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Simultaneous quantification of flavonoids and triterpenoids in licorice using HPLC

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Abstract

Numerous bioactive compounds are present in licorice (Glycyrrhizae Radix), including flavonoids and triterpenoids. In this study, a reversed-phase high-performance liquid chromatography (HPLC) method for simultaneous quantification of three flavonoids (liquiritin, liquiritigenin and isoliquiritigenin) and four triterpenoids (glycyrrhizin, 18α-glycyrrhetinic acid, 18β-glycyrrhetinic acid and 18β-glycyrrhetinic acid methyl ester) from licorice was developed, and further, to quantify these 7 compounds from 20 different licorice samples. Specifically, the reverse-phase HPLC was performed with a gradient mobile phase composed of 25 mM phosphate buffer (pH 2.5)–acetonitrile featuring gradient elution steps as follows: 0 min, 100:0; 10 min, 80:20; 50 min, 70:30; 73 min, 50:50; 110 min, 50:50; 125 min, 20:80; 140 min, 20:80, and peaks were detected at 254 nm. By using our technique, a rather good specificity was obtained regarding to the separation of these seven compounds. The regression coefficient for the linear equations for the seven compounds lay between 0.9978 and 0.9992. The limits of detection and quantification lay in the range of 0.044–0.084 and 0.13–0.25 μg/ml, respectively. The relative recovery rates for the seven compounds lay between 96.63 ± 2.43 and $103.55 \pm 2.77\%$. Coefficient variation for intra-day and inter-day precisions lay in the range of 0.20–1.84 and 0.28–1.86%, respectively. Based upon our validation results, this analytical technique is a convenient method to simultaneous quantify numerous bioactive compounds derived from licorice, featuring good quantification parameters, accuracy and precision.
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Keywords: Licorice; Flavonoids; Triterpenoids; Simultaneous quantification

1. Introduction

Licorice (Glycyrrhizae Radix) is the dried root of the plant *Glycyrrhiza uralensis* Fisch. The plant is distributed from the north-east to the north-west of mainland China, and throughout central Asia and southern Europe [1]. Licorice has been a most-important ingredient of Chinese traditional medicine used from ancient times, which was found in Chinese traditional prescriptions at the rate of 60%. The traditional pharmacological effects of licorice such as an epinephrine-like action, the inhibition of pectic acid secretion and histamine-induced ulceration, detoxification and anti-inflammatory action, have been well verified [1,2].

Numerous bioactive compounds are present in licorice. Glycyrrhizin exhibited anti-inflammatory activity and to suppress the acute hepatic injury elicited by CCl_4 [3,4]. 18β-Glycyrrhetinic acid inhibited renal 11β-hydroxy-steroid dehydrogenase type-2 activity which causes renal sodium retention and potassium loss [5]. Glycyrrhetinic acid was a high potency anti-*Helicobacter pylori* compound, inhibiting 79.3% of strains at MIC ≤50 mg/l [6]. Both glabrene and isoliquiritigenin derived from licorice roots were revealed having tyrosinase inhibitory effect which is a key enzyme in melanin biosynthesis [7]. The consumption, of glabridin, an isoflavan isolated from *Glycyrrhiza globra* roots, has been demonstrated to result in a 53% reduction in copper ion-induced oxidation [8]. Both liquiritigenin and isoliquiritigenin, two triterpenoids found in licorice, have been shown xanthin-oxidase activity [9]. Two flavonoids, glycyrrhisoflavanone and glycyrrhisoflavone, isolated from Xi-bei licorice and licochalcones A and B, isolated from Xinjiang licorice, have been reported to exhibit potent radical-scavenging activity [10,11].

Triterpenoids such as glycyrrhizin, glycyrrhetinic acid (a glycoside of glycyrrhizin), uralsaponin, licorice saponines and

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glycyrrhetinic acid methyl ester and flavonoids such as liquiritin, liquiritigenin, isoliquiritin, isoliquiritigenin, neoliquritin and isolicoflavonol are the major compounds found in licorice [1,12–16]. From a review of the literature, numerous studies have been attempted to quantify these compounds in licorice. Most of the studies were focused on single compound examination, of which glycyrrhizin appearing the most-frequently examined compound [17–21]. Simultaneous two-flavonoids quantification such as liquiritin and isoliquiritigenin have also been discussed [22]. One triterpenoid (glycyrrhizin) and two flavonoids (18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid) were analyzed for, simultaneously, by Tsai and Chen [23]. Six-flavonoids quantification was reported by Yoneda et al. for which apioliquiritin, liquiritin, apioisoliquiritin, isoliquiritin, liquiritigenin and ononin to be analyzed in a single method [24], however, numerous flavonoids and triterpenoids quantification using a single method would appear, to not have been reported previously. Simultaneously quantification flavonoids and triterpenoids from licorice in a single operation is much more convenient than using several separate procedures, especially for routine analysis or for a large number of samples. One of the greatest advantage is the simplification the overall analytical process, for example, by reducing the frequency of changing the separation system and the number of mobile phase preparations. Moreover, most of the literature-revealed analytical methods did not appear to have validated the involved analytical methodology.

