Journal of Chromatographic Science 2013;51:99–106
doi:10.1093/chromsci/bms111 Advance Access publication July 23, 2012

Article

High-Performance Liquid Chromatography–Electrospray Ionization Time-of-Flight Mass Spectrometry Analysis of *Radix Saposhnikoviae* for Metabolomic Research

Yue-Yue Li[1†], Xin-Xia Wang[1†], Liang Zhao[1], Hai Zhang[1], Lei Lv[1], Gui-chen Zhou[1], Yi-Feng Chai[2] and Guo-Qing Zhang[1*]

[1]Department of Pharmacy, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, P. R. China, and [2]Department of Pharmaceutical Analysis, School of Pharmacy, Second Military Medical University, Shanghai, P. R. China

*Author to whom correspondence should be addressed. Email: gqzhang@smmu.edu.cn
†Both authors contributed to this work equally.

Received 13 February 2012; revised 27 April 2012

In this study, metabolite profiling of *Radix Saposhnikoviae* from different geographical locations was performed using high-performance liquid chromatography–electrospray ionization time-of-flight mass spectrometry (HPLC–ESI-TOFMS) and multivariate statistical analysis technique. Principle component analysis (PCA) of the data shows that these samples could be roughly separated into three groups: Guan Fangfeng, Kou Fangfeng and Chuan Fangfeng. The potential chemical markers were discovered through the loading plot of PCA. Based on accurate mass measurements and subsequent fragment ions of TOFMS after in-source collision induced dissociation, as well as matching of empirical molecular formulae with those of published components in the in-house chemical library, 10 potential markers, such as 4′-*O*-glucosyl-5-*O*-methylvisamminol, cimifugin, prim-*O*-glucosylcimifugin and 3′-*O*-angeloylhammaudol, were tentatively identified and partially verified by the available reference standards. The results of this study indicate that it is an effective and novel approach to identify traditional Chinese medicine (TCM) from different sources, and that performing quantity determination of corresponding marker compounds could optimize the quality control of TCM.

Introduction

Radix Saposhnikoviae (RS), which is called Fang feng in Chinese, refers to the dried roots of *Saposhnikovia divaricata* (Turcz.) Schischk. As a traditional Chinese medicine (TCM), RS has been widely utilized for the treatment of pyrexia, rheumatism, headache and convulsion over thousands of years of Chinese clinical practice (1–3). Phytochemical studies show that abundant compounds exist in the roots of *S. divaricata*, such as coumarins, chromones, alkapolyalkynes and polysaccharides, among which coumarins and chromones are the primary components of RS (4–7). It has been reported that the quality of RS from different areas is quite different according to their different growth environment, such as culture condition, climate, soil or sowing and process methods (8–13). Currently, RS's qualities are being assessed by determining the concentration of several pharmacological active constituents such as 4′-*O*-glucosyl-5-*O*-methylvisamminol, prim-*O*-glucosylcimifugin, cimifugin and sec-*O*-β-D-glucosylhammauol (14–23); it is difficult to identify the geographical origin of RS utilizing the existing analytical methods. The curative effects of TCM are principally based on the synergic effects of their multi-targeting, multi-ingredient preparations, in contrast to modern pharmacology and drug development that often focus on a single chemical entity (24). Therefore, the method, employing a few markers or pharmacologically active constituents to assess the quality and authenticity of the complex preparations, has a number of severe challenges.

Metabolomics is an interdisciplinary tool that includes a quantitative exhaustive profiling of all metabolites contained in a target organism through the use of high throughput instruments (25–31), such as high-performance liquid chromatography–electrospray ionization time-of-flight mass spectrometry (HPLC–ESI-TOFMS). HPLC–ESI-TOFMS is a feasible method for establishing metabolic profiling of TCM for its inherent characteristics of accurate mass measurements and high resolution. Moreover, its high sensitivity ensures that the components of minor amounts will not be excluded, ensuring a detailed and complete metabolic profiling. Metabolomics enables the sample classification of diverse biological status, origin or quality in samples, by means of chemometrics, such as principal component analysis (PCA). Xie (32) applied ultra-performance liquid chromatography (UPLC)–QTOFMS to perform the metabolite profiling of five medicinal Panax herbs; Xie differed them by PCA, through the loading plot of which chemical markers were further identified.

