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Simultaneous quantification of eight major constituents in Herba *Siegesbeckiae* by liquid chromatography coupled with electrospray ionization time-of-flight tandem mass spectrometry

Zhen Jiang [a], Qing-Hong Yu [b], Yan Cheng [a], Xing-Jie Guo [a,*]

[a] Department of Analytical Chemistry, College of Pharmacy, Shenyang Pharmaceutical University, 103 Wenhua Road, Chenyang 110016, Liaoning Province, PR China
[b] Department of Rheumatology and Immunology, Zhujiang Hospital, Southern Medical University, 253 Industry Road, 510515 Guangdong, PR China

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ABSTRACT

A simple and reliable high-performance liquid chromatography coupled with electrospray ionization time-of-flight tandem mass spectrometry method was developed and validated for the determination of the major diterpenoids and flavonoids in the aerial parts of Herba *Siegesbeckiae*, including Kirenol, hythiemoside B, *ent*-16β,17,18-trihydroxy-kauran-19-oic acid, *ent*-17,18-dihydroxy-kauran-19-oic acid, *ent*-16β,17-dihydroxy-kauran-19-oic acid, 16α-hydro-*ent*-kauran-17,19-dioic acid, Rhamnetin, 3′,4′-dimethoxy quercetin. The separation of eight compounds was performed on a Waters Symmetry Shield TM RP18 column (250 mm × 4.6 mm i.d., 5 μm) with gradient elution using a mobile phase consisting of 0.1% aqueous formic acid and acetonitrile containing 0.1% formic acid in selected ion monitoring mode. All calibration curves showed good linearity ($r > 0.999$) within the test ranges. The precision was evaluated by intra- and inter-day tests, which revealed relative standard deviation (RSD) values less than 3.7%. The recoveries for the quantified compounds were between 97.4 and 101.2% with RSD values below 2.4%. According to the literatures, this study represents the first investigation of the simultaneous analysis of multiple components and the method can be applied to determine the amounts of the major compounds in Herba *Siegesbeckiae*.

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1. Introduction

The plants of the genus *Siegesbeckiae* (Compositae) are annual herbs widely distributed in tropical, subtropical, and temperate parts of the world. Three species of *Siegesbeckiae* (*Siegesbeckiae orientalis* L., *Siegesbeckiae pubescens* Makino, *Siegesbeckiae glabrescens* Makino) grow in China, and their aerial parts have been used as a traditional Chinese medicine, "Xi-Xian", for the treatment of rheumatic arthritis, hypertension, malaria, neurasthenia, and hepatitis [1]. Extracts of Herba *Siegesbeckiae* (HS) have been reported to exhibit antioxidant [2], antiallergic [3] and antifertility effects [4]. In the past few decades, systematic chemical studies have been performed, and HS is known to contain a large number of compounds, including diterpenoids, flavonoids, steroids and fatty acids. Pharmacological studies have suggested that characteristic diterpenoids are the main bioactive constituents of HS, a series of *ent*-pimarane and *ent*-kaurane diterpenoids from the herbs have been reported to be responsible for the antifertility [4], anti-inflammatory [5] and PTP1B inhibitory activities [6]. Flavonoids were also the bioactive compositions, Kim et al. suggested that flavonoids in HS provided the activity of inhibiting the production of NO in the lipopolysaccharide (LPS)-induced microglia [7]. Therefore, the quality control of HS should be focused on the determination of the diterpenoids and flavonoids.

To our knowledge, only several papers employed high pressure liquid chromatography-ultraviolet (HPLC-UV) to determine Kirenol or Darutigenol in HS [8–10]. And very few literatures were referred to the quantitative assay of *ent*-pimarane and *ent*-kaurane diterpenoids. An intractable problem is that most diterpenoids found in HS are not detectable by HPLC-UV analysis for the lack of an UV chromophore. Consequently, it is particularly difficult to simultaneously determine these diterpenoids with HPLC-UV. By contrast, MS is a sensitive and selective technique that allows for detection of the constituents of no UV absorption, and TOF-MS allows the generation of mass information with greater accuracy and precision. Due to high sample throughput, HPLC–TOF-MS methods are suitable for the analysis of traditional Chinese medicine (TCM) especially for low-abundance and complex compounds, which are difficult to analyze by conventional isolation.