Based on the above-mentioned purpose, the aim of the current study, a novel reversed-phase high-performance liquid chromatography (HPLC) method for simultaneous quantification of three flavonoids (liquiritin, liquiritigenin and isoliquiritigenin) and four triterpenoids (glycyrrhizin, 18α-glycyrrhetinic acid, 18β-glycyrrhetinic acid and 18β-glycyrrhetinic acid methyl ester) from licorice was developed. Chemical structures of these seven compounds are revealed in Fig. 1. In order to validate an HPLC quantitative method, the specificity, linearity, accuracy, precision and limits of detection and quantification of the methodology were investigated. The specific content of these 3 flavonoids and 4 triterpenoids in 20 licorice samples using our technique was also examined.

2. Experimental

2.1. Chemicals and reagents

Liquiritin standard was purchased from Wako (Osaka, Japan), glycyrrhizin from Nacalai Tesque (Kyoto, Japan), isoliquiritigenin and 18α-glycyrrhetinic acid from Sigma–Aldrich (Seelze, Germany, USA); liquiritigenin, 18β-glycyrrhetinic acid and 18β-glycyrrhetinic acid methyl ester from Extrasyntèse (Genay, France). The purity of these seven compounds were more than 98%. Acetonitrile was HPLC grade and was purchased from Tedia (Fairfield, OH, USA), sodium dihydrogen phosphate and phosphoric acid were acquired from Merck (Darmstadt, Germany). All other chemicals were reagent grade.

2.2. Apparatus and chromatographic conditions

A Hitachi HPLC system (Tokyo, Japan) consisted of a L-7100 pump, equipped with a multi-solvent delivery system and a L-7400 ultraviolet (UV) detector. The column was a Mightysil RP-18GP, 5 μm, 4.6 mm in internal diameter (i.d.) and 250 mm in length (Kanto, Tokyo, Japan).

The mobile phase was composed of 25 mM phosphate buffer (pH 2.5)–acetonitrile featuring gradient elution steps as follows: 0 min, 100:0; 10 min, 80:20; 50 min, 70:30; 73 min, 50:50; 110 min, 50:50; 125 min, 20:80; 140 min, 20:80. The mobile phase was filtered under vacuum through a 0.45 μm membrane filter before use. The flow rate of mobile phase was 1.2 ml/min with UV absorbance detection at 254 nm. The operating temperature was maintained at 40 °C.

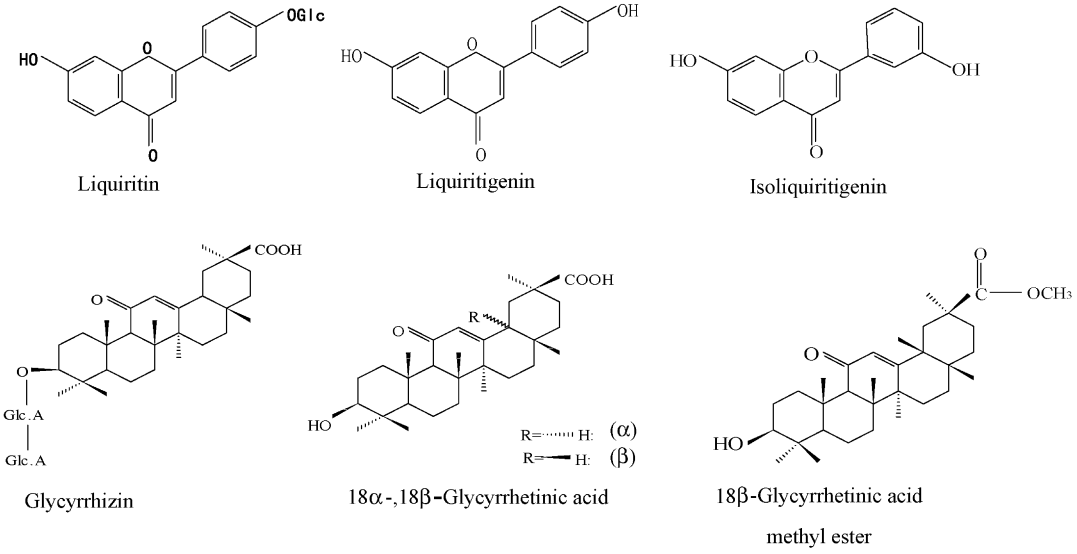

Fig. 1. Chemical structures of flavonoids and triterpenoids in licorice.

