Research Paper

Simultaneous Quantification of Six Major Flavonoids From *Fructus sophorae* by LC-ESI-MS/MS and Statistical Analysis

L. CHANG, X. X. ZHANG, Y. P. REN, L. CAO, X. R. ZHI AND L. T. ZHANG*
Department of Pharmaceutical Analysis, School of Pharmacy, Hebei Medical University, Shijiazhuang, Hebei, PR China

A new, sensitive and selective high-performance liquid chromatography–tandem mass spectrometric method has been developed for the determination of six major flavonoids including sophoricoside, genistin, genistein, rutin, quercetin, kaempferol in *Fructus sophorae*. Principal component analysis and hierarchical clustering analysis were used to classify and differentiate these samples. Chromatographic separation was performed on a C_{18} column with linear gradient elution of methanol and 0.05% acetic acid (v/v) at a flow rate of 0.8 ml/min. The detection was accomplished in the negative mode using multiple-reaction monitoring. The total run time was 8.0 min. Full validation of the assay was carried out including linearity, precision, accuracy, recovery, limit of detection and limit of quantification. The validated method was successfully applied to the simultaneous determination of these active components in *Fructus sophorae*. The results demonstrated that the quantitative difference in content of six active compounds was useful for chemotaxonomy of many samples from different sources and the standardization and differentiation of many similar samples. Simultaneous quantification of bioactive components by high-performance liquid chromatography–tandem mass spectrometric method coupled with chemometric techniques would be a well-acceptable strategy to comprehensively control the quality of *Fructus sophorae*.

Key words: Flavonoids, *Fructus sophorae,* quantification, LC-MS/MS, principal components analysis, hierarchical clustering analysis

Traditional Chinese Medicine, commonly referred to as "Chinese Medicine" or simply "TCM", is one of the oldest forms of medical treatment and one of the most commonly used in the world. *Fructus sophorae* also known as HuaiJiao and *Styphnolobium japonicum* (L.) Schott (Leguminosae), is the dried ripe fruits and used as herbal ingredient used in TCM for its hemostatic properties[1,2]. Modern pharmacological and clinical studies have shown that some components in *Fructus sophorae* possessed antifertility action, hemostatic properties, anticancer, antitumor, antiobesity, antioxidation effects, and played important roles in the treatment of hypertension and hemorrhoids[1,3-5]. Nowadays, it has been more and more widely used in modern functional foods for improving health.

Pharmacological studies on *Fructus sophorae* have revealed that it contains flavonoids, alkaloids, terpenoids, amino acid, saccharide, phospholipids and others[3]. Specifically, flavonoids are the major active components. Sophoricoside, genistin, genistein, rutin, quercetin and kaempferol are the six main flavonoids that have been found to be active[1,3]. (fig. 1). Among these analytes, sophoricoside and genistin are structural isomers, and genistein is the hydrolysis product of them[6]. At present, many related drugs of *Fructus sophorae* such as Huai jiao tea, Huai jiao pills and Huai jiao capsules have already been produced and used in clinical treatment[7]. Therefore, a quality control of *Fructus sophorae* would be of great significance. On account of different sources and climatic conditions, its chemical constituents may vary substantially. Therefore, in order to further effectively utilize it and enhance the clinical safety, simultaneous quantitative analysis of active components is more reliable and accurate method for the quality control of traditional Chinese medicine.

Principal component analysis (PCA) is a useful statistical technique that has found application