* Corresponding author. Tel.: +86 24 23986285; fax: +86 24 23986285.
E-mail address: gxjhyz@yahoo.com.cn (X.-J. Guo).

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In this paper, HPLC–ESI-TOF-MS method was proposed for the quantification of the major diterpenoids and flavonoids including Kirenol (1), hythiemoside B (2), *ent*-16β,17,18-trihydroxy-kauran-19-oic acid (3), *ent*-17,18-dihydroxy-kauran-19-oic acid (4), *ent*-16β,17-dihydroxy-kauran-19-oic acid (5), 16α-hydro-*ent*-kauran-17,19-dioic acid (6), Rhamnetin (7), 3′,4′-dimethoxy quercetin (8) in five different sources of HS. According to the literatures, this study represents the first investigation of the simultaneous analysis of multiple components in HS.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade acetonitrile was purchased from Concord Technology Co., Ltd. (TianJin, China). Distilled water was prepared from demineralized water in our laboratory. Analytical reagent grade formic acid was obtained from Kermel Chemical Reagent Co., Ltd. (TianJin, China). The aerial parts of HS from five sources, including *S. pubescens* Makino from Hebei (two batches, samples A and B purchased from Anguo Province Zhenyu Chinese Medicine Pieces Company Limited) and Henan (sample C gathered from Xin County Henan Province), *S. orientalis* L. from Hunan (sample D bought from Anhui Jioujioutong Pharmaceutical Company Limited) and *S. glabrescens* Makino from Yunnan (sample E bought from Ynnan Qiancaoyuan Pharmaceutical Company Limited), were authenticated by Qi-shi Sun, Professor of Pharmacognosy. Compounds 1–8 (Fig. 1) were isolated and purified from the extract of *S. pubescens* Makino (sample A) in our laboratory, and identified by ^{1}H NMR, ^{13}C NMR and comparison with those reported in the literatures among which compounds 7 and 8 were not yet reported from SH [11–17]. The purities of the above ingredients using as standards were above 98% by LC analysis.

2.2. Sample preparation

The dried powders (1 g) of HS were extracted by refluxing with 10 ml of methanol for 1 h, subsequently the extraction was repeated two additional times. The combined extract was filtered, and the filtrate was concentrated to a final volume of 10 ml using a rotary evaporator at 45 °C. The sample was passed through a 0.45 μm filter.

2.3. HPLC conditions

HPLC-MWD analysis was carried out on an Agilent 1200 series HPLC system (Agilent Technologies, USA), consisting of a G1376A Capillary Pump, a G1316A Thermostat Column Compartment and a G1367B autosampler. A Symmetry Shield TM RP18 column (250 mm × 4.6 mm i.d., 5 μm, Waters) was used for chromatographic separation, the column temperature was set at 30 °C. A linear gradient system consisted of mobile phase A (0.1% formic acid aqueous solution) and mobile phase B (acetonitrile containing 0.1% formic acid). The gradient elution profile was as follows: 0–10 min, 70% A; 10–12 min, 70–62% A; 12–22 min, 62% A; 22–25 min, 62–50% A; 25–28 min, 50–38% A; 28–40 min, 38% A. The flow rate was kept at 1.0 ml/min. The injection volume was 2 μl.

