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Simultaneous determination of six index constituents and comparative analysis of four ethnomedicines from genus *Gentiana* using a UPLC-UV-MS method

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ABSTRACT: Many species from genus *Gentiana* (Gentianaceae) have a long history of applications as folk medicines in the world. A simple rapid UPLC-UV-MS/MS method has been developed and validated for the simultaneous determination of six index constituents (gentiopicroside, swertiamarin, loganic acid, sweroside, mangiferin and ferulic acid) from the four ethnomedicines (*G. rigescens* Franch. ex Hemsl., *G. rhodantha* Franch. ex Hemsl., *G. scabra* Bunge and *G. farreri* Balf. f.). The UPLC analysis was performed on Shim-Pack XR-ODS III (150 × 2.0 mm, 2.2 μm). The mobile phase was consisted of acetonitrile–0.1% formic acid water using gradient elution. The wavelength 242 nm was chose for the four iridoids as well as mangiferin and 320 nm was set for ferulic acid. Mass spectrometry was applied for identification and quantification for analytes with low concentration. All the regression equations revealed a good linear relationship ($R^2 > 0.9993$). Accuracy and precision were all within the required limits. The chromatogram fingerprints analysis combined with principal component analysis showed the similarity values of the four species were <0.788 while the similarity values of *G. scabra* Bunge and *G. rigescens* Franch. ex Hemsl. were >0.993, which provided reasonable foundation for utilization and clinical application of the four ethnomedicines. This developed method appears to be a useful tool for quality control of the four ethnomedicines. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: UPLC-UV-MS/MS; ethnomedicines; *Gentiana*; fingerprints; principal component analysis

Introduction

Many *Gentiana* species have been employed as folk medicines to treat disease and maintain health since the ancient times in the word (Aberham *et al*., 2007; Cao and Wang, 2010; State Pharmacopoeia Commission, 2010; Wang *et al*., 2012; Yang, 1991; Yang *et al*., 2010). Among them, roots and rhizomes of *G. scabra* Bunge and *G. rigescens* Franch. ex Hemsl. as original materials of traditional Chinese medicine 'Longdan' in *Chinese Pharmacopoeia* are used as hepatoprotective agents in northeast and southwest China, respectively (State Pharmacopoeia Commission, 2010). Tibetan and the Miao nationality living in southwest China (Tibet, Yunnan and Guizhou provinces) commonly utilized the aerial parts of *G. rhodantha* Franch. ex Hemsl. for treatment of hepatitis, jaundice and cough (Wu *et al*., 2011). The aerial parts of *G. farreri* Balf. f. are the traditional Tibetan medicine to treat pneumonia and fever. Because of the remarkable medicinal functions, plants of the genus *Gentiana* have been a hot topic in phytochemistry and pharmacology since the 1960s and exhibited chemical diversity (Wang *et al*., 2012; Yang *et al*., 2010). Additionally, more research demonstrated this genus is a rich source of iridoids and phenols such as gentiopicroside, swertiamarin, loganic acid, sweroside and mangiferin, which are commonly regarded as major constituents and likely to responsible for the different therapeutic effect of these folk medicines (Chen *et al*., 2013b Ikeshiro and Tomita, 2007; Kakuda *et al*., 2002; Ma *et al*., 1994; Xu *et al*., 2006, 2007, 2008, 2011; Yang *et al*., 2012; Zhang *et al*., 2009). Therefore, the multicomponent analysis could be developed as an important approach for the quality evaluation of ethnomedicines from genus *Gentiana*.

Recently, the chromatographic fingerprints combined with chemometrics methods have been widely used for the analysis and quality control of medicine (Avula *et al*., 2013, 2014; Chen *et al*., 2008, 2013a; Shan *et al*., 2014; Yu *et al*., 2013). Chen *et al*. (2008) developed an HPLC-UV method combined with fingerprints and multivariate analysis for quality control and original discrimination of *Ganoderma lucidum* (Leyss. ex Fr.) Karst. Shan *et al*. (2014) used fingerprints and chemometrics

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Abbreviations used: MRM, multiple reaction monitoring; PCA, principal component analysis.

methods based on chemical characteristics and bioactivity to evaluate *Panax ginseng* C.A. Meyer in different ages and seasons.

To date, several analytical methods based on NIR, HPLC-UV, LC-MS and LC-NMR have been published for quality control of *Gentiana* species (Cao and Wang, 2010; Chuang *et al*. 2014; Pan *et al*. 2014; Wei *et al*. 2012; Wolfender *et al*. 1997). However, to the best of our knowledge no report is available on simultaneous determination of the six inconstituents (**1**, loganic acid; **2**, swertiamarin; **3**, mangiferin; **4**, gentiopicroside; **5**, sweroside; and **6**, ferulic acid) in the four species (Fig. 1) by UPLC-MS/MS. In the present study, an accurate, specific and reliable method was developed and LC fingerprints combined with principal component analysis (PCA) were carried out to distinguish the four ethnomedicines (*G. rigescens* Franch. ex Hemsl., *G. rhodantha* Franch. ex Hemsl., *G. scabra* Bunge and *G. farreri* Balf. f.), which appears to be a useful tool for quality control of the four ethnomedicines.