In this study, a rapid and effective method was developed for characterization of the multiple constituents in RS by HPLC–ESI-TOFMS, and then multivariate statistical analysis methods were used to analyze samples, finding out chemical markers for differentiation of RS from different areas. It is the first time that HPLC–ESI-TOFMS was applied in combination with chemometrics methods for the analysis of RS from different origins, which optimizes the quality control method for RS.

Experimental

Plant materials and reagent

Thirty-eight samples of RS were collected in producing areas from eight provinces (Table I), including the species of Guan Fangfeng, Kou Fangfeng and Chuan Fangfeng. All samples were authenticated by Professor Baokang Huang from the Department of Parmacognosy, the Second Military Medical University (Shanghai, China). The dried root was crashed into flocci with a pulverizer. Methanol (Fisher, Waltham, MA) and formic acid (Tedia, Fairfield, OH) were HPLC grade. Ultrapure water was used for all analyses. Other chemicals were of analytical grade from Sinophar Chemical Reagent Co. Standards of 4′-*O*-glucosyl-5-*O*-methylvisamminol, prim-*O*-glucosylcimifugin, cimifugin and sec-*O*-D-glucosylhammaudol (all purity > 98%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Table I
Sample List

Sample	Sample	Province of collection
1–5	Guan Fangfeng	Heilongjiang
6–10		Jilin
11–16		Neimenggu
17–20	Kou Fangfeng	Hebei
21–23		Gansu
24–26		Shanxi
27, 28		Anhui
29–38	Chuan Fangfeng	Sichuan

Sample preparation

Pulverized powder of RS (0.15 g) was weighed accurately and extracted with 20 mL methanol at the reflux temperature for 2 h in a water bath. After cooling to room temperature, extra methanol was added to account for the loss of methanol through evaporation during the extraction, and then the supernatant was filtrated through a syringe filter (nylon, 0.22 μm).

HPLC conditions

HPLC analysis was performed on an Agilent 1200 Series (Agilent, Waldbronn, Germany) equipped with a binary pump, micro degasser, well-plate autosampler and thermostated column compartment. The chromatographic separation was performed on an Agilent Zorbax Extend-C18 (5 μm, 250 ×4.6 mm i.d.) column. The mobile phases consisted of solvent A (0.1% formic acid in water, v/v) and solvent B (0.1% formic acid in methanol, v/v). The optimized HPLC elution conditions were: 0–5 min, 5–40% B; 5–13 min, 40–60% B; 13–23 min, 60–85% B; 23–33 min, 85–100% B. The analysis time was 35 min. The column temperature was room temperature, the flow rate was 1 mL/min, split ratio was 1:3 and injection volume was 10 μL.

ESI-TOFMS conditions

Mass spectrometry was performed on an Agilent 6220 TOFMS operating in positive-ion mode. The capillary and skimmer voltages were set to 4,000 and 60 V, respectively. The drying gas (350°C, 8 L/min) and nebulizing gas (35 psig) was nitrogen. MS spectra were acquired in full scan analysis over an m/z range of 100–1,000 using extended dynamic range and stored in centroid mode. The fragmentor voltages of 180 and 350 V were chosen for metabolic fingerprint acquisition and identification of potential chemical markers, respectively. To maintain mass accuracy during the run time, a reference mass solution containing reference ions at m/z 121.0509 and 922.0098 was used in positive ionization mode. The operating software was MassHunter Workstation Software (version B.02.00).

Evaluation of instrument stability and method reproducibility

A mixed sample, which was prepared by blending aliquot 38 samples together, represented the average status of the RS samples. This sample was used during HPLC–ESI-TOFMS method development and validation and in the actual analysis, during which it was injected once after every five extracted samples to monitor the retention time variation and mass accuracy (a total of eight times). After this, the Masshunter data (MHD) files of the chromatograms were analyzed by Mass Profiler Software. The statistical analysis result of repeated sampling data reflects the method reliability and operation status of equipment.