***Address for correspondence**
E-mail: zhanglantong@263.net

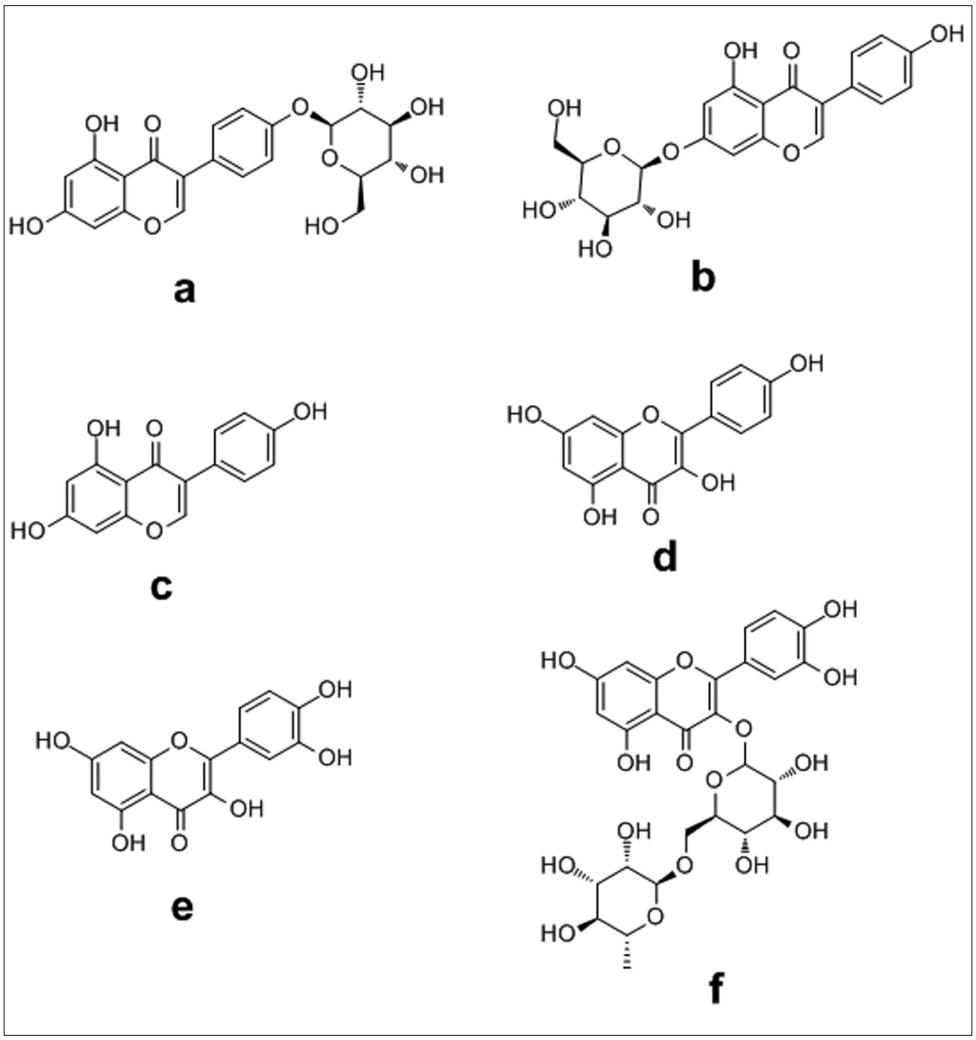

Fig. 1: The structures of the analytes.
The chemical structures (monitored transitions, declustering potential and collision energy) for (a) sophoricoside (431.1/267.9 amu, -85V, -43eV), (b) genistin (431.1/267.9, -72V, -38eV), (c) genistein (269.0/133.0 amu, -61V, -44eV), (d) kaempferol (284.9/93.0 amu, -70V, -52eV), (e) quercetin (301.0/150.9 amu, -55V, -32eV), (f) rutin (609.2/300.0 amu, -75V, -51eV).

in fields such as face recognition and image compression. It is a way of identifying patterns in data, and expressing the data in a way to highlight their similarities and differences[8]. Hierarchical clustering analysis (HCA) is a statistical method for finding relatively homogeneous clusters of cases based on measured characteristics[9,10]. PCA and HCA were performed according to the contents of *Fructus sophorae* to classify and differentiate the samples and to evaluate and control its quality better.

In present study, we firstly developed an accurate and simple high-performance liquid chromatography–tandem mass spectrometric method (HPLC–MS/MS) method for simultaneous determination of six major components in *Fructus sophorae*. In addition, for the isomeride pair sophoricoside and genistin with similar fragmentation pathway, the molecular polarities will be used to differentiate them from each other[11,12]. Thirty batches of *Fructus sophorae* from different sources were compared by HPLC–MS/MS combined with PCA and HCA to elucidate the difference among various samples in order to distinguish genuine medicinal material from other familiar species in different places.

MATERIALS AND METHODS

Fructus sophorae samples (No. 1-30) originated from different provinces were purchased from the local Chinese herb stores. All the voucher specimens were deposited in the Department of Pharmaceutical Analysis, Hebei Medical University. Methanol and acetic acid (HPLC-grade) were purchased from

Dikma Technologies Inc., Lake Forest, CA, USA. Purified water was obtained from Wahaha (Hangzhou Wahaha Group Co. Ltd., Hangzhou, China). Analytical grade dehydrated ethanol (Tianjin Chemical Corporation,Tianjin, China) were used for the sample preparation. Sophoricoside (11061521), genistin (11080316), genistein (11012521), kaempferol (11042524) were purchased from Shanghai Tauto Biotech Co., Ltd, China. Rutin (100080-200707) was obtained from National Institute for the Control of Pharmaceutical and Biological Products and quercetin was provided by the Department of Pharmaceutical Analysis, Hebei Medical University. The purities of the above ingredients were more than 98% according to LC analysis.

