralensis* ($55.0 \pm 11.7\%$), while *G. inflata* contained 6.4% and *G. glabra* only $4.5 \pm 2.1\%$. These results are in agreement with previously reported findings.⁷ No significant trend was observed for levels of liquiritigenin+isoliquiritigenin in the licorice species.

Five commercial licorice dietary supplements were analyzed using the UHPLC-MS/MS standard addition method (Table 3). The label of sample BC625 indicated that the product contained *G. glabra* root powder, and the presence of glabridin (**12**) supported this claim. However, sample BC625 also contained ~33 mg/g licochalcone A (Table 3) indicative of *G. inflata* as well as a trace amount of licoricidin (**13**) and 26% of liquiritin+isoliquiritin (**1,2**) indicating that *G. uralensis* was also present. Dietary supplement BC741 was labeled as containing licorice without any species designation, and based on the data in Tables 3 and 4, BC741 also contained all three species. In particular, licochalcone A (**11**) and glycyrrhetic acid (**14**) indicated *G. inflata*, 20% liquiritin+isoliquiritin (**1,2**) showed that *G. uralensis* was present; and 79% apiosides **3–5** along with a trace amount of glabridin indicated the presence of *G. glabra*. Capsules of BC736, although labeled as *G. glabra* root powder, contained <0.2 mg/g glabridin (Table 3). The additional presence of 57% apiosides **3–5**, 21% liquiritin+isoliquiritin (**1, 2**) (Table 4) and a significant amount of licochalcone A (70.66 mg/g; Table 3) indicated that BC736 was actually a mixture of all three licorice species.

BC737 (capsules) and BC740 (tea) were blends of multiple plant materials both claiming to contain licorice root. The low abundance of the licorice compounds, made both NMR and HPLC-UV techniques inapplicable.⁷ Using the new UHPLC-MS/MS method, the characteristic licorice compounds **1–9** confirmed that these two products contained licorice. The apioside (**3–5**) content (94.4/94.2%; Table 4) suggested that BC737 and BC740 probably contained *G. glabra*, which was confirmed by the measurement of glabridin (**12**) in BC737.

Sensitive and accurate quantitative UHPLC-MS/MS analysis of botanical dietary supplements is useful for both chemical standardization and simultaneous phytochemical identification. In this application, UHPLC-MS/MS analysis was used to detect the presence of licorice and to identify the specific *Glycyrrhiza* species utilized in complex botanical dietary supplements mixtures. At the same time, this method provided quantitative analysis of 14 licorice secondary metabolites belonging to multiple classes of compounds including chalcones, isoflavonoids, triterpenes, flavanones, and saponins, that may be used for the further chemical standardization of these products. Few other licorice methods measure such a diverse set of compounds, and those that do, such as Wei, *et al.*,²² require much longer separation times (80 min compared with 15 min) and have much narrower linear ranges (1 – 2 orders of magnitude for the UV absorbance method of Wei, *et al.*, compared with 3 – 4 orders of magnitude for UHPLC-MS/MS). The method of standard addition, not always used for quantitative analysis using tandem mass spectrometry, also prevented matrix effects, which are known to interfere with the HPLC-MS/MS analysis of several licorice constituents.²²

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ABBREVIATIONS USED

HPLC	high-performance liquid chromatography
NMR	nuclear magnetic resonance
LOD	limit of detection
LLOQ	lower limit of quantitation
SRM	selected reaction monitoring
UHPLC-MS/MS	ultrahigh-pressure liquid chromatography tandem mass spectrometry

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$R_1=H$, $R_2=\beta$ -D-glc: **1** Liquiritin, **2** Isoliquiritin

$R_1=H$, $R_2=\beta$ -api-(1 $\rightarrow$ 2)- β -D-glc: **3** Liquiritin apioside, **4** Isoliquiritin apioside

$R_1=\beta$ -api-(1 $\rightarrow$ 2)- β -D-glc, $R_2=H$: **5** Licuraside

$R_1=H$, $R_2=H$: **6** Liquiritigenin, **7** Isoliquiritigenin

Figure 1. Chemical structures of 14 licorice components measured using UHPLC-MS/MS

Api: apinose, gluA: glucuronic acid, glc: glucose.