2.3. Plant materials

Twenty samples of licorice were used for this study. Sample numbers 8, 9 and 10 were cultivated in a local farm and harvested following 1 year of growth. The other 17 samples were obtained from various local herbal markets. Sample number 3 was used for validation of the analytical method, and also for limit of detection (LOD) and limit of quantification (LOQ) determinations. These 20 licorice samples were used for the quantification of the above-mentioned 3 flavonoids and 4 triterpenoids in licorice.

2.4. Preparation of licorice sample

A total of 10 ml of 80% methanol (aqueous) was added to 0.4 g licorice powder (passed through a 60-mesh screen) in a covered test tube, and the mixture was extracted at 60 °C for 6 h with supplementation of extraction solvent throughout the duration of the process. Subsequent to centrifugation of the mixture at 9000 rpm and 4 °C for 15 min, the supernatant was filtered through a 0.45 μm membrane filter before use.

2.5. Standard solutions preparation

All stock solutions of the three flavonoids (liquiritin, liquiritigenin and isoliquiritigenin) and the four triterpenoids (glycyrrhizin, 18α-glycyrrhetinic acid, 18β-glycyrrhetinic acid and 18β-glycyrrhetinic acid methyl ester) were prepared with methanol at a concentration of 1.0 mg/ml, and stored at −18 °C until use.

2.6. Validation of the analytical method

Validation of the analytical method for the three flavonoids and four triterpenoids derived from licorice was undertaken, the method being examined for specificity, linearity, accuracy, precision, LOD and LOQ.

For specificity validation, a standard solution was composed of three flavonoids (liquiritin, liquiritigenin and isoliquiritigenin) at the concentration of 10 μg/ml and four triterpenoids (glycyrrhizin, 18α-glycyrrhetinic acid, 18β-glycyrrhetinic acid and 18β-glycyrrhetinic acid methyl ester) at the concentrations of 8–40 μg/ml to be prepared with methanol. Licorice sample solution was prepared as described above. Eighty percent methanol was used as a blank solution (control). A volume of 10 μl of standard, sample or blank solution was injected into the HPLC column and analyzed using an HPLC method as described above.

For linearity validation, standard solutions of liquiritin (10–180 μg/ml), liquiritigenin (5–90 μg/ml), isoliquiritigenin (2.5–80 μg/ml), glycyrrhizin (20–200 μg/ml), 18α-glycyrrhetinic acid (10–320 μg/ml), 18β-glycyrrhetinic acid (4-72 μg/ml) and 18β-glycyrrhetinic acid methyl ester (5–90 μg/ml) were prepared. A volume of 10 μl of standard solution was injected into the HPLC column and analyzed using an HPLC method as described above. Triplicate analyses were performed for all samples and on 3 different days. The standard curves for the seven compounds were analyzed using the linear least-squares regression equation derived from the peaks area from the chromatogram versus concentrations of the seven compounds.

For accuracy validation, liquiritin (40, 80 and 120 μg/ml), liquiritigenin (19, 38 and 57 μg/ml), isoliquiritigenin (4, 8 and 12 μg/ml), glycyrrhizin (60, 120 and 180 μg/ml), 18α-glycyrrhetinic acid (50, 100 and 150 μg/ml) and 18β-glycyrrhetinic acid (4, 8 and 12 μg/ml) were prepared and mixed with licorice sample solution at a ratio of 1:1 (v/v). A volume of 10 μl of the mixture was injected into the HPLC column and analyzed using an HPLC method as described above. Triplicate analyses were performed on 3 different days. Sample recovery rate for the three flavonoids and the four triterpenoids from licorice sample solutions were calculated according to the following equation:

$$\text{Recovery}\,(\%) = \frac{2 \times \text{measured compound concentration} - \text{compound concentration in licorice sample solution}}{\text{compound's theoretical concentration}} \times 100,$$

The coefficient of variation (CV) was calculated as the standard deviation (S.D.) to the mean value from the results of triplicate testing.

For precision validation, liquiritin (80 μg/ml), liquiritigenin (38 μg/ml), isoliquiritigenin (8 μg/ml), glycyrrhizin (120 μg/ml), 18α-glycyrrhetinic acid (100 μg/ml), 18β-glycyrrhetinic acid (8 μg/ml) and 18β-glycyrrhetinic acid methyl ester (40 μg/ml) were prepared and 10 μl was injected into the HPLC column. Concentrations of the three flavonoids and the four triterpenoids from the experiments were individually calculated using the linear regression equation of the standard curve for the seven compounds. Triplicate analyses were conducted for all samples. The intra-day and inter-day precision values were obtained by triplicate analyses for each day and also per day over a 3-day period, respectively.