Blank solvent (methanol) was injected between analyses to monitor inter-sample cross-over effect.

Establishment of composition database and peak assignment

To screen the reported chemical constituents in RS, all components reported in the literatures on RS were summarized in a Microsoft Office Excel table to establish a database in which the formula and the exact molecular weight of each known chemical compound were calculated. We matched the empirical molecular formula with that of published known compounds in the database. The empirical molecular formula was deduced and shortlisted by comparing the accurately measured mass value $[M + H]^+$ or $[M + Na]^+$ to the exact mass value of the putative deprotonated molecular ions at the mass accuracy of less than 3 ppm.

Multivariate statistical analysis

We obtained the total ion chromatograms (TIC) from HPLC–ESI-TOFMS in Agilent Qualitative Analysis software, and extracted the components of each TIC as MHD files for further analysis. Then we arranged all samples' components into one large matrix, which included the common and specific components among different samples with corresponding retention time, molecular weight and peak area. If a compound was not detected in the sample, the ion intensity was documented as 0.001 in the final data table. We aligned the retention time, mass of compounds and the normalization of peak area, and each chromatography dataset was normalized to the total sum of the integrals to partially compensate for differences in concentrations.

The two-dimensional matrix was imported to SIMCA-P software 11.0 (Umetrics, Umea, Sweden) for PCA. PCA can visualize the differences existing within the data while identifying the significant variables that influence the discrimination in quality (33). The data sets were mean-centered and par-scaled beforePCA (34, 35). According to the score plots of PCA, we could analyze the first few principal components that contain the primary information, observing the classification of samples. The loading plots of PCA provide intuitional perspective of the ingredients' distribution and reveal which play the primary role in the classification of samples.

Univariate data analyses

Important chemical markers that contributed significantly to the clustering of observations were further subjected to univariate data analyses. Statistical differences between classes were compared against the Guan Fangfeng group using repeated measure for one-way analysis of variance (ANOVA). $P < 0.05$ was considered to be statistically significant.

Results and Discussion

Development of the extraction method

To improve the efficiency of extraction, we tried extraction methods that included direct ultrasonic extraction, soaking overnight followed by ultrasonic extraction and reflux extraction in a water bath. Finally, we chose reflux as the extraction method because it could extract the maximal amount of ingredients in RS. The related parameters in the extraction procedure such as the solvent, time and repeated times were also optimized. Methanol was the best extraction solvent among methanol, ethanol and 70% ethanol, which allowed for the maximal value of peaks on the chromatogram. The crude flocculent sample was extracted with methanol for 30 min, 1 h and 2 h, one or two times. It was found that the method of highest yield of extraction was reflux in a water bath for 2 h.

Development of HPLC–ESI-TOFMS method

Optimization of HPLC method

Several columns from Agilent technologies including Zorbax Extend-C_{18} (5 μm, 250 × 4.6 mm i.d.), Zorbax Eclipse XDB-C_{18} (5 μm, 150 × 4.6 mm i.d.), Zorbax SB-C_{18} (3.5 μm, 100 × 3.0 mm i.d.) and Zorbax Eclipse XDB-C_{18} (1.8 μm, 50 × 3.0 mm i.d.) were tested in the study. The gradient program and the flow rate were translated to corresponding equivalent conditions by Method Translator software from Agilent Technologies. The result showed that the best resolution and peak shape of the constituents were achieved on the Zorbax Extend-C18 column (5 μm, 250 × 4.6 mm i.d.). To get a better peak signal response, the acid modifier, formic acid, was added to the mobile phase and the effect of different concentrations was tested (0.1%, 0.05% and 0.01%), and then 0.1% HCOOH was chosen because of its best performance.