Experimental

Chemicals and plant materials

The HPLC-grade solvent (acetonitrile and formic acid) were purchased from Tedia and Dikmapure (USA), respectively. The pure water for the UPLC analysis was purified by a Milli-Q system from Millipore (USA). The standard compounds (**1–6**) were provided by Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

The aerial parts of *G. rhodantha* Franch. ex Hemsl. and *G. farreri* Balf. f. were collected in Guizhou and Tibet Provinces China, respectively. The roots and rhizomes of *G. scabra* Bunge and *G. rigescens* Franch. ex Hemsl. were obtained from Inner Mongolia and Yunnan Provinces of China, respectively. All the samples (Table 1) were collected in November 2012 and authenticated by Professor Hang Jin (Institute of Medicinal Plants, Yunnan Academy of Agricultural Sciences).

Sample preparation

All samples were ground into powder and then sieved through a 60 mesh stainless steel sieve before extraction. A total of 0.25 g of each sample powder was extracted with 10 mL 70% methanol for 30 min under ultrasonic once and filtered through a paper filter. The solution was stored at 4 °C and filtered through a 0.22 μm membrane filter (Millipore, USA) before injection into the UPLC system. The injection volume was 3 μL.

Instrumentation and chromatographic conditions

All analyses were performed on Shimadzu UPLC system equipped with a degasser, binary gradient pumps, a column oven, an autosampler and UV detector. Chromatographic separation was carried out on Shim-Pack

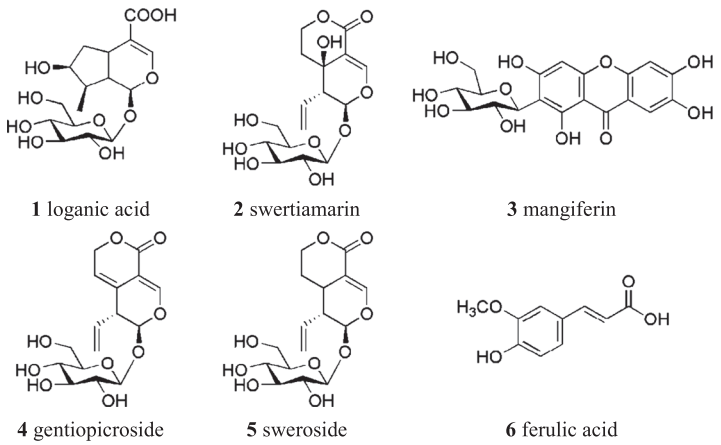

Figure 1. Structures of the six index constituents.

Table 1. Summary for the sample sources

No.	Sources	No.	Sources
S1	*G. rhodantha* Franch. ex Hemsl.	S11	*G. rigescens* Franch. ex Hemsl.
S2	*G. rhodantha* Franch. ex Hemsl.	S12	*G. rigescens* Franch. ex Hemsl.
S3	*G. rhodantha* Franch. ex Hemsl.	S13	*G. rigescens* Franch. ex Hemsl.
S4	*G. rhodantha* Franch. ex Hemsl.	S14	*G. rigescens* Franch. ex Hemsl.
S5	*G. rhodantha* Franch. ex Hemsl.	S15	*G. rigescens* Franch. ex Hemsl.
S6	*G. farreri* Balf. f.	S16	*G. scabra* Bunge
S7	*G. farreri* Balf. f.	S17	*G. scabra* Bunge
S8	*G. farreri* Balf. f.	S18	*G. scabra* Bunge
S9	*G. farreri* Balf. f.	S19	*G. scabra* Bunge
S10	*G. farreri* Balf. f.	S20	*G. scabra* Bunge

G., *Gentiana*.

XR-ODS III (150 × 2.0 mm, 2.2 μm). The mobile phase was consisted of acetonitrile (A)–0.1% formic acid water (B) using gradient elution with the flow rate 0.25 mL/min as follows: 12% A at 0–2.5 min; 12–15% A at 2.5–7.3 min; 15–32% A at 7.3–12 min; 32–78% A at 12–16 min; and 12% A at 16–20 min. The column temperature was kept at 40 °C. The detection wavelength was set at 242 nm for the four iridoids as well as mangiferin and at 320 nm for ferulic acid. The Shimadzu LCMS-8030 triple quadrupole mass spectrometer (Japan) equipped with an electrospray ionization source. The parameters were set as follows: nebulizing gas and drying gas were nitrogen at a flow rate of 3.0 and 15.0 L/min, respectively; the interface voltage was set to 3.5 kV; desolvation line temperature was 250 °C and the heat block temperature was 400 °C. All mass spectra were acquired in both negative and positive ion modes and recorded from *m/z* 100 to 500. Multiple reaction monitoring (MRM) acquisition mode was used for quantification of analytes with low concentration. Data acquisition was performed using Shimadzu LabSolution software.