2.4. MS conditions

The HPLC system was coupled to an Daltonic micrOTOF-Q mass spectrometer equipped with electrospray ionization (ESI) interface (Bruker, Germany) in negative ion mode. The MS operating conditions were optimized as follows: the dry gas temperature was set at 240 °C, the flow rate was 6.0 l/min, the nebulizer pressure was set at 1.2 bar, the capillary voltage at −4.5 kV and collision energy at −10.0 eV. Quantification was obtained by using selected ion

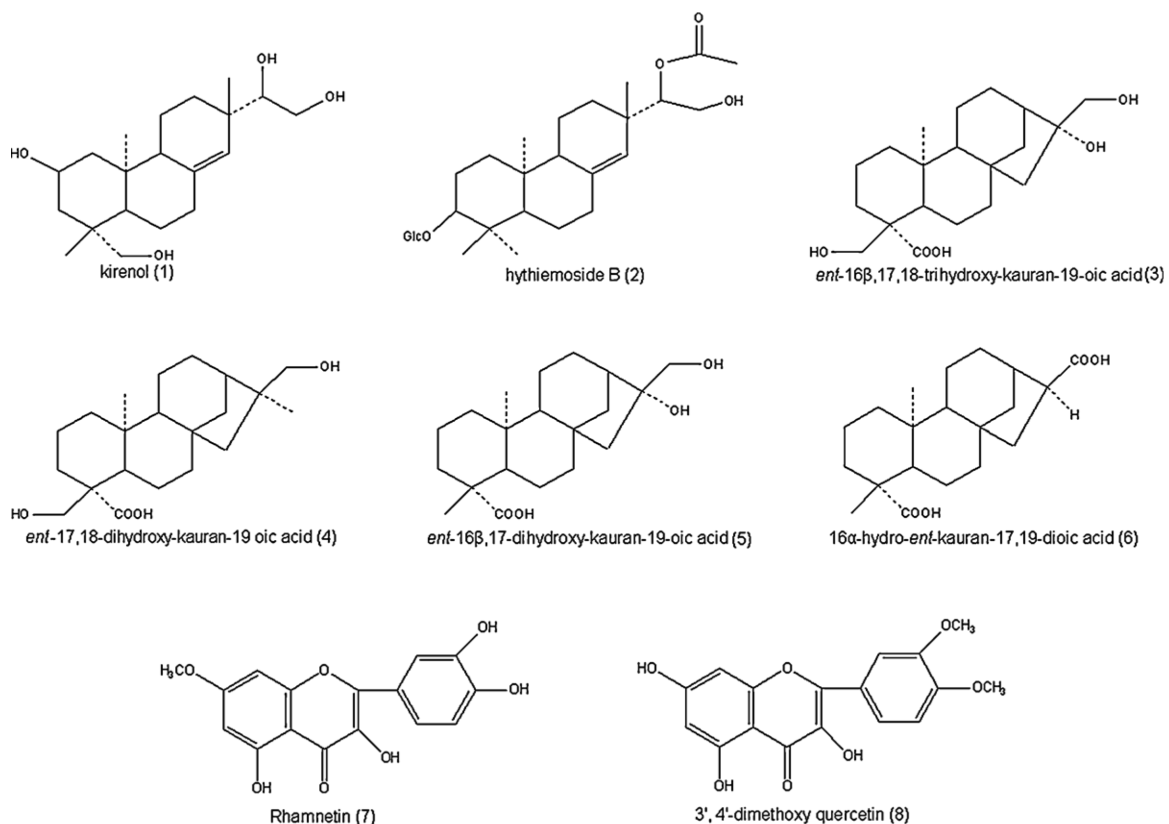

Fig. 1. Structures of compounds 1–8.

monitoring (SIM) mode with negative ion mode at m/z 373.21 for 1, 561.28 for 2, 351.21 for 3, 335.22 for 4 and 5, 333.21 for 6, 315.05 for 7 and 329.06 for 8. The data recorded was processed with Bruker Datonics Data Analysis software (Bruker, Germany).

2.5. Standard preparation and calibration curves

Stock standard solutions of eight compounds (1 mg/ml) were prepared in methanol respectively. Standard mixture solutions were prepared in methanol at various concentration levels in the range of 0.30–200 μg/ml. All solutions were filtered prior to analysis through a 0.45 μm syringe filter. The calibration curve for each compound was constructed by plotting the peak area versus the analyte concentration. The solutions were stored at 4 °C before use.