Figure 2. UHPLC-MS/MS chromatograms of *G. glabra* extract (BC694), *G. uralensis* extract (BC629), *G. inflata* extract (BC711) and standard licorice compound mixture (medium quality control).

Figure 3. Calibration curve for isoliquiritin in *G. uralensis* extract (BC629)

The calibration curve was extrapolated to the left, and the concentration of isoliquiritin was the negative intercept with the x-axis. [isoliquiritin] = 236.41 ng/mL

Figure 4. Linear and quadratic calibration curves for licochalcone A in *G. inflata* extract (BC711)

(A) Linear regression analysis (blue line) suggests that the licochalcone A concentration is 1740.5 ng/mL, whereas non-linear fitting (orange line) results in the more accurate concentration of 1409.0 ng/mL. (B) Using an inverse calibration curve with quadratic fitting as described by Meija *et al.*³⁰ yields a licochalcone A concentration of 1290.0 ng/mL.

Table 1

UHPLC Retention Times (RT), MS/MS Selected Reaction Monitoring (SRM) Transitions, Collision Energies (CE), and Concentrations (ng/mL) of the 14 Licorice Standards Spiked into Each Sample for Standard Addition. The First SRM Transition for Each Compound was Used as the Quantifier and the Other SRM Transitions were Used as Qualifiers During UHPLC-MS/MS.

#	Name	RT (min)	SRM transitions <i>m/z</i> (polarity)	CE (V)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
1	Liquiritin	3.24	417>255 (-) 417>135 (-)	17 28	1.0	4.8	9.6	24.0	48.0	95.9	239.8	479.5	959.0	1918.0
2	Isoliquiritin	4.90	417>255 (-) 417>135 (-)	17 28	0.9	4.5	9.0	22.5	44.9	89.7	224.3	448.6	897.1	1794.2
3	Liquiritin apioside	3.25	549>255 (-) 549>135 (-)	29 41	0.9	4.4	8.8	22.0	44.0	88.0	220.0	440.0	880.0	1760.0
4	Isoliquiritin apioside	4.73	549>255 (-) 549>135 (-)	29 41	0.2	1.2	2.4	6.0	11.9	23.8	59.4	118.8	237.5	475.0
5	Licuraside	4.92	549>255 (-) 549>135 (-)	29 41	0.7	3.7	7.5	18.7	37.4	74.8	186.9	373.8	747.6	1495.2
6	Liquiritigenin	5.17	255>119 (-) 255>135 (-)	22 14	1.0	4.8	9.6	23.9	47.8	95.5	238.8	477.5	955.0	1910.0
7	Isoliquiritigenin	7.44	255>119 (-) 255>135 (-)	22 14	1.0	4.8	9.6	23.9	47.8	95.5	238.8	477.5	955.0	1910.0
8	HBMA	1.22	209>165 (-) 209>121 (-)	814	0.9	4.5	9.0	22.5	45.0	90.0	225.0	450.0	900.0	1800.0
9	Glycyrrhizin	8.74	821>351 (-) 821>113 (-)	41 54	1.0	4.8	9.5	23.8	47.5	95.0	237.5	475.0	950.0	1900.0
10	Glycycomarin	9.95	367>308 (-) 367>297 (-)	26 24	1.0	4.9	9.7	24.3	48.5	97.0	242.5	485.0	970.0	1940.0
11	Licochalcone A	10.79	367>93 (-) 339>121 (+) 339>297 (+) 339>93 (+)	39 15 15 15	0.9	4.3	8.6	21.5	43.0	86.0	215.0	430.0	860.0	1720.0
12	Glabridin	11.59	323>201 (-)	24	1.0	4.9	9.8	24.5	49.0	98.0	245.0	490.0	980.0	1960.0