Optimization of mass condition

Positive electrospray ionization (ESI^+) was selected as the ionization mode for the TOFMS experiments because the metabolites were detected with greater ion intensities using this ionization mode, and hence this mode provided richer information on the metabolites. Furthermore, no metabolites ionized only in the negative polarity. TIC of several typical samples from different regions are displayed in Figure 1. Through visual observation, we could see the significant differences in the number of peaks and peak abundance among the samples.

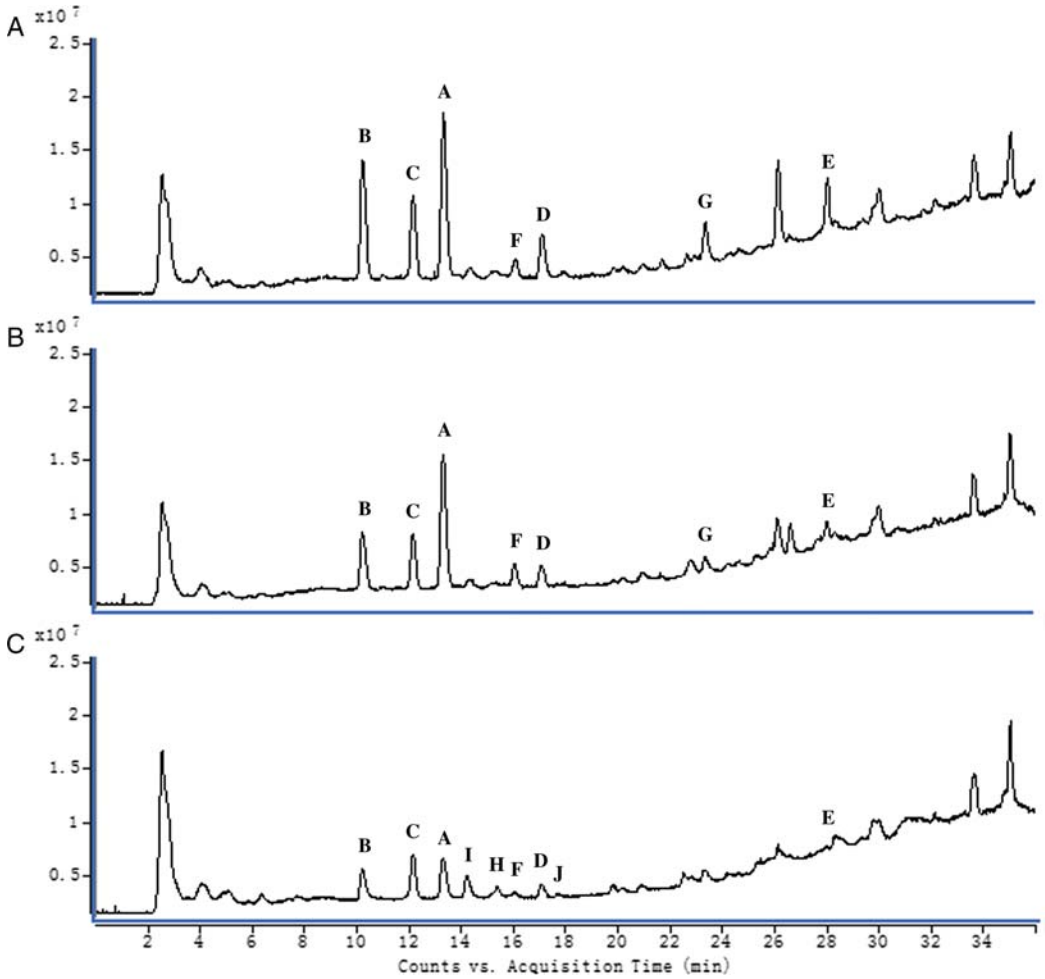

Figure 1. HPLC–ESI-TOFMS TIC chromatograms of RS from different provinces: Heilongjiang (A); Hebei (B); Sichuan (C). The marker ions were labeled A–J at peak apex and were sorted according to their importance for separating samples.