2.7. LOD and LOQ

The LOD and LOQ for the analytical method were also evaluated. Ten-fold dilutions of the three flavonoids and the four triterpenoids were made with methanol, and were then analyzed using an HPLC method as described above. Again, triplicate analyses were conducted for all samples tested. Linear regression equations for the three flavonoids and the four triterpenoids were individually derived from concentrations and peaks area for the seven compounds. The LOD and LOQ were calculated according to the following equations:

$$\text{LOD} = \frac{3.3\sigma}{S}; \qquad \text{LOQ} = \frac{10\sigma}{S}$$

where σ is the mean standard deviation and S is the slope of the same equation, respectively.

2.8. Quantification of flavonoids and triterpenoids in licorice samples

The specific quantity of the 3 flavonoids and the 4 triterpenoids contained in the 20 licorice samples was determined using our specialized HPLC method as described above. The moisture content of the 20 samples was determined by drying the sample in an oven at 105 °C for 24 h. The flavonoids and triterpenoids content in licorice samples was established dry weight basis.

3. Results and discussion

3.1. Specificity validation

The three flavonoids and four triterpenoids present in the licorice samples were separated by a reversed-HPLC method, featuring gradient elution of phosphate buffer–acetonitrile and UV absorbance detection at 254 nm. The results of HPLC chromatograms are shown in Fig. 2. The control for sample testing, no peak was observed (data not shown), indicating the dissolving solvent (80% methanol, aqueous) did not interfere with the results for the seven compounds separation. As demonstrated by the results shown in Fig. 2A, a good separation effect for these seven compounds was obtained with our methodology, retention times for liquiritin, liquiritigenin, isoliquiritigenin, glycyrrhizin, 18α-glycyrrhetinic acid, 18β-glycyrrhetinic acid and 18β-glycyrrhetinic acid methyl ester being, respectively, 15.88, 28.97, 53.70, 63.96, 106.68, 110.53 and 130.82 min. Chromatogram for the licorice sample solution is shown in Fig. 2B, a good separation effect also being apparent, complete separation peaks for liquiritin, liquiritigenin, isoliquiritigenin, glycyrrhizin and 18α-glycyrrhetinic acid being demonstrated. The 18β-glycyrrhetinic acid found in the test sample solution being lowly, therefore, peak of this compound was not apparent (Fig. 2B), while the sample concentration was increased,