An Agilent 1200 liquid chromatography system (Agilent Technologies, USA) equipped with a quaternary solvent delivery system, an autosampler, and a column compartment was used for all experiments. Detection was performed using a 3200 QTRAP system from Applied Biosystems/ MDS Sciex (Applied Biosystems, USA), a hybrid triple quadrupole linear ion trap mass spectrometer equipped with Turbo V sources, and a Turbo Ion Spray interface.

HPLC–MS/MS conditions:

The chromatographic separation was performed on a Diamonsil C_{18} column (150×4.6 mm, 5 μm). A linear gradient elution of eluents A (methanol) and B (0.05% acetic acid) was used for the separation. The elution program was optimized as follows: 0-1.5 min, linear change from A-B (35:65, v/v) to A-B (75:25, v/v); 1.5-6 min, linear change from A-B (75:25, v/v) to A-B (95:5, v/v); and 6-8 min, isocratic elution A-B (95:5, v/v); The flow rate was 0.8 ml/min, the injection volume was 10 μl and the column temperature was maintained at 25º.

The ESI interface operated in the negative mode was used. The ion spray voltage was set to −4500 V, and the turbo spray temperature was kept at 650º. Nebulizer gas (gas 1) and heater gas (gas 2) was set at 60 and 65 arbitrary units, respectively. The curtain gas was kept at 25 arbitrary units and interface heater was on. Nitrogen was used in all cases. Multiple reaction monitoring (MRM) was employed for determination. The precursor-to-product ion pairs, declustering potential (DP) and collision energy (CE) for each analyte were given in legends to fig. 1. The dwell time of each ion pair was 60 milliseconds. Other parameters were also optimized for maximum abundance of the ion of interest by the automatic tuning procedure of the instrument. All data was controlled and synchronized by Analyst software (Versions 1.4.2) from Applied Biosystems/ MDS Sciex.

Preparation of standard solutions:

The standard stock solutions of genistein (0.92 mg/ml), rutin (1.03 mg/ml), quercetin (0.41 mg/ml), kaempferol (0.223 mg/ml) were prepared in methanol and sophoricoside (1.16 mg/ml) and genistin (1.01 mg/ml) were prepared in 50% methanol. They were stored away from light at 4º. Working solutions were prepared by appropriate dilution and admixture of the stock solution.

Sample preparation:

The dry plant samples were ground to powder by a pulverizer and sieved through a 45-mesh size before use. 0.2 g of powder was placed in 50 ml capped conical flask and extracted with 30 ml of 70% ethanol in an ultrasonic ice-water bath for 45 min. The extracted solution was adjusted to the original weight by adding 70% ethanol. Then the supernatant was diluted and filtered through a 0.45 um microporous membrane before LC injection of 10 μl.

Validation of the assay:

Calibration curves were constructed by plotting the peak area against the corresponding concentration of the standard solutions.[13] For each target constituent, limit of detection (LOD) and limit of quantification (LOQ) were determined by serial dilution of standard solution until the signal to noise ratio (S/N ratio) for each compound got to 3 and 10, respectively.

Both instrument precision and the method precision were tested. The instrument precision was evaluated by analysing the mixture solution at the concentration of 58.0 ng/ml for sophoricoside, 505 ng/ml for genistin, 69.0 ng/ml for genistein, 51.5 ng/ml for rutin, 123 ng/ml for quercetin, 11.5 ng/ml for kaempferol in six replicate injections under the optimal conditions. For the precision of the method, the intra-day precision was examined by analysing six replications prepared from sample 3 within one day, while the inter-day precision was determined over three consecutive days.

The recovery test was used to evaluate the accuracy of this method. Accurate amounts of 6 flavonoids (high, middle and low) were added to 0.1 g of *Fructus sophorae* (SX-3), and then extracted and analyzed as described above. The percentage recoveries were calculated according to the following equation: Recovery(%)=(observed amount-original amount)/added amount×100%, and RSD(%)=(SD/mean)×100%. In order to investigate the stability of the sample solutions, the sample solution prepared from sample 3 was stored at 4º and analyzed every 12 h within 48 h.

Peak identification:

Identification of each analyte is a prerequisite for successful quantification. For structural identification, the information-dependent acquisition (IDA) method was used to trigger the enhanced product ion (EPI) scans by analyzing MRM signals[14]. According to the comparison of retention time, parent and product ions with standards in MRM-IDA-EPI spectra, all the peaks of target compounds were unambiguously identified. The retention time of sophoricoside, genistin, genistein, rutin, quercetin and kaempferol is 4.59, 4.28, 5.56, 4.47, 5.27 and 5.86, respectively.