Validation procedure

The method was validation by linearity, the limit of detection (LOD) and quantification (LOQ), precision and accuracy. Because of the different solubilities of standards, margiferin was dissolved in 50% methanol at 2 mg/mL, and gentiopicroside and other standards were prepared at concentrations of 2.5 and 1 mg/mL in methanol, respectively. Each standard solution, including seven different concentration levels, was injected in triplicate for establishment of calibration curve. Calibration curves established by UPLC-MS/MS were prepared for quantification of analytes with low concentration (**3** in *G. farreri* Balf. f.; **4** and **5** in *G. rhodantha* Franch. ex Hemsl.) and the calibration curves using UPLC-UV method were applied quantification of other analytes. The LOD and LOQ under the present chromatographic conditions were determined by serial dilution of standard solution at a S/N (signal-to-noise ratio) of 3 and 10, respectively.

Inter- and intra-day variation was used to determine the precision of the method including measurements of retention times and peak areas by analyzing known concentrations of standard solutions on three consecutive days in triplicate and six times (0, 4, 8, 12, 16, 20 and 24 h) during a single day, respectively. The recovery test was chosen to evaluate the accuracy of the method, and then three different amounts (low, medium and high spike) of each standard solution were spiked to the S3 and S6 extract. The recovery rates of UPLC-UV method were obtained by spiking the four iridoid standards to S6 as well as mangiferin to S3. The recovery rates of UPLC-MS/MS method were obtained by adding the standards (gentiopicroside and sweroside added to S3; mangiferin added to S6). The recovery rate (*R*) was calculated using the amount added of standards, and actual amount (measured amount-original amount) obtained by UPLC-UV-MS/MS analysis:

$$R(\%) = [(\text{measured amount-original amount})/\text{amount added}] \times 100\%$$

Statistical analysis

The chromatogram fingerprints analysis was carried out by Similarity Evaluation System for Chromatographic Fingerprint of Tradition Chinese Medicine developed by the Chinese Pharmacopoeia Committee (Version 2004 A). The reference fingerprint chromatograms were obtained by comparison of chromatograms of each sample, and then the similarity values between the reference fingerprints and the sample chromatograms together with the common peak data (retention time and peak areas) were calculated. Additionally, principal component analysis based on the common peak areas was performed by software SIMCA-P^{+}10.0.

Results and discussion

Optimization of extraction procedure

In order to find an efficient ultrasonic extraction procedure, several extraction solvent ratios and time tests were performed. Different methanol–aqueous ratios (0, 30, 50, 70, 80, 90 and 100%, v/v) were screened to obtain the best extraction. The results showed that the yields of mangiferin began to reduce when the methanol–aqueous ratio was >50% and the yields of the four iridoids were potently increased when extractions were performed with 70% methanol–aqueous solutions. The best extraction solvent was proved to be 70% methanol aqueous solutions. Additionally, different extraction time was also investigated to optimize the extraction procedure. All sample powder (0.25 g) was extracted by 70% methanol aqueous solutions (10 mL) for 15, 25, 35, 45, 55 and 60 min. The results showed that 35 min was found to be the best extraction time and a longer time was not necessary.

Optimization of LC-MS conditions

Several experiments were carried out to optimize chromatographic conditions. Different mobile phases (acetonitrile–water and methanol–water) were investigated to obtain a better separation and the results showed that the acetonitrile–water system for the six indexes was better than the methanol–water system in separation ability. Additionally, 0.1% formic acid was added to the mobile phase to enhance resolution and eliminate peak tailing of the six compounds while enhancing the intensity of adducted molecular ions $[M+HCOO]^-$ and protonated molecular ions $[M+H]^+$ in the mass spectrometer. Gradient elution programs with 0.25 min/mL could ensure satisfactory separation of each compound and maintain reasonable analytical time. Various chromatographic columns Shim-Pack Various XR-ODS III (150 × 2.0 mm, 2.2 μm and 75 × 2.0 mm, 1.6 μm) were tested to optimize chromatographic conditions. Although the chromatographic column Shim-Pack Various XR-ODS III (75 × 2.0 mm, 1.6 μm) had the shorter run time, it did not show better performance in samples with complex constituents. Shim-Pack Various XR-ODS III (150 × 2.0 mm, 2.2 μm) was chosen for this application.

In order to determine optimal chromatographic conditions, all the analytes were detected in both positive and negative ionization modes. Under positive ionization modes, **5** and **6** exhibited protonated molecular ions $[M+H]^+$. The other compounds except **3** $[M-H]^-$ displayed adducted molecular ions $[M+HCOO]^-$. MRM acquisition mode was used for quantification purposes and all the MRM settings were auto-optimized (Table 2).

Method validation

The developed method for determination of the six indexes was validated in terms of linearity, precision and accuracy when compared with corresponding standard. All calculations were carried out using Shimadzu LabSolution software (Shimadzu, Japan).

The standard solutions were prepared and diluted with methanol to appropriate concentrations for establishment of the calibration curve. These calibration curves were plotted with seven different contents and revealed a good linear relationship ($R^2 > 0.9993$). The calibration curves for UPLC-UV and MS were in the range from 5 to 3050 and from 0.05 to 8 μg/mL, respectively.