3. Results and discussion

3.1. Extraction procedure

In order to obtain quantitative extraction of the investigated compounds, the extraction method (ultrasonication, reflux), kind of solvent (50% ethanol–water, 80% ethanol–water, ethanol, methanol, and acetone), solvent volume (5, 10 and 20 ml), extraction time (30 min, 1 and 2 h) and extraction period (once, twice and three times) were optimized. The results showed that reflux extraction was more effective for extraction. Solvent volume should be over the medicinal material but not too much, so 10 ml solvent was appropriate. The influence of other extraction procedure is presented in Fig. 4. Based on the above investigation, the optimal condition for the extraction of HS was selected as 1.0 g dried powder sample to be extracted with 10 ml methanol under refluxing for three times (1 h per time).

3.2. Optimization of the chromatographic conditions

The selection of mobile phase was a critical factor in achieving good chromatographic behavior and appropriate ionization. The effectiveness of HPLC separation was tested using both the standard solution and the extract from HS. The effect of different mobile phase compositions on chromatographic separation was compared. Acetonitrile–water possessed better resolution and peak shape than methanol–water. Several mobile phase additives, such as formic acid, glacial acetic acid, ammonium formate and ammonium acetate, were used to achieve better resolution of the analytes. It was found that the good signal intensity, resolution and peak shape were achieved when formic acid was added to both acetonitrile and aqueous solution. But with the adding amount of formic acid increasing, the signal intensity had the downward trend. Ultimately, 0.1% formic acid added to mobile phase was suitable. To sum up the above arguments, we determined that the optimal mobile phase consisted of A (0.1% aqueous formic acid) and B (acetonitrile containing 0.1% formic acid).

The eight components were analyzed by MS in ESI negative ion mode. Positive ion mode was also tested, the sensitivity obtained was satisfactory for 1 and 2, but not for the others. Total ion chromatogram (TIC) in negative ion mode is shown in Fig. 2, it can be seen from Fig. 2 that the eight compositions cannot be completely separated. In recent years, more and more LC–MS^2 methods were reported for the determination of traditional Chinese medicine, but in this work the fragment ions of 1, 2, 4–6 were instable for the quantitative analysis. So the quantitative assay was carried out by SIM. In ESI negative ion mode $[M+Cl]^-$ for compounds 1 and 2 and $[M-H]^-$ for compounds 3–8 were considered better stability and higher abundance. The analytes were identified by comparing their retention time and accurate m/z data with standard solutions containing the corresponding compound. The typical chromatograms for the standard mixtures analyzed using the optimum chromatographic condition are shown in Fig. 3A.

3.3. Calibration curves, limits of detection and quantification

Calibration curves were constructed for each analyte by using a series of standard working solutions. Six concentrations for each analyte were analyzed for three consecutive days. The linear regression equation was obtained by plotting the peak areas versus the concentration of each analyte. The linearity of all calibration curves was determined by calculating the correlation coefficients. A summary of the calibration studies for the eight analytes is presented in Table 1.

The limit of detection (LOD), defined as the lowest detectable concentration of an analyte, was determined by serially dilution of the standard solution for each analyte to give a signal-to-noise ratio of about 3:1. The LOD for each analyte can be seen in Table 1. The limit of quantification (LOQ), defined as the lowest measurable analyte concentration, was achieved by serial dilution of the standard solution for each analyte to give a signal-to-noise ratio of about 10:1. The LOQ for each compound is shown in Table 1.

3.4. Method validation

Method precision was checked by intra-day and inter-day variability. The samples (A) were prepared as described in Section 2.2. For intra-day variability test, the samples were analyzed in six replicates for once a day, while for inter-day variability test, the samples were examined for consecutive 3 days. The RSD was taken as a measure of precision. From the results obtained, the present method was found to be acceptable precision and accuracy, with the intra-day variability RSD values between 0.85% and 3.22% and the inter-day variability RSD values between 1.10% and 3.68%.