Statistics:

The effect of origin in *Fructus sophorae* on the total amount of those analytes was analyzed by PCA using SPSS (SPSS for Windows 13.0, SPSS Inc., USA) software[14,15]. The HCA of Samples 1-30 was performed using SPSS software. A method called Ward was applied and square Euclidean distance was selected as a measurement.

RESULTS AND DISCUSSION

Various extraction methods, solvents and times were evaluated to obtain the best extraction efficiency[16]. Ultrasonic bath extraction with the advantages such as convenience, rapidness and less solvent is a commonly used extraction method for quantitative analysis of traditional Chinese medicine[17]. We investigated different extraction methods, the results revealed that the ultrasonic bath extraction efficiency was similar to refluxing extraction. But the former was a more convenient method, so further experiments were carried out by ultrasonic bath extraction. Then we used different extraction solvent, including water, ethanol (40, 60, 70, 80 and 100%; v/v), and methanol. The results suggested that 70% ethanol displayed the highest extraction efficiency. We also considered the influence of extraction time. A comparative study on different extraction time of 30, 45 and 60 min was conducted at ambient conditions using ultrasonic bath extraction. The results showed that the 6 flavonoids were almost completely extracted within 45 min. So, extraction with 70% ethanol in an ultrasonic bath for 45 min was adopted.

First, for developing the method, the optimization of precursor ions and product ions of the analytes for MRM mode analysis by syringe pump infusion at a flow rate of 10 µl/min was a prerequisite for successful quantification[18]. The standard solutions of the analytes were infused into the mass spectrometer separately to obtain MS/MS fragment ions and to optimize mass parameters such as DP and CE. Then it was found that negative ESI could offer higher sensitivity and better peak reproducibility than positive ESI. In the full scan mass spectra, the deprotonated molecular ions $[M-H]^-$ of sophoricoside, genistin, genistein, rutin, quercetin, kaempferol (m/z, 431.1, 431.1, 269.0, 609.2, 301.0 and 284.9) were stable and exhibited higher abundance. Thus $[M-H]^-$ were chosen as the precursor ions for MS/MS fragmentation analysis. DP is one of the most important mass spectrometer parameters impacting ion response and was optimized in order to obtain the maximum sensitivity. In MS/MS analysis, only precursor ion was isolated and then dissociated into product ions. Several fragment ions of the analytes were observed in the product ion scan mode. Then the predominant fragment ions were chosen in MRM mode for quantification and the most suitable collision energy was also obtained by observing the maximum response for the MS/MS monitoring fragment ion.

Among the six flavonoids, sophoricoside and genistin are structural isomeride, and genistein is the hydrolysis product of them. So the selection of the LC conditions was the key in order to obtain chromatograms with better resolution of adjacent peaks, especially when similar components were analyzed. In view of achieving higher peak responses and shorter analysis time of target compounds in chromatograms, the effect of different mobile phase compositions was compared. There were no obvious differences between methanol-water and acetonitrile-water. Because of the high-toxicity and high-price of acetonitrile, methanol–water was chosen. Besides several mobile phase additives such as ammonium acetate (0.5, 1, and

2 mmol/l), formic acid (0.01, 0.05 and 0.1%) and acetic acid (0.01, 0.05 and 0.1%) were used to achieve the high sensitivity[14,17]. It was also found that eluent A (methanol) and B (0.05% v/v acetic acid) was beneficial for enhancing the ionization of compounds detected in negative electrospray interface mode and could guarantee sharp peak shape and reproducible retention time. When the flow rate was set at 0.8 ml/min, the resolution was greatly improved. Compared with previous reports, the total run time in our study was shorter and the 6 compounds could be separated within 8.0 min. And no significant peaks interfering with the analytes were observed. The typical extract ions chromatograms of standards and sample and the product ion scan spectra are shown in fig. 2.

The linearity, regression, and linear ranges of 6 analytes were determined using HPLC-MS method. Excellent linearity was achieved in these specified concentration ranges with the correlation coefficients higher than r≥0.9904. The LODs and LOQs for each compounds were less than 3.12 ng/ml and 12.5 ng/ml, which showed a high sensitivity. The detailed descriptions of the regression equation, the linear range, LOD and LOQ for each compound were shown in Table 1.