Table 2. LC-MS/MS parameters (multiple reaction monitoring) for the selected analytes (Q_1, precursor ion mass; CE, collision cell energy; and Q_3, product-ion mass)

Analyte	R_t (min)	Q_1 (*m/z*)	Q_3 (*m/z*)	Q_1 Pre-bias (V)	CE (eV)	Q_3 Pre-bias (V)
Loganic acid	2.541	421.15 $[M+HCOO]^-$	375.25	30.0	10	26.0
			213.20	30.0	25	22.0
Swertiamarin	4.625	419.15 $[M+HCOO]^-$	179.20	30.0	15	19.0
			119.25	30.0	30	22.0
Mangiferin	5.736	421.10 $[M-H]^-$	301.00	30.0	20	20.0
			331.05	30.0	25	23.0
Gentiopicroside	6.270	401.15 $[M+HCOO]^-$	179.15	30.0	15	11.0
			89.20	30.0	20	16.0
Sweroside	6.632	359.15 $[M+H]^+$	197.20	−26.0	−10	−21.0
			127.15	−26	−30	−23
Ferulic acid	11.027	195.05 $[M+H]^+$	177.00	−14	−10	−19
			163.00	−14	−20	−30

R_t, Retention time.

Table 3. Linear regression data of standards

Analyte	Regression equation	Linearity range (μg/mL)	R^2	Limit of detection (μg/mL)	Limit of quantitation (μg/mL)
UV					
Loganic acid	$y = 7188.27x - 5831.52$	5–500	0.9998	0.24	0.71
Swertiamarin	$y = 5296.17x - 8249.73$	12–500	0.9995	0.11	0.52
Mangiferin	$y = 22015.9x - 263443$	20–2000	0.9996	0.12	0.38
Gentiopicroside	$y = 10246.6x + 100822$	30–3050	0.9999	0.15	0.47
Sweroside	$y = 7280.82x + 3311.17$	5–100	0.9997	0.25	0.79
MS					
Mangiferin	$y = 137308x + 86125.4$	0.08–8	0.9993	0.018	0.067
Gentiopicroside	$y = 121337x + 5780.64$	0.07–7.5	0.9999	0.021	0.073
Sweroside	$y = 272679x + 4834.71$	0.05–8	0.9996	0.009	0.042

Table 4. Intra- and inter-day precision (RSDs) of method

Analytes	Intra-day precision (RSD, %)						Inter-day precision (RSD, %)	
	Day 1		Day 2		Day 3			
	R_t	P_a	R_t	P_a	R_t	P_a	R_t	P_a
UV								
Loganic acid	0.68	1.13	0.72	1.27	0.83	1.22	0.65	1.58
Swertiamarin	0.81	1.53	0.74	1.42	0.64	1.09	0.78	0.96
Mangiferin	0.76	1.21	0.47	1.14	0.58	1.28	0.56	1.22
Gentiopicroside	0.46	0.97	0.82	1.34	0.44	1.85	0.66	0.98
Sweroside	0.37	0.62	0.37	0.76	0.31	0.84	0.41	1.25
MS								
Mangiferin	0.53	1.09	0.44	1.03	0.55	1.1	0.37	1.07
Gentiopicroside	0.47	1.12	0.51	0.88	0.41	1.03	0.62	1.15
Sweroside	0.48	0.87	0.37	0.92	0.31	0.82	0.45	0.94

R_t, Retention time; P_a, peak area.

Table 5. Recovery study of the method

Analytes	Original amount (μg/mL)	Amount added (μg/mL)	Measured amount (μg/mL)	Recovery (%)	RSD (%)
UV					
Loganic acid	169.12 (S6)	85	255.32	101.41	0.98
		170	339.74	100.36	1.77
		255	423.59	99.76	1.72
Swertiamarin	160.49 (S6)	80	241.19	100.88	1.34
		160	319.76	99.54	1.58
		240	404.27	101.57	1.33
Mangiferin	496.54 (S3)	250	749.54	101.20	1.53
		500	1007.83	102.26	1.22
		750	1244.09	99.67	1.34
Gentiopicroside	348.28 (S6)	175	527.2	102.24	0.92
		350	701.43	100.90	0.87
		525	870.01	99.38	1.01
Sweroside	16.40 (S6)	8	24.58	102.25	1.67
		16	32.74	102.13	1.03
		32	48.96	101.75	1.75
MS					
Mangiferin	2.47 (S6)	1.25	3.73	100.45	0.93
		2.5	5.01	101.60	0.88
		5	7.56	101.80	1.11
Gentiopicroside	0.21 (S3)	0.1	0.31	102.50	1.26
		0.2	0.41	102.00	1.17
		0.3	0.52	102.33	1.32
Sweroside	2.79 (S3)	1.4	4.20	100.92	0.81
		2.8	5.60	100.50	1.17
		4.2	6.95	99.07	0.93

Table 6. Contents (mg/g) of the six index constituents in the four ethnomedicines