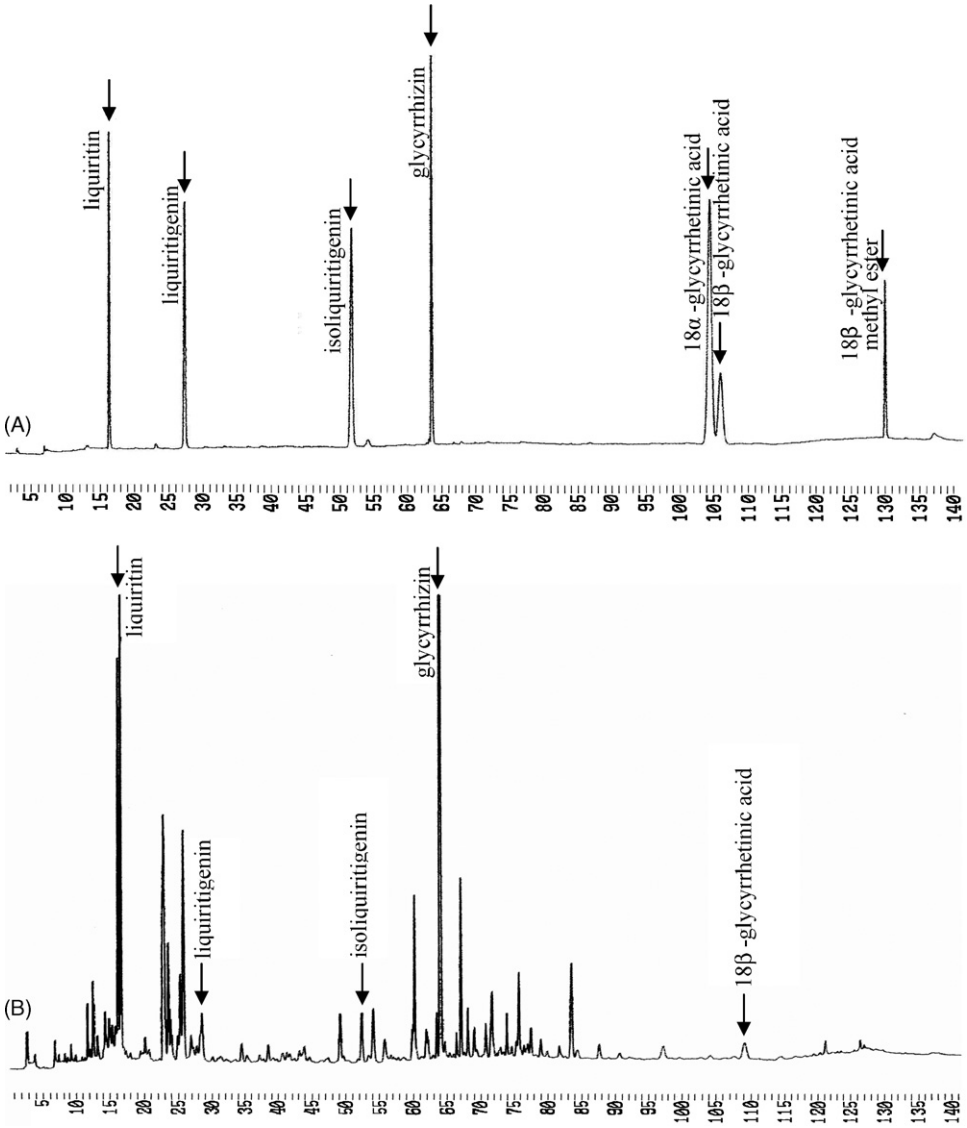

Fig. 2. Specificity validation for the HPLC analytical method for flavonoids and triterpenoids deriving from licorice: (A) standard solutions for the three flavonoids and the four triterpenoids and (B) licorice sample solution.

Table 1
Parameters of quantification for flavonoids and triterpenoids from licorice

Compound	Wavelength (nm)	Linear range (μg/ml)	Regression equation	r^2	LOD[a] (μg/ml)	LOQ[b] (μg/ml)
Liquiritin	254	10–180	$Y = 4453.66X - 6154.26$	0.9991	0.056	0.17
Liquiritigenin	254	5–90	$Y = 9215.45X - 12836.04$	0.9990	0.053	0.16
Isoliquiritigenin	254	2.5–80	$Y = 15175.53X - 21416.44$	0.9985	0.058	0.18
Glycyrrhizin	254	20–200	$Y = 6043.42X - 37757.17$	0.9978	0.048	0.15
18α-Glycyrrhetinic acid	254	10–320	$Y = 9623.42X - 38721.82$	0.9992	0.079	0.24
18β-Glycyrrhetinic acid	254	4–72	$Y = 14760.87X - 34968$	0.9973	0.044	0.13
18β-Glycyrrhetinic acid methyl ester	254	5–90	$Y = 12205.19X - 18081.11$	0.9984	0.084	0.25

[a] Limit of detection (LOD): the individual slope (S) and mean standard deviation (σ) were analyzed and the LOD was calculated by the equation = $3.3\sigma/S$.
[b] Limit quantitation (LOQ): was calculated by the equation = 10σ/S.

the peak of 18β-glycyrrhetinic acid was apparent in the chromatogram (data not shown). Further, 18β-glycyrrhetinic acid methyl ester was not present in the test sample, and hence a peak for this compound was not apparent in the chromatogram.

As shown in Fig. 2, this reversed-phase HPLC method has been validated revealing good specificity for the analysis of the three flavonoids and the four triterpenoids contained in licorice.

The mobile phase used in our study was composed of 0.25 mM phosphate buffer and acetonitrile with a gradient elution. The separation process for the three flavonoids and the four triterpenoids from licorice was performed within 130 min. In fact, separation of a mixture of these seven compounds using the phosphate–acetonitrile mobile phase system would not have required 130 min. However, the total running time of 130 min was empirically required for 80% methanol licorice extract which contained numerous flavonoids, triterpenoids and other compounds, for which these seven compounds to be involved. Simultaneous quantification of all these compounds in a single operation is much more convenient than using several separate procedures, especially for routine analysis or for a large number of samples. One of the greatest advantages is the simplification of the overall analytical process, for example, by reducing the frequency of changing the separation system, reducing the number of mobile phase preparations, and reducing the number of times the calibration curves have to be made, as well as the number of sample injections. Simultaneous separation of the flavonoids, triterpenoids and other compounds present in the 80% methanol licorice extract requires a special technique. As demonstrated by the results shown in Fig. 2B, we could find many of the compounds present within the retention times of 2–30 min and 45–85 min. Further, liquiritigenin, isoliquiritigenin and 18α-glycyrrhetinic acid were present in the 80% methanol licorice extract at very low levels, with very small absorption peaks. For these reasons, it was necessary to increase the capacity factor (k' value) of the separation system in order to delay the elution of the compounds. We therefore set a 100% phosphate buffer as the initial mobile phase for elution of the compounds with the highest polarity. The compounds to be eluted then followed in sequence according to their polarity, from high to low. Thus, a multiple-gradient elution program was performed. Our separation technique allowed us to obtain a good separation effect (Fig. 2B). Most of compounds present could be effectively separated from each other. Although this separation process took about 130 min, which appears rather long for a separating operation, the technique has the advantage of combining two quantification processes in a single procedure, significantly reducing the total analysis time.