For the instrument precision, the RSD of the investigated components was less than 2.13%. The overall intra-and inter-day precisions (RSD) for the investigated components were less than 2.07 and 2.44%, respectively. The average recovery was

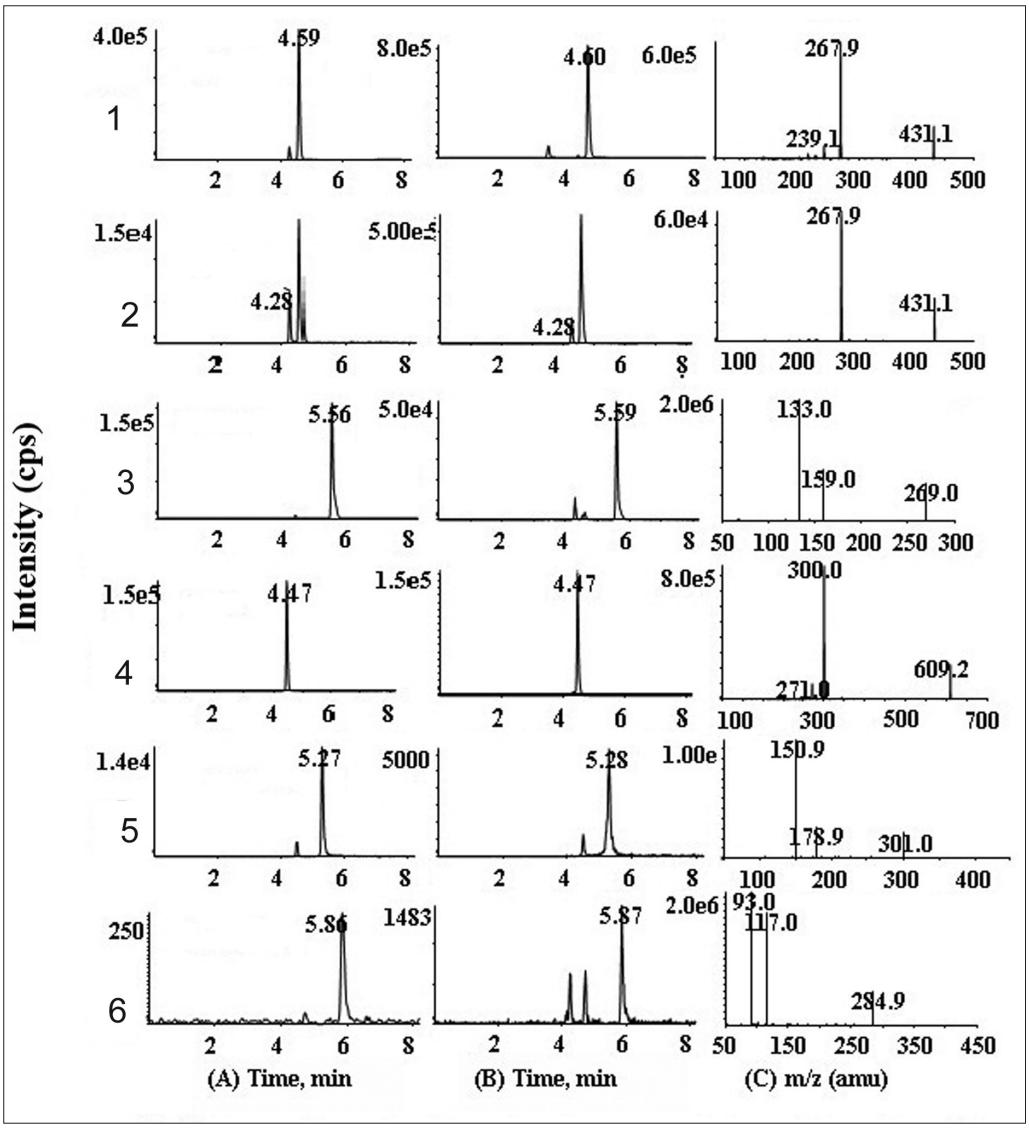

Fig. 2: Representative ions chromatograms.
Representative extract ions chromatograms of standard solution (A) and the *Fructus sophorae* sample (B), and the product ion scan spectra (C) for 1. sophoricoside, 2. genistin, 3. genistein, 4. rutin, 5. quercetin, 6. kaempferol.

in the range of 96.24-103.34% with RSD ranging from 0.92 to 2.83%. The results indicate that the method was accurate and reproducible (Table 2). All analytes were found to be stable with 48 h (RSD<2.30%) when the solution was stored at 4º (Table 2).

In this study, the established analytical method was applied to determine 6 flavonoids in the 30 batches of *Fructus sophorae* samples. The analytical results are shown in Table 3. The total contents of 6 analytes ranged from 84.971 to 153.781 mg/g in *Fructus sophorae*, which indicated that *Fructus sophorae* samples from various sources were obviously different. For *Fructus sophorae* samples, the samples bought from Shaanxi (mean content 128.690 mg/g) and Hebei (mean content 128.981 mg/g) Province contained more flavonoids than the samples from other places. In addition, sophoricoside was the highest component whose mean content was 78.969 mg/g in *Fructus sophorae*.