#	Name	RT (min)	SRM transitions <i>m/z</i> (polarity)	CE (V)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
13	Licoricidin	13.20	323>135 (-)	18										
			423>203 (-)	27	1.0	4.8	9.5	23.8	47.6	95.2	238.1	476.2	952.4	1904.8
			423>233 (-)	21										
			423>391 (-)	21										
14	Glycyrrhethinic acid	14.30	469>425 (-)	37	1.0	4.9	9.7	24.3	48.5	97.0	242.5	485.0	970.0	1940.0
			469>355 (-)	44										

Table 2

Limit of Detection (LOD), Linear Range, Coefficients of Determination (R^2), Accuracy and Precision for the UHPLC-MS/MS Analysis of 14 Liocorice Compounds. Accuracy and Precision were Evaluated Using Low, Medium and High Quality Controls (QC), Expressed as Coefficient of Variation (CV).

#	Analyte	LOD (ng/mL)	Linear range (ng/mL)	R^2	QC (ng/mL)	Accuracy CV% (n=3)	Precision CV%	
							intraday (n=4)	Interday (n=6)
1	Liquiritin	0.12	0.48–3836	0.9998	239.8	1.7	4.5	5.1
							3.2	6.2
							5.1	3.8
2	Isoliquiritin	0.09	0.22–4485.5	0.9996	224.3	1.7	8.2	7.7
							5.9	3.6
							3.4	3.8
3	Liquiritin apioside	0.44	0.88–4400	0.9992	220.0	3.8	8.7	7.4
							2.1	7.6
							6.5	6.9
4	Isoliquiritin apioside	0.24	0.48–2375	0.9991	118.8	8.0	7.8	6.4
							3.7	6.4
							4.6	5.0
5	Licuraside	0.19	0.37–5980	0.9996	373.8	6.4	5.8	6.3
							2.3	4.6
							4.6	5.7
6	Liquiritigenin	0.10	0.24–3820	0.9995	238.8	1.9	6.4	5.8
							3.6	6.2
							5.9	3.8
7	Isoliquiritigenin	0.10	0.24–3820	0.9998	238.8	8.2	4.2	5.8
							5.0	6.2
							5.3	3.8
8	HBMA	2.2	9.0–4500	0.9995	22.5	3.9	3.9	6.8

#	Analyte	LOD (ng/mL)	Linear range (ng/mL)	R ²	QC (ng/mL)	Accuracy CV% (n=3)	Precision CV%	
							intraday (n=4)	Interday (n=6)
						4.7	6.4	2.4
						4.2	4.8	2.0
9	Glycyrrhizin	0.95	5.0–3800	0.9988	9.5	5.4	9.4	7.0
						6.3	2.9	7.5
						3.1	5.9	6.1
10	Glycycomarin	0.05	0.12–3880	0.9999	2.4	5.0	0.8	2.6
						3.9	2.5	6.2
						3.9	5.9	3.8
11	Licochalcone A	0.04	0.11–215 (linear) 86–4300 (quadratic)	0.9987 0.9994	2.2 215.0 860.0	7.2	6.0	4.0
						6.0	7.3	3.6
						6.1	5.2	7.7
12	Glabridin	0.49	0.98–3920	0.9995	2.5 245.0 980.0	5.9	5.8	5.6
						2.7	1.9	5.2
						2.3	5.6	2.4
13	Licoricidin	2.4	4.8–4762	0.9989	4.8 238.1 952.4	2.8	6.6	8.5
						7.5	3.8	8.6
						4.6	6.8	8.7
14	Glycyrrhetic acid	0.49	0.97–3880	0.9998	2.4 242.5 970.0	9.8	3.5	6.0
						4.8	5.7	7.0
						5.8	6.2	7.0

Table 3

Quantitative Analysis of Licorice Compounds in Botanical Samples. Results are Expressed as mg/g Extract.