3.2. Quantification parameters

Ouantification parameters for the three flavonoids and four triterpenoids using the above-described analytical HPLC method were examined. The results are presented in Table 1. Further, three separated calibration curves for the seven compounds obtained on different days by plotting the peaks area from the chromatogram versus concentrations of compound were found to be linear when evaluated by linear regression analysis. The standard curves for liquiritin, liquiritigenin, isoliquiritigenin, glycyrrhizin, 18α-glycyrrhetinic acid, 18β-glycyrrhetinic acid and 18β-glycyrrhetinic acid methyl ester were linear in the ranges of 10–180, 5–90, 2.5–80, 20–200, 10–320, 4–72 and 5–90 μg/ml, respectively. Good regression coefficients (r^2) for the three flavonoids' and four triterpenoids' linear regression equations exhibited, of which lying in the ranges of 0.9985–0.9991 and 0.9973–0.9992, respectively.

The limit of detection of the test compound represents the lowest concentration of the test compound that is able to be detected by the HPLC instrument and the analytical method, whereas the limit of quantification represents the lowest concentration of the test compound that can be quantified with acceptable precision and accuracy by the instrument and analytical method. The results of the LOD and LOQ analyses for the three flavonoids and the four triterpenoids ranged from 0.044 to 0.084 and 0.13 to 0.25 μg/ml, respectively, indicating that the analytical method for the quantification of these seven selected compounds deriving from licorice exhibited good sensitivity.

3.3. Accuracy validation

Following the spiking of the three flavonoids and the four triterpenoids into the licorice sample solution, recoveries for these seven compounds were examined in order to validate the accuracy of the analytical method used. Triplicate analyses were made over 3 different days. The results are included in Table 2. Good related recovery rates for the seven compounds were obtained, which ranged from 96.63 ± 2.43 to $103.55 \pm 2.77\%$ with variation coefficients ranging from 0.36 to 4.11%, of which being less than 5%, indicating that the analytical method for the

Table 2
Validation of accuracy of analytic method for flavonoids and triterpenoids from licorice

Marker substance	Spiked level (μg/ml)	Recovery[a] (%)			Mean (%)	CV[b] (%)
		1	2	3		
Liquiritin	40	97.14	98.77	102.69	99.54 ± 2.85	2.87
	80	101.56	98.43	100.86	100.29 ± 1.64	1.64
	120	103.94	104.07	101.66	103.22 ± 1.35	1.31
Liquiritigenin	19	99.24	100.16	93.03	97.48 ± 3.88	3.98
	38	99.44	99.65	100.14	99.74 ± 0.36	0.36
	57	97.99	100.40	98.07	98.82 ± 1.37	1.39
Isoliquiritigenin	4	101.84	99.42	105.96	102.41 ± 3.31	3.23
	8	98.91	98.94	105.63	101.16 ± 3.87	0.38
	12	100.22	100.74	97.23	99.40 ± 1.89	1.90
Glycyrrhizin	60	100.70	103.88	104.56	103.05 ± 2.06	1.99
	120	99.21	98.09	98.71	98.67 ± 0.56	0.57
	180	101.97	101.65	100.99	101.54 ± 0.49	0.49
18α-Glycyrrhetinic acid	50	98.12	93.83	97.95	96.63 ± 2.43	2.51
	100	97.16	97.22	103.60	99.33 ± 3.70	3.73
	150	99.08	103.09	101.28	101.15 ± 2.01	1.99
18β-Glycyrrhetinic acid	4	103.32	106.43	100.91	103.55 ± 2.77	2.67
	8	103.59	97.49	98.97	100.02 ± 3.18	3.18
	12	96.45	92.76	100.69	96.64 ± 3.97	4.11

[a] All values are mean ± S.D. obtained by triplicate analyses.
[b] Coefficient of variation = (S.D./mean) × 100%.

quantification of the three flavonoids and the four triterpenoids from licorice exhibited quite good accuracy.

From the results of Hurst et al. [20], 93–105% recoveries of glycyrrhizic acid and a regression coefficient of $r = 0.99$ for the regression equation were obtained using a reversed-phased HPLC method which isocratically eluted with a mobile phase of methanol/water/acetic acid (60/34/6, v/v/v).

3.4. Precision validation

Both the intra-day and inter-day precision levels for our analytical method for the three flavonoids and four triterpenoids were evaluated, such values being obtained, respectively, by triplicate analyses during 1 day and also obtained per day over a 3-day period. The results of such investigations are summarized in Table 3, the coefficient of variation of the intra-day and inter-day precision values for the three flavonoids and four triterpenoids ranging from 0.20 to 1.84 and 0.46 to 1.86%, respectively. All of the values were lower than 2%. The corresponding results indicated that the analytical method for quantification of the three flavonoids and the four triterpenoids deriving from licorice revealed good precision.