A recovery study was performed to validate the accuracy of the developed method. The samples (0.5 g) of HS (A) were spiked with different concentration levels (50, 100 and 150%) of known amounts of the compounds 1–8. The spiked samples were extracted with 10 ml methanol following the procedure for sample preparation as described above. The recovery was determined by comparing the amount of analyte added to the sample and the amount of analyte detected during LC–TOF-MS analysis. The recoveries (Table 2) of the eight analytes were in the range of 97.4–101.2%, with RSDs less than 2.4%. The accuracy of the proposed method was therefore found to be sufficient for the determination of the eight compounds in the extract of the herb.

3.5. Matrix effect

Evaluation of the matrix effects on the results of quantitative determination of multiple components in TCMs is an important and often overlooked element. Matrix effects occur when matrix compounds co-eluting with the analytes alter the ionization efficiency of the electrospray interface. The postextraction addition is an effective method for providing favorable results even with variable matrices [18]. The sample of HS (A) was extracted as described in Section 2.2. Next, 5 ml of the extract was spiked with a one-fold mixed standard solution at three concentration levels (50, 100 and 150%), and another 5 ml of the extract was diluted one fold with methanol. Triplicate samples were prepared at each level. The matrix effect was calculated by the formula: matrix effect (%) = $(a - b)/c \times 100\%$, where a is the peak area of the analyte in

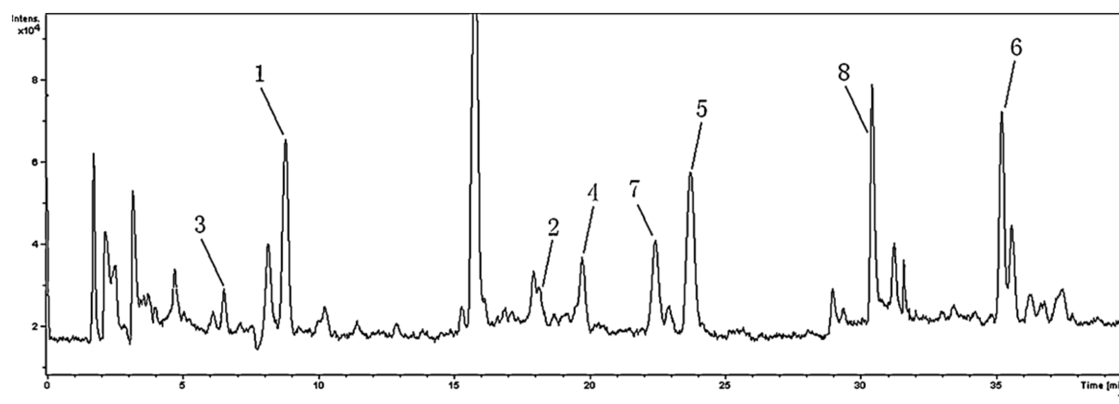

Fig. 2. Total ion chromatogram in negative ion mode of the exact of HS.