PCA is an unsupervised clustering method that does not require any knowledge of the data set and acts to reduce the dimensionality of multivariate data while preserving most of the variance therein[15]. In our study, we used PCA on the analytical data of all the 30 samples. First, two principal components PC_1 and PC_2 are used to provide a convenient visual aid for identifying inhomogeneity in the data sets. The specific contribution of analytes was studied by a loadings plot from PCA[14]. The factor loadings plot (fig. 3) helps us to determine what the components represent. Sophoricoside, genistin, genistein and kaempferol are most highly correlated as the first component, while the second component is most highly correlated with rutin and quercetin. Fig. 4 shows the principal component projection plot of PC_1 and PC_2 (over 80% of variance explained) for the 30 *Fructus sophorae* samples. From the results, we can classify these samples into four groups (indicated I to IV). For the PC_1 and PC_2, the samples from Shaanxi and Hebei are higher than others, but the samples from Shaanxi are more stable than the samples from

TABLE 1: REGRESSION DATA, LOD, AND LOQ FOR THE INVESTIGATED COMPOUNDS

Components	Regression equation[a]	Linear range (ng/ml)	r	LOD[b] (ng/mL)	LOQ[c] (ng/mL)
Sophoricoside	Y=1560X+31400	12.39-3716.64	0.9904	0.36	1.45
Genistin	Y=140X+9510	100.19-5009.60	0.9966	3.12	12.5
Genistein	Y=2650X+6160	1.47-147.20	0.9967	0.15	0.53
Rutin	Y=593X+31800	98.88-4944.00	0.9905	0.32	1.29
Quercetin	Y=2050X-106	3.28-328.00	0.9991	0.76	3.08
kaempferol	Y=643X+208	0.75-29.88	0.9992	0.29	0.68

[a]Y, peak area and X, concentration (ng/mL); [b]LOD (S/N=3). [c]LOQ (S/N=10), LOD=Limit of detection, LOQ=Limit of quantification

TABLE 2: INTRA- AND INTER-ASSAY, ACCURACY AND STABILITY OF THE SIX ACTIVE COMPONENTS

Compounds	Precision (*n*=6)		Accuracy (*n*=6)					Stability
	Intra-day RSD%	Inter-day RSD%	Original (µg)	Spiked (µg)	Found (µg)	Recovery (%)[a]	RSD% (*n*=6)[b]	(48h, *n*=3) RSD(%)
Sophoricoside	1.45	1.83	10184.20	5121.50	15143.35	96.83	2.34	1.56
				10243.00	20238.73	98.16	1.29	
				20486.00	30213.36	97.77	1.83	
Genistin	1.97	2.31	1416.70	700.00	2103.89	98.17	2.03	2.21
				1400.00	2776.52	97.13	1.22	
				2800.00	4276.62	102.14	1.38	
Genistein	2.07	2.44	282.50	149.20	427.60	97.25	1.64	2.30
				298.40	588.45	102.53	2.83	
				596.80	856.86	96.24	0.92	
Rutin	1.37	1.85	2395.80	1155.75	3541.96	99.17	1.26	1.34
				2311.50	4763.01	102.41	1.14	
				4623.00	6905.54	97.55	2.53	
Quercetin	0.96	1.74	20.30	10.00	29.94	96.39	1.37	1.22
				20.00	39.78	97.41	1.53	
				40.00	60.72	101.04	1.76	
Kaempferol	1.05	1.88	29.60	15.30	45.09	101.26	2.42	1.83
				30.60	59.67	98.28	1.93	
				61.20	92.84	103.34	1.67	

[a]Recovery (%)=(Observed amount-original amount)/added amount×100; [b]RSD(%)=(SD/mean)×100

Hebei when total content are similar. These samples are different from each other from Hebei. fig. 4 indicates that origin is highly related to the quality of products. Apparently, different places were essential reasons resulting in the obvious variation of the compounds.

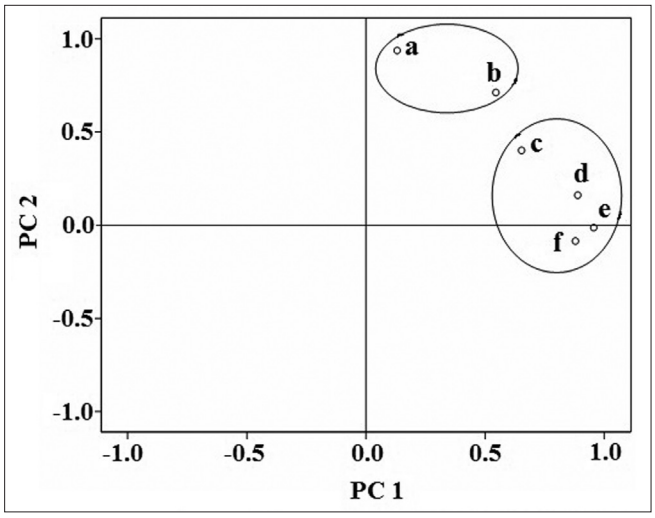

Fig. 3: Loadings plot of PC1 against PC2.
Loadings plot of PC1 against PC2 for the six components of *Fructus sophorae*. (a) rutin, (b) quercetin, (c) genistin, (d) kaempferol, (e) genistein, (f) sophoricoside.