Analytes	Loganic acid	Swertiamarin	Mangiferin	Gentiopicroside	Sweroside	Ferulic acid
S1	2.678	ND	19.963	0.007[a]	0.122[a]	ND
S2	2.561	ND	18.967	0.006[a]	0.119[a]	ND
S3	2.822	ND	21.870	0.008[a]	0.128[a]	ND
S4	2.821	ND	21.709	0.008[a]	0.111[a]	ND
S5	2.777	ND	21.126	0.008[a]	0.100[a]	ND
S6	6.773	6.324	0.102[a]	13.258	0.657	ND
S7	7.137	6.477	0.100[a]	14.030	0.701	ND
S8	6.938	6.452	0.101[a]	13.681	0.705	ND
S9	6.865	6.382	0.107[a]	13.446	0.684	ND
S10	7.239	6.552	0.102[a]	13.879	0.697	ND
S11	5.862	1.598	ND	34.362	0.280	ND
S12	0.699	2.096	ND	39.860	0.250	ND
S13	1.104	2.553	ND	42.269	0.226	ND
S14	0.555	2.323	ND	40.002	0.321	ND
S15	1.189	2.479	ND	29.852	0.456	ND
S16	3.083	1.954	ND	19.762	0.387	ND
S17	3.097	1.994	ND	20.258	0.420	ND
S18	3.062	1.955	ND	19.891	0.408	ND
S19	3.149	2.023	ND	20.259	0.402	ND
S20	3.136	2.022	ND	20.526	0.381	ND

ND, Undetectable. [a] Determinated by UPLC-MS/MS method.