3.5. Flavonoids and triterpenoids content of licorice

Quantification of the presence of the 3 flavonoids and 4 triterpenoids from 20 licorice samples was undertaken using the above-mentioned analytical method. The results are as indicated in Table 4. Of these seven compounds, glycyrrhizin was the most abundant in licorice (1.212 ± 0.054 to 40.733 ± 2.009 mg/g), followed, in descending order, by liquiritin (0.451 ± 0.016 to 30.729 ± 1.552 mg/g), liquiritigenin

Table 3
Validation of precision of analytic method for flavonoids and triterpenoids from licorice

Marker substance	Theoretical concentration (μg/ml)	Intra-day precision[a] ($n = 3$)		Inter-day precision[b] ($n = 9$)	
		Measured concentration (μg/ml)	CV[c] (%)	Measured concentration (μg/ml)	CV (%)
Liquiritin	80	82.82 ± 0.74	0.89	81.35 ± 0.79	0.97
Liquiritigenin	38	38.81 ± 0.18	0.46	38.56 ± 0.11	0.28
Isoliquiritigenin	8	8.09 ± 0.05	0.64	8.08 ± 0.14	1.75
Glycyrrhizin	120	118.44 ± 2.18	1.84	120.93 ± 2.25	1.86
18α-Glycyrrhetinic acid	100	101.22 ± 0.20	0.20	100.46 ± 1.66	1.66
18β-Glycyrrhetinic acid	8	8.52 ± 0.09	1.03	8.24 ± 0.07	0.87
18β-Glycyrrhetinic acid methyl ester	40	39.43 ± 0.00	1.79	39.51 ± 0.18	0.46

[a] All values are mean ± S.D. obtained by triplicate analyses.
[b] All values are mean ± S.D. to be obtained by triplicate analyses for 3 days.
[c] Coefficient of variation = (S.D./mean) × 100%.

Table 4
Flavonoids and triterpenoids content (mg/g, dried matter) in licorice samples

Sample number	Liquiritin	Liquiritigenin	Isoliquiritigenin	Glycyrrhizin	18α-Glycyrrhetinic acid	18β-Glycyrrhetinic acid
1	7.174 ± 0.241[a]	0.818 ± 0.035	0.264 ± 0.009	9.209 ± 0.164	0.119 ± 0.006	0.055 ± 0.003
2	13.303 ± 0.699	1.189 ± 0.023	0.202 ± 0.006	17.240 ± 0.385	0.475 ± 0.015	0.070 ± 0.003
3	11.177 ± 0.202	0.731 ± 0.038	0.368 ± 0.007	19.051 ± 0.312	0.336 ± 0.015	0.084 ± 0.004
4	8.503 ± 0.353	0.503 ± 0.016	0.094 ± 0.005	16.962 ± 0.576	0.101 ± 0.002	0.057 ± 0.003
5	14.038 ± 0.270	0.299 ± 0.016	0.203 ± 0.011	17.301 ± 0.845	0.211 ± 0.007	0.075 ± 0.002
6	14.796 ± 0.302	0.320 ± 0.013	0.236 ± 0.011	16.418 ± 0.301	0.281 ± 0.005	0.073 ± 0.001
7	8.497 ± 0.301	1.490 ± 0.079	0.291 ± 0.007	12.790 ± 0.311	0.002 ± 0.000	0.058 ± 0.002
8	5.371 ± 0.172	1.038 ± 0.023	0.228 ± 0.009	7.881 ± 0.141	0.145 ± 0.006	0.130 ± 0.005
9	0.928 ± 0.050	0.128 ± 0.007	0.198 ± 0.009	1.212 ± 0.054	0.189 ± 0.009	0.106 ± 0.001
10	0.451 ± 0.016	0.108 ± 0.006	0.081 ± 0.002	2.581 ± 0.111	0.038 ± 0.001	0.042 ± 0.0008
11	11.199 ± 0.280	0.584 ± 0.011	0.262 ± 0.003	17.609 ± 0.327	0.158 ± 0.007	0.062 ± 0.0003
12	20.484 ± 1.197	2.174 ± 0.022	0.416 ± 0.014	29.074 ± 0.663	0.086 ± 0.0001	0.033 ± 0.002
13	22.449 ± 0.643	1.415 ± 0.065	0.262 ± 0.004	25.872 ± 0.262	0.087 ± 0.004	0.029 ± 0.002
14	15.874 ± 0.533	1.752 ± 0.020	0.488 ± 0.005	20.492 ± 0.782	0.085 ± 0.0002	0.023 ± 0.001
15	9.040 ± 0.179	1.188 ± 0.007	0.396 ± 0.013	16.496 ± 0.097	0.079 ± 0.0009	0.038 ± 0.001
16	18.919 ± 1.068	0.534 ± 0.028	0.073 ± 0.003	21.296 ± 0.793	0.068 ± 0.001	0.016 ± 0.0008
17	13.682 ± 0.531	1.900 ± 0.094	0.776 ± 0.018	22.993 ± 0.606	0.062 ± 0.0004	0.044 ± 0.005
18	30.729 ± 1.552	1.494 ± 0.076	0.337 ± 0.014	40.733 ± 2.009	0.100 ± 0.003	0.031 ± 0.001
19	13.042 ± 0.259	1.549 ± 0.075	0.325 ± 0.014	18.090 ± 0.316	0.042 ± 0.002	0.015 ± 0.000
20	9.619 ± 0.447	0.816 ± 0.002	0.489 ± 0.012	19.670 ± 0.244	0.083 ± 0.001	0.035 ± 0.002