HCA was performed on the analytical data of all the 30 samples. The clustering results illustrated as a dendrogram are presented in fig. 5. Thirty tested *Fructus sophorae* samples could be successfully grouped into two main clusters or domains (A and B) by using Ward method. All samples from Shaanxi (1-6) and part samples from Hebei (7, 8, 10) were in cluster A and the other samples were in cluster B, which was further divided into two subgroups (B1 and B2). All samples from Henan (13-18) and Anhui (22-24) were in subgroup B1 (B1-1). In PCA, all samples from Shaanxi were in group I; all samples from Henan and Anhui were in group II. The results were consistent for PCA and HCA.

TABLE 3: CONTENT OF THE SIX ACTIVE COMPONENTS IN *FRUCTUS SOPHORAE* (*n*=3)

Code	Source	Content (mg/g)						
		Sophoricoside	Genistin	Genistein	Rutin	Quercetin	Kaempferol	Sum
SX-1	Shaanxi	86.275[a]	9.421	2.573	20.654	0.346	0.217	119.486
SX-2	Shaanxi	87.316	13.307	2.976	21.564	0.329	0.257	125.749
SX-3	Shaanxi	101.842	14.167	2.825	23.958	0.203	0.296	143.291
SX-4	Shaanxi	96.113	10.542	3.079	29.515	0.161	0.264	139.674
SX-5	Shaanxi	82.574	10.426	1.986	26.327	0.432	0.301	122.046
SX-6	Shaanxi	88.626	10.642	2.053	19.654	0.543	0.376	121.894
HB-1	Hebei-baoding	96.875	15.521	3.506	36.806	0.684	0.389	153.781
HB-2	Hebei-baoding	87.326	10.507	4.070	16.074	0.414	0.418	118.809
HB-3	Hebei-baoding	70.626	13.394	0.748	49.056	0.452	0.086	134.362
HB-4	Hebei-shijiazhuang	90.626	13.395	3.748	32.055	0.452	0.385	140.661
HB-5	Hebei-shijiazhuang	73.853	10.961	1.172	23.538	0.203	0.302	110.029
HB-6	Hebei-shijiazhuang	72.765	8.694	1.078	33.256	0.345	0.108	116.246
HN-1	Henan-zhengzhou	87.153	13.680	1.476	19.653	0.087	0.089	122.138
HN-2	Henan-zhengzhou	83.515	10.014	1.601	9.772	0.056	0.102	105.060
HN-3	Henan-zhengzhou	75.467	9.274	1.362	15.265	0.078	0.089	101.535
HN-4	Henan-nanyang	82.435	9.466	1.554	9.955	0.072	0.107	103.589
HN-5	Henan-nanyang	67.983	8.456	1.253	10.055	0.122	0.099	87.968
HN-6	Henan-nanyang	86.457	9.076	1.062	20.732	0.118	0.083	117.528
SD-1	Shandong	72.634	10.014	1.246	22.473	0.273	0.291	106.931
SD-2	Shandong	72.222	7.014	0.314	18.646	0.057	0.040	98.293
SD-3	Shandong	70.626	13.395	0.748	29.056	0.452	0.086	114.363
AH-1	Anhui	81.038	9.780	1.024	19.107	0.106	0.063	111.118
AH-2	Anhui	72.325	8.735	1.082	14.237	0.103	0.096	96.578
AH-3	Anhui	84.846	8.873	0.906	22.217	0.095	0.103	117.040
LN-1	Liaoning	58.975	9.421	0.568	20.946	0.067	0.068	90.045
LN-2	Liaoning	60.623	8.056	0.601	15.543	0.094	0.054	84.971
GS-1	Gansu	66.562	10.244	0.903	19.386	0.075	0.101	97.271
GS-2	Gansu	68.642	9.738	0.879	20.943	0.086	0.064	100.352
GD-1	Guangdong	73.372	5.961	0.756	26.180	0.292	0.083	106.644
GD-2	Guangdong	69.373	5.961	0.757	26.180	0.293	0.083	102.647