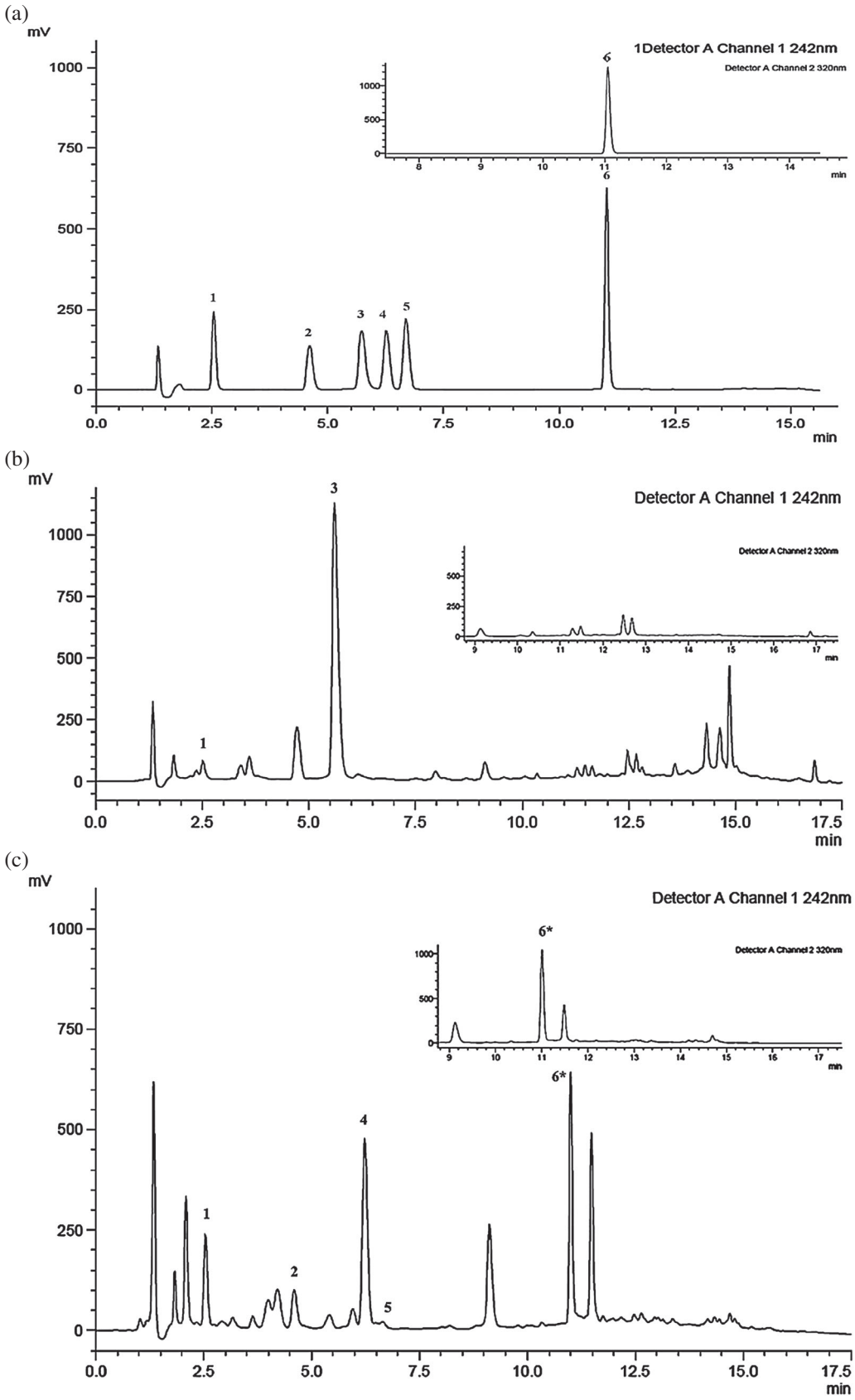

Figure 2. Chromatograms at 242 and 320 nm for standards and samples. (a) Standards; (b) *Gentiana rhodantha* Franch. ex Hemsl.; (c) *G. farreri* Balf. f.; (d) *G. rigescens* Franch. ex Hemsl.; and (e) *G. scabra* Bunge.

The LODs (S/N = 3) and LOQs (S/N = 10) for these indexes were determined by serial dilution of standard solution using the described UPLC-MS/MS conditions. Linearity data, LODs and LOQs are summarized in Table 3.

Recovery and precision data are displayed in Tables 4 and 5. The inter- and intra-day relative standard deviations (RSD) of retention time were <1.1% and the RSD of retention time peak areas were <2%. Recovery for all analytes in the low to high concentration range was in the range of 99.54–102.50% with RSD values no more than 2%. Therefore, the developed method is sensitive, precise and accurate for simultaneous quantitative evaluation of the six compounds.

Quantification

The six indexes were confirmed by comparison of their retention times, UV spectrum data and precursor/product-ion pairs obtained by MRM acquisition mode. A wavelength of 320 nm was selected for analysis of ferulic acid and 242 nm was chosen for iridoids as well as mangiferin. Compounds **1** and **4** were detected in the four *Gentiana* species while their contents ranging from 0.007–42.269 mg/g differed from each other. Among them, compound **4** was the major constituent in the four *Gentiana* species except *G. rhodantha* Franch. ex Hemsl. with high concentration of mangiferin. However, mangiferin was