Botanical sample	Liquiritin	Isoliquiritin	Liquiritin apioside	Isoliquiritin apioside	Licurside	Liquiritigenin	Isoliquiritigenin	HBMA	Glycyrrhizin	Glycycomarin	Licochalcone A	Glabridin	Licoricidin	Glycyrrhetic acid
Code	1	2	3	4	5	6	7	8	9	10	11	12	13	14
<i>G. glabra</i>	BC289	1.92	0.64	24.81	6.77	8.95	0.62	16.14	31.40	<LOD ^a	0.06	10.62	<LOD	<LLOQ ^b
	BC694	1.27	0.23	38.98	10.19	6.50	0.38	13.20	19.69	<LOD	<LOD	7.18	<LOD	0.10
	BC695	2.39	0.44	64.30	17.44	4.28	0.38	17.28	24.14	<LOD	0.03	3.60	<LOD	<LLOQ
	BC726	1.76	0.37	55.39	12.93	13.87	0.12	16.60	18.34	<LOD	<LOD	2.27	<LOD	<LLOQ
	BC731	2.94	0.48	38.09	10.30	6.53	0.37	12.67	16.28	<LOD	<LOD	6.44	<LOD	<LLOQ
	BC732	8.14	1.39	82.06	22.13	12.21	1.05	22.97	17.98	<LOD	<LOD	5.68	<LOD	<LLOQ
<i>G. uralensis</i>	BC624	52.77	8.54	21.94	4.25	0.85	1.49	20.09	28.07	1.20	0.06	<LOD	2.13	<LLOQ
	BC629	51.82	9.46	49.35	13.82	0.64	0.84	7.76	9.35	1.25	<LOD	<LOD	2.13	<LLOQ
	BC689	32.50	2.78	10.30	1.21	0.53	0.49	12.93	45.07	0.87	0.03	<LOD	0.21	0.04
	BC716	24.89	4.48	22.46	4.86	2.46	0.49	18.78	24.67	1.54	<LOD	<LOD	<LLOQ	0.11
	BC725	23.22	5.08	27.52	5.96	0.59	0.62	16.39	21.14	1.49	0.03	<LOD	1.66	0.06
<i>G. inflata</i>	BC711	13.25	1.84	77.24	20.07	1.68	1.51	50.98	14.49	0.05	82.33	<LOD	<LOD	1.86
Commercial licorice dietary supplements	BC625	21.50	3.39	57.82	14.57	3.65	1.15	16.99	21.75	<LOD	32.68	1.01	<LLOQ	0.86
	BC736	1.92	0.54	4.89	1.29	0.35	0.89	1.04	4.37	0.23	70.66	0.18	<LLOQ	2.68
	BC737	0.39	0.06	8.34	2.26	1.27	0.08	2.25	4.10	<LOD	0.04	0.54	<LOD	<LOD
	BC740	0.11	0.01	1.54	0.41	0.30	<LOD	0.28	0.43	<LOD	<LOD	<LOD	<LOD	<LOD
	BC741	23.32	3.01	78.18	19.92	3.99	0.30	19.29	20.92	<LOD	9.48	<LLOQ	<LLOQ	0.06

^a LOD=limit of detection

^b LLOQ=lower limit of quantitation

Table 4

Comparison of Liquiritin+Isoliquiritin (**1,2**), Liquiritin Apioside+Isoliquiritin Apioside+Licuraside (**3,4,5**) and Liquiritigenin+Isoliquiritigenin (**6,7**). Results are Expressed as Percentage of the Total Amount of Compounds **1–7**.

	Sample Code#	1,2 (%)	3,4,5 (%)	6,7 (%)
<i>G. glabra</i>	BC289	5.7	90.5	3.8
	BC694	2.6	96.0	1.5
	BC695	3.2	96.2	0.7
	BC726	2.5	97.3	0.2
	BC731	5.8	93.1	1.1
	BC732	7.5	91.3	1.2
<i>G. uralensis</i>	BC624	63.4	27.9	8.7
	BC629	48.6	50.6	0.9
	BC689	71.0	24.2	4.8
	BC716	49.0	49.7	1.3
	BC725	43.1	51.9	5.0
<i>G. inflata</i>	BC711	6.4	82.8	10.8
Commercial licorice dietary supplements	BC625	23.5	71.9	4.5
	BC736	21.4	56.7	21.8
	BC737	3.6	94.4	2.1
	BC740	5.1	94.2	0.7
	BC741	20.4	79.1	0.5