Stability of instrument and reproducibility of methods

The analysis results of repeated injection of the mixed sample showed that a total of 95 compounds were extracted from it and 89% percent of these existed in at least six chromatograms. The compounds with higher abundance could be extracted from all eight chromatograms. The standard deviation (SD) value of the retention time (RT) was less than 0.16%, showing that the reproducibility of the RT was excellent in the system. The relative standard deviation (RSD) value of the peak area was less than 1.59%, illustrating that the precision and reproducibility of peak integration were acceptable. The SD value of molecular weight was less than 0.0025, showing the great accuracy of the mass measurement, which enabled more accurate and reliable matching of compounds. Overall, the method was reliable and could provide accurate statistical analytic data.

Multivariate statistical analysis and characteristic markers exploring

To compare the difference between RS from different locations, an unsupervised pattern recognition method, PCA, was performed. PCA uses an *N*-dimensional vector approach to separate samples on the basis of the cumulative correlation of all metabolite data and then identifies the vector (eigenvector) that yields the greatest separation among samples without requiring prior knowledge of the data sets (36). After Pareto scaling with mean-centering, a two-component PCA model was established and cumulatively explained 54.8% of data variation. As shown in Figure 2A, the score plot of the first two principal components demonstrated that the determined samples clearly clustered into three groups including Guan Fangfeng, Kou Fangfeng and Chuan Fangfeng, indicating that RS from different locations are significantly different in the content of ingredients.

To find the potential chemical markers for the discrimination between different kinds of RS, we analyzed the relationship between categories and possible marker compounds by contrasting the score plot with the loading plot (Figure 2B), because they had a direct geometric link. For example, the diamond points that were primarily located in the fourth quadrant of the score plot represented samples of Guan Fangfeng; accordingly, in the loading plot, the marked points at the same place that were far from the origin of coordinate contribute more to the differences among Guan Fangfeng and others. Separately, these were Compounds A (RT 13.39 min, m/z 453.1757), B (RT 10.29 min, m/z 469.1710), C (RT 12.17 min, m/z 307.1182), D (RT 17.18 min, m/z 439.1605), E (RT