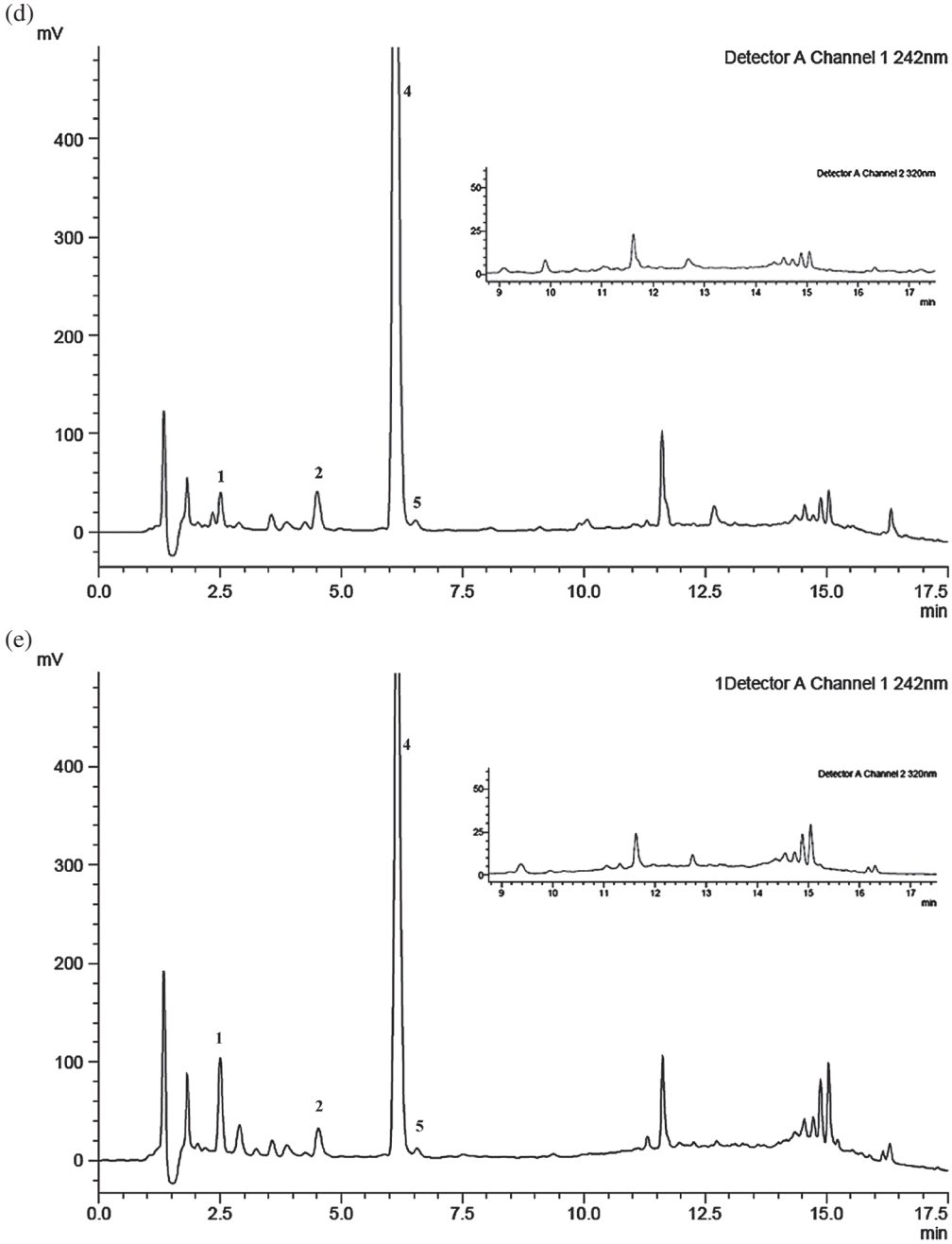

Figure 2. (Continued)

undetected in *G. rigescens* Franch. ex Hemsl. and *G. scabra* Bunge. Compound **6** was not found in all of these samples. Interestingly, compound **6** could be confirmed by UPLC-UV according to the retention time and UV spectrum data in *G. farreri* Balf. f. samples. Nevertheless, this conclusion was denied when reconfirmed by mass data, which indicated that the retention time and UV spectrum data could not be regarded as exactly qualitative information for some compounds. The contents (mg/g) of the six indexes in the four *Gentiana* species are summarized in Table 6.

Fingerprint analysis and PCA

LC-UV fingerprint analysis performed by the chromatogram fingerprints analysis was carried out by the Similarity Evaluation System and then the fingerprints were matched automatically. The reference fingerprint chromatograms and the common peak dates which were confirmed by the RSDs of all peak relative retention time (<1%) were obtained by comparing these chromatograms (S1–S20). The fingerprint chromatograms are displayed in Figs. 2 and 3. Furthermore, the similarity values between the reference fingerprint and the sample chromatograms were calculated by this software. The results showed that the similarity values (10 common peaks) of the 20 samples were between 0.59 and 0.788, which impaired the chemical diversity existing in the four *Gentiana* species when performed in this chromatogram condition. The similarity values of *G. rigescens* Franch. ex Hemsl. and *G. scabra* Bunge were >0.993, which would provide a reasonable explanation for the two species recorded as original materials of traditional Chinese medicine 'Longdan' in the *Chinese Pharmacopoeia*.

According to the common peak dates obtained by fingerprint analysis, PCA was applied for distinguishing the four *Gentiana* species. The PCA results could explain 94.055% of total variance with 67.696% in principal component 1 (PC1) and 24.359% in PC2 (Fig. 4). Figure 4 exhibited 20 samples divided into four groups and the four *Gentiana* species could be distinguished. However, the distance of *G. rigescens* Franch. ex Hemsl. and *G. scabra* Bunge in 2D and 3D PCA scores plots are close, even S15 was classified incorrectly into *G. scabra* Bunge group, which could provide more evidence on clinical application of the two species.

In conclusion, this developed UPLC-MS/MS method for the simultaneous determination of the six indexes in the four ethnomedicines from genus *Gentiana* with good precision, accuracy and linearity has been carried out for the first time. This method combined with fingerprint analysis and PCA could successfully distinguish the four *Gentiana* species, which could provide a reasonable foundation for utilization and clinical application.