[a]Average of duplicates

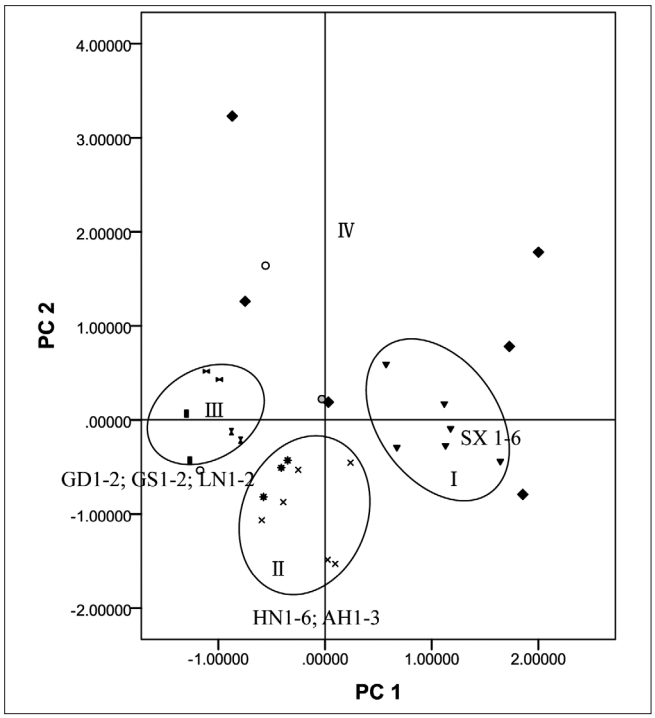

Fig. 4: Principal components analysis scores plot.
Principal components analysis scores plot for the 30 tested samples of *Fructus sophorae*. AH-1 to 3 (✳), GD-1 and 2 (⋈), GS-1 and 2 (⧗), HB-1 to 6 (◆), HN-1 to 6 (×), LN-1 and 2 (▮), SD-1 (⊙), SD-2 and 3 (○), SX-1 to 6 (▼)

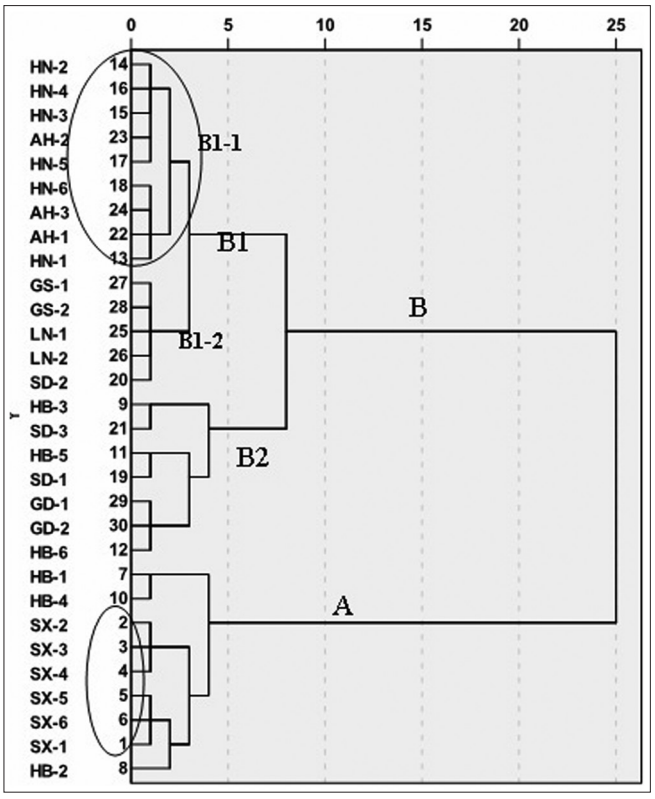

Fig. 5: Dendrograms of hierarchical cluster analysis.
Dendrograms of hierarchical cluster analysis for the 30 tested samples of *Fructus sophorae* using Ward method.

Genuine medicinal materials are generally produced in specific regions and with long history, cultivated and processed by skillful techniques and shows excellent quality and evidently curative effects[19]. The genuine place of *Fructus sophorae* is Shaanxi. The samples from Shaanxi are higher and more stable in content. The genuine medicinal materials (Shaanxi) could be distinguished from other general samples. The PCA and HCA further confirmed the excellent quality of genuine medicinal materials.

In this study, a selective, rapid and sensitive HPLC–MS/MS method has been developed and validated to quantify six constituents in *Fructus sophorae*. And we accomplished the separation of isomeride pair sophoricoside and genistin as well as their aglycone (genistein) for the first time. According to quantitative analysis, PCA and HCA, the quality origined from Shaanxi province (genuine medicinal materials) were the best and the most stable. The proposed method in this paper is particularly suitable for the routine analysis of *Fructus sophorae* and its quality control.

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