[a] Data are mean ± standard deviation obtained by triplicate analyses.

(0.108 ± 0.006 to 2.174 ± 0.022 mg/g) and isoliquiritigenin (0.073 ± 0.003 to 0.776 ± 0.018 mg/g). 18α-Glycyrrhetinic acid (0.002 ± 0.000 to 0.475 ± 0.015 mg/g) and 18β-glycyrrhetinic acid (0.015 ± 0.000 to 0.130 ± 0.005 mg/g) revealed the lowest abundance for the 20 licorice samples, 18β-glycyrrhetinic acid methyl ester not being detected in any of the 20 licorice samples. Glycyrrhizin and liquiritin were found in sample numbers 8, 9 and 10, from the 1-year-old licorice samples, revealing lowly content. The other 17 samples, were obtained from the local herbal market, to have been commonly harvested following 3 years of cultivation. Such samples revealed a much more-substantial glycyrrhizin and liquiritin content than was the case for the 1-year-old samples. From results such as these, it would appear that the glycyrrhizin and liquiritin content of the 1-year-old roots of the *Glycyrrhizae* plant had not reached a peak. Not dissimilar results were reported by Hayashi et al. [14], for which the glycyrrhizin content of the 1-year-old and 3-year-old roots of *Glycyrrhiza glabra* L. featured ranges of 0.75–5.3 and 5.0–17.0 mg/g, respectively.

As the results of Yoneda et al. [24] indicated, the glycyrrhizin, liquiritin and liquiritigenin content of 49 licorice samples ranged from, respectively, 24.6 to 41.0, 5.4 to 10.9 and 0.1 to 0.5 mg/g. A field survey of the habitat of *G. glabra* in East Anatolia and Turkey, where these plants were endemic, was undertaken, the glycyrrhizin content of 86 such samples ranging from 16 to 69 mg/g [21]. The glycyrrhizin content of the roots of *G. glabra*, as reported in the study conducted in Turkey, was found to vary from 11 to 80 mg/g, depending upon individual plant variation [25]. Liquiritigenin and isoliquiritigenin contained in the cork layer and woody part of *G. glabra* L. has been reported to be of the order of 4.2 mg/g (liquiritigenin) and 2.1 mg/g (isoliquiritigenin) in the cork layer and 15.8 mg/g (liquiritigenin) and 6.0 mg/g (isoliquiritigenin) in the woody part of the plant, both these two compounds revealing substantially greater content in the woody part than was the case for the cork layer [13]. The isoliquiritigenin content of the 1-year-old and 3-year-old roots of *G. glabra* L. lay in the range of 0.25–1.5 and 1–3 mg/g, respectively [14]. In general, by comparing our results with those of other workers, the glycyrrhizin and liquiritin content of licorice samples were similar to the results of other workers, although our reported liquiritigenin content appeared to be somewhat greater than the results reported by Yoneda et al. [24], whereas the isoliquiritigenin content revealed by our study was lower than that reported by Hayashi et al. [14].

4. Conclusion

The simultaneous quantification of three flavonoids (liquiritin, liquiritigenin and isoliquiritigenin) and four triterpenoids (glycyrrhizin, 18α-glycyrrhetinic acid, 18β-glycyrrhetinic acid and 18β-glycyrrhetinic acid methyl ester) from licorice using a reversed-phase high-performance liquid chromatography method was successfully developed and used herein. Such a technique features convenient, which is very applicable for the simultaneous accurate quantification of numerous bioactive compounds found in licorice, especially for routine analysis or for a large number of samples. Good validation support for this analytical methodology as regards specificity, accuracy, precision and quantification parameters. The results of this study, suggest a relatively convient technique for the simultaneous quantification of numerous bioactive compounds found in licorice.

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