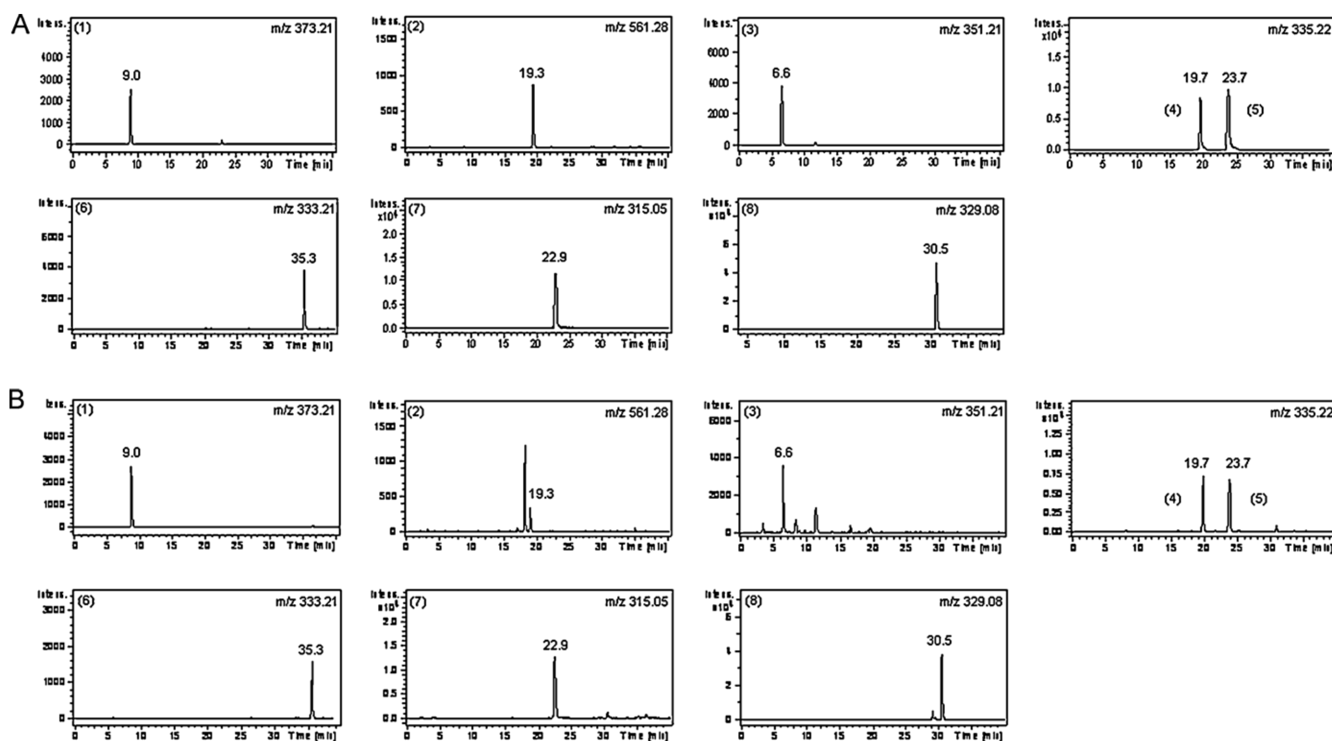

Fig. 3. Representative SIM chromatograms of standard solutions (A) and sample solutions (B).

the spiked sample matrix, b is the peak area of the analyte in the unspiked sample matrix and c is the peak area of the standard solution at the same concentration [19]. No matrix effect is observed when the matrix effect (%) is equal to 100%. In this study, the matrix effects of the eight compounds were in the range of 98.1–100.7%, indicating that no matrix effect was observed.

3.6. Sample analysis

The optimum conditions were applied to the determination of compounds 1–8 in HS from five different sources. The typical chromatograms obtained are shown in Fig. 3B. The quantitative analyses were performed by means of the external standard methods. The

Table 1
Calibration parameters of LC analysis for the 8 compounds of HS.

No.	Regression equation	Linear range (μg/ml)	r	Slope RSD (%)	Y-intercept RSD (%)	LODs (μg/ml)	LOQs (μg/ml)
1	$y = 141.18x + 70.761$	10–200	0.9999	1.7	1.8	3.3	8.0
2	$y = 1829.4x + 65.05$	0.50–10	0.9998	1.6	1.8	0.20	0.50
3	$y = 4343.9x - 24.581$	0.50–10	0.9999	1.1	1.2	0.07	0.20
4	$y = 3531.7x - 336.92$	3.0–60	0.9999	1.2	1.4	0.12	0.30
5	$y = 2499.5x - 364.7$	3.0–60	0.9998	1.2	1.5	0.20	0.50
6	$y = 4061.7x + 392.9$	3.0–60	0.9999	1.4	1.5	0.07	0.20
7	$y = 36{,}781x + 745.8$	0.50–10	0.9999	1.1	1.2	0.04	0.10
8	$y = 52{,}356x + 124.79$	0.30–6.0	1.0000	1.6	1.8	0.04	0.10

Note: The relationship between peak area and analyte concentration is expressed as linear regression lines ($y = ax + b$), where y is the peak area measured by TOF-MS, x is the concentration of the analytes. The correlation coefficient is r.