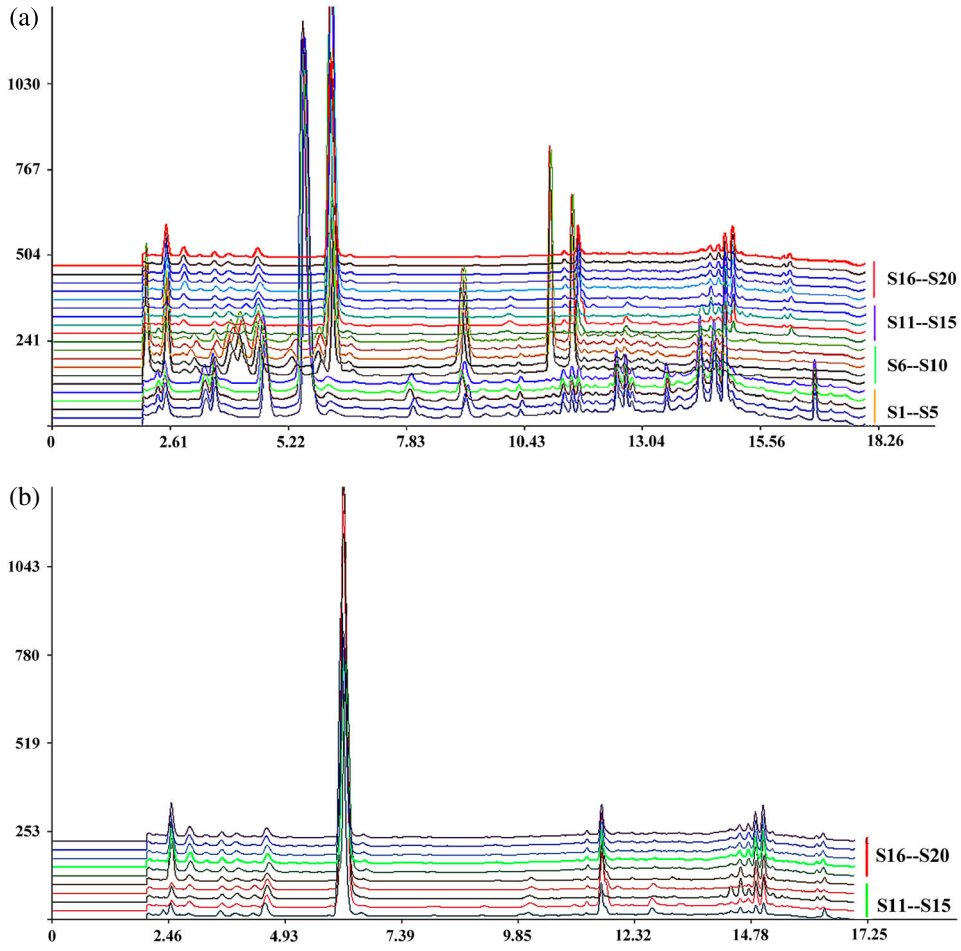

Figure 3. Fingerprint chromatograms at 242 nm. (a) Fingerprint chromatograms of the four species; (b) fingerprint chromatograms of *G. rigescens* Franch. ex Hemsl. and *G. scabra* Bunge.

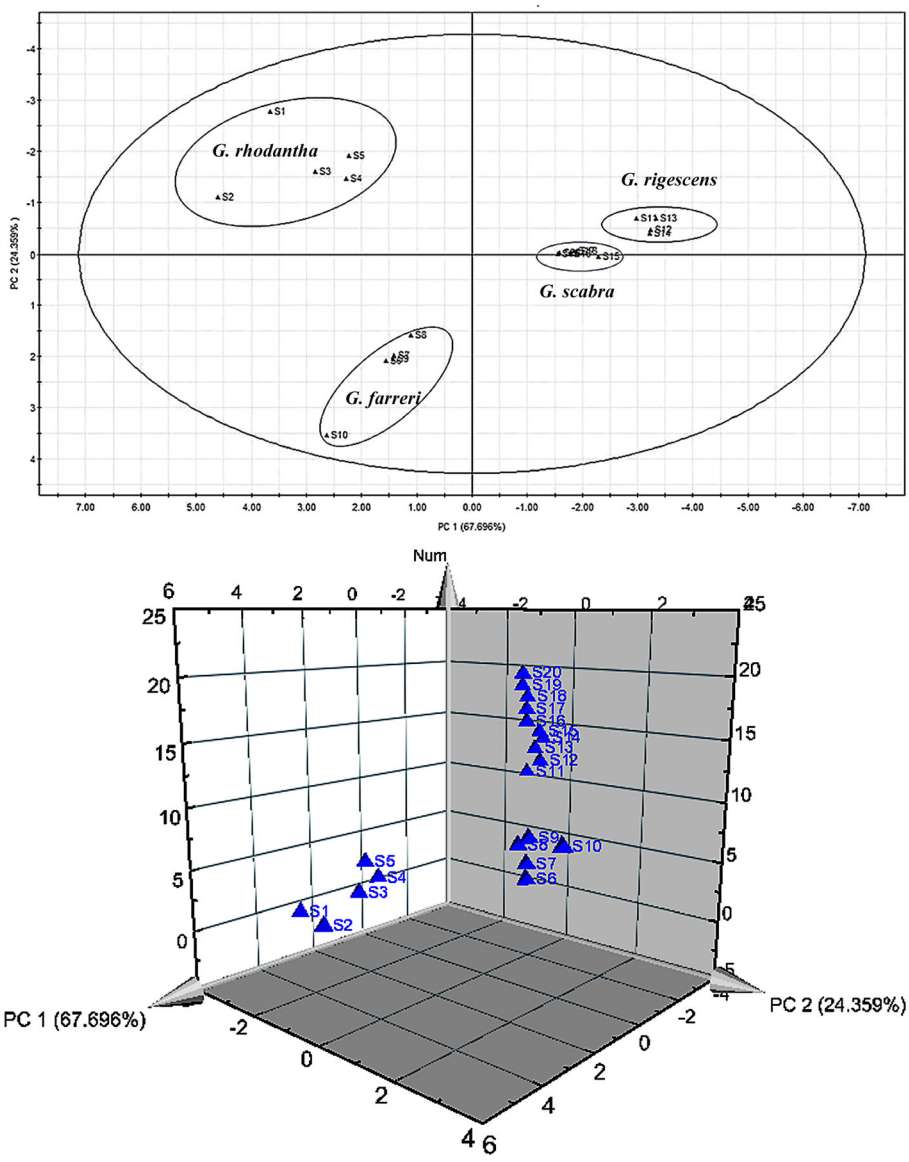

Figure 4. The principal component analysis (PCA) 2D and 3D scores plots for 20 samples using relative peak areas of the 10 common peaks as input data.

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