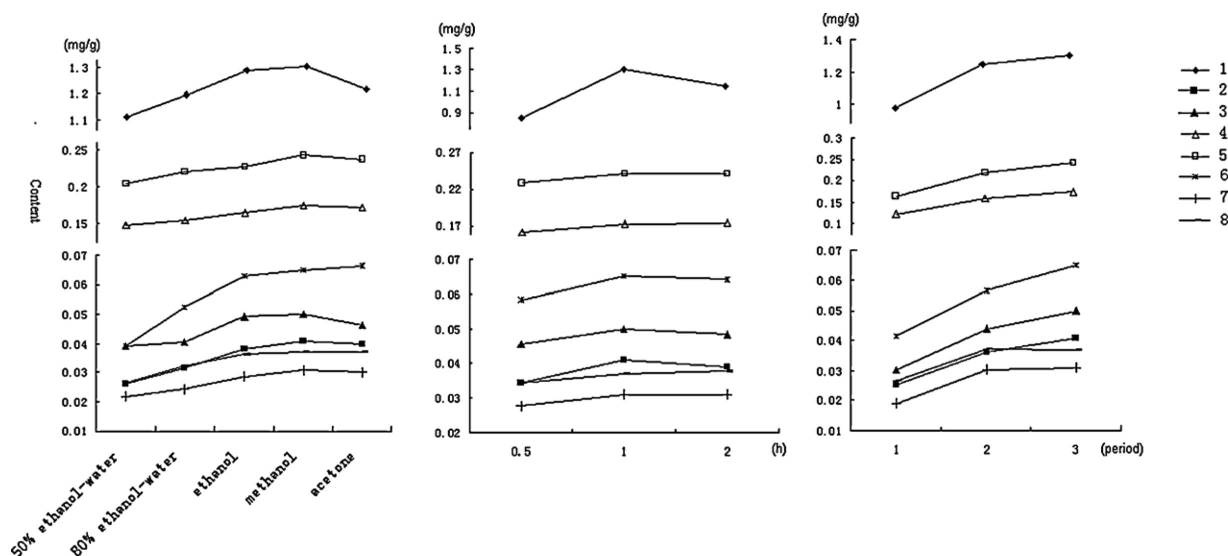

Fig. 4. Effect of extraction solvent (4-1), extraction time (4-2) and extraction period (4-3) on extraction efficiency of the eight compounds.

contents of the eight compounds in HS from different sources are listed in Table 3. The quantitative analytical results indicated that the variations of their contents were great. Compound 1 was the most prevalent component in samples A–C and E, compounds 2 and 3 were inexistence or not detected in sample of D, compounds 2, 3, 5 were inexistence or not detected in sample of E. Although the differences of the content between the samples from different sources were obvious, it is difficult to distinguish the sources of the

Table 2
Recoveries of the eight analytes of HS ($n = 3$).

Compounds	Original (mg)			Spiked (mg)	Found (mg)			Recovery (%)	RSD (%)
	1st	2nd	3rd		1st	2nd	3rd		
1	0.6606	0.6397	0.6539	0.3250	0.9783	0.9622	0.9801		
	0.6643	0.6432	0.6628	0.6500	1.3238	1.3021	1.3115	100.3	1.31
	0.6411	0.6580	0.6738	0.9750	1.6252	1.6360	1.6676		
2	0.0208	0.0201	0.0206	0.0104	0.0310	0.0304	0.0303		
	0.0209	0.0202	0.0209	0.0208	0.0412	0.0412	0.0412	98.3	2.36
	0.0201	0.0207	0.0212	0.0312	0.0516	0.0511	0.0522		
3	0.0254	0.0246	0.0251	0.0128	0.0379	0.0369	0.0378		
	0.0255	0.0247	0.0254	0.0265	0.0516	0.0514	0.0512	98.3	1.39
	0.0246	0.0253	0.0259	0.0385	0.0624	0.0636	0.0635		
4	0.0879	0.0850	0.0869	0.0434	0.1319	0.1297	0.1306		
	0.0883	0.0855	0.0881	0.0869	0.1743	0.1729	0.1749	101.2	1.34
	0.0852	0.0874	0.0895	0.1303	0.2171	0.2201	0.2240		
5	0.1228	0.1189	0.1215	0.0606	0.1822	0.1761	0.1809		
	0.1235	0.1195	0.1232	0.1211	0.2403	0.2398	0.2406	97.4	1.64
	0.1192	0.1223	0.1252	0.1816	0.2997	0.3007	0.3001		
6	0.0330	0.0319	0.0326	0.0163	0.0496	0.0475	0.0486		
	0.0332	0.0321	0.0331	0.0326	0.0656	0.0655	0.0662	100.0	2.16
	0.0320	0.0328	0.0336	0.0489	0.0803	0.0821	0.0831		
7	0.0157	0.0152	0.0156	0.0077	0.0232	0.0226	0.0234		
	0.0158	0.0153	0.0158	0.0154	0.0313	0.0305	0.0308	98.2	1.87
	0.0153	0.0157	0.0160	0.0231	0.0381	0.0379	0.0385		
8	0.0188	0.0182	0.0186	0.0092	0.0281	0.0275	0.0278		
	0.0189	0.0183	0.0188	0.0183	0.0373	0.0365	0.0372	99.9	1.24
	0.0182	0.0187	0.0191	0.0274	0.0452	0.0462	0.0458		

Table 3
The contents of eight compounds in HS from different sources ($n = 3$).

Sample number	1		2		3		4		5		6		7		8	
	Contents (mg/g)	RSD (%)	Contents (mg/g)	RSD (%)	Contents (mg/g)	RSD (%)	Contents (mg/g)	RSD (%)	Contents (mg/g)	RSD (%)	Contents (mg/g)	RSD (%)	Contents (mg/g)	RSD (%)	Contents (mg/g)	RSD (%)
A	1.302	0.53	0.041	1.20	0.050	1.50	0.173	0.38	0.242	0.35	0.065	1.70	0.031	0.47	0.037	0.84
B	1.084	0.74	0.052	1.00	0.050	1.70	0.166	0.56	0.259	1.40	0.062	1.30	0.055	0.54	0.030	0.54
C	0.521	0.66	0.040	0.93	0.013	2.00	0.067	0.68	0.093	0.98	0.056	1.00	0.006	1.20	0.004	2.20
D	0.482	0.34	[a]	–	[a]	–	0.429	1.20	0.050	0.78	0.520	0.69	0.087	0.49	0.034	0.99
E	0.567	0.47	[a]	–	[a]	–	0.116	0.39	[a]	–	0.230	0.88	0.007	0.93	0.019	1.10

[a] Trace or not detected.

samples. Generally, the variations were based on internal factors such as genetic variation and plant origin as well as external factors including seasonal, environmental factors, harvest time, and storage conditions.

4. Conclusions

A HPLC–TOF-MS method has been developed for the simultaneous determination of eight major diterpenes and flavonoids extracted from the aerial parts of HS for the first time. The validation data indicated that this method is reliable and can be applied to determine the contents of eight compounds in HS from different sources. This valuable information concerning the concentration of these pharmacologically active constituents in HS could be of great importance for the quality assessment and should therefore be useful for the guidance of clinical use. Also this HPLC–TOF-MS assay supplies a gap of simultaneous determination of multiple constituents in HS.

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