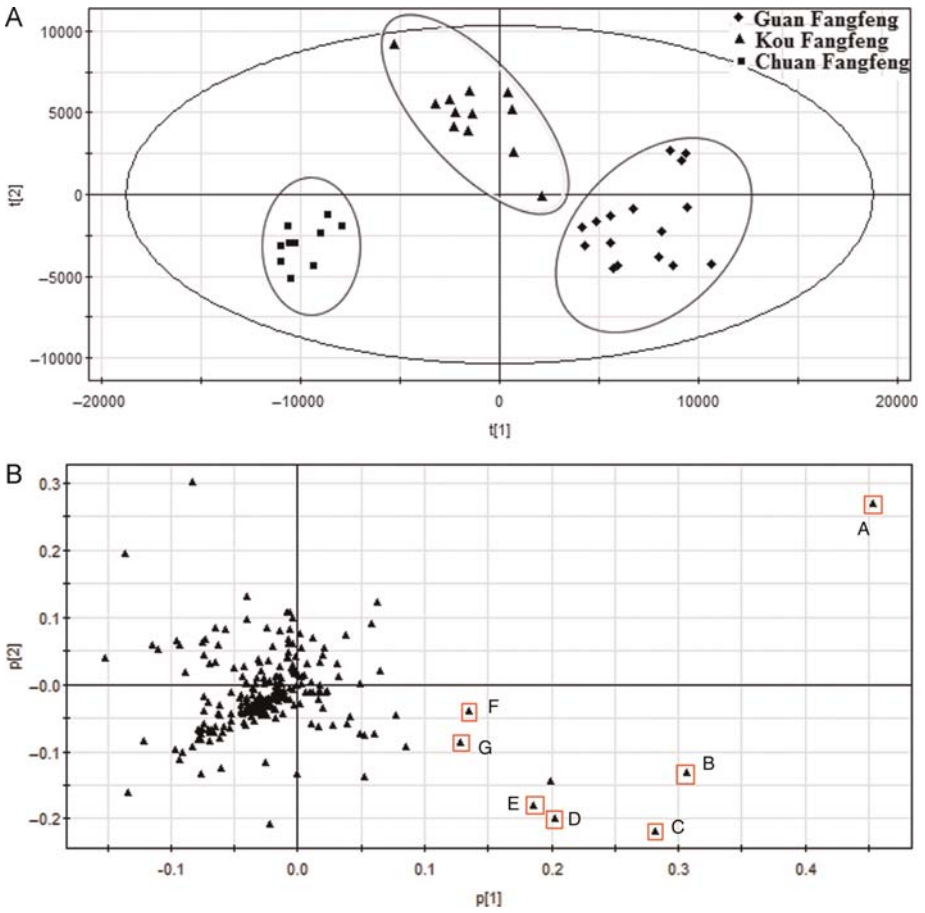

Figure 2. PCA plots of sample analysis: PCA scores plot, PC1 versus PC2 (A); PCA loading plot, PC1 versus PC2 (B). The points marked with letters represent potential chemical markers of Guan Fangfeng. The dots marked with diamonds, triangles and squares represent Guan Fangfeng, Kou Fangfeng and Chuan Fangfeng samples, respectively in Figure 2A.

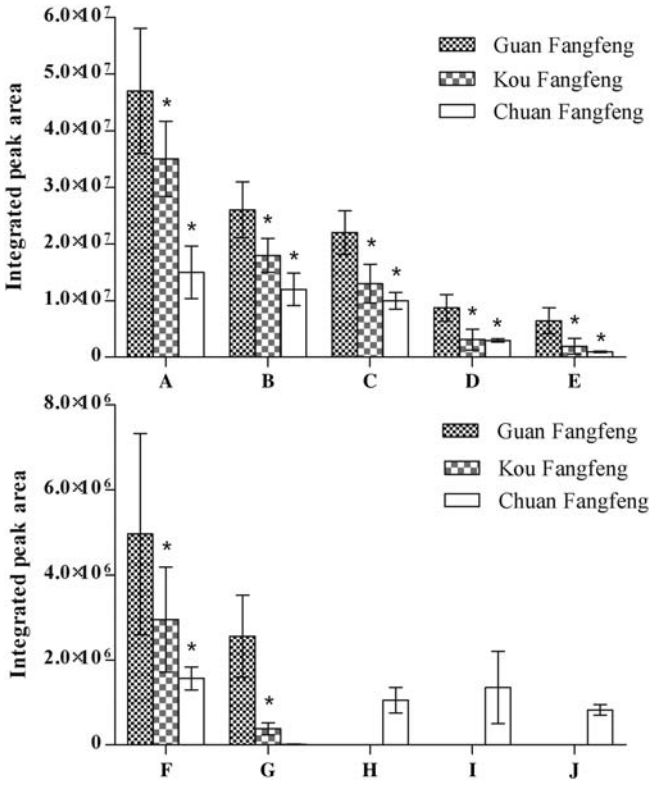

Figure 3. Integrated ion intensity plots of the potential marker ions: A (RT 13.39 min, m/z 453.1757), B (RT 10.29 min, m/z 469.1710), C (RT 12.17 min, m/z 307.1182), D (RT 17.18 min, m/z 439.1605), E (RT 28.11 min, m/z 359.1495), F (RT 16.12 min, m/z 291.1230), G (RT 23.43 min, m/z 329.1387), H (RT 15.51 min, m/z 187.0390), I (RT 14.28 min, m/z 247.0967) and J (RT 17.81 min, m/z 217.0496) against the three groups ($p < 0.05$ when compared to the Guan Fangfeng group).

28.11 min, m/z 359.1495), F (RT 16.12 min, m/z 291.1230) and G (RT 23.43 min, m/z 329.1387). After this, we examined the integrated ion intensity plots of the potential chemical markers, which are shown in Figure 3 (all values were expressed as mean ± SD among different groups). As shown in Figure 3, Compound A (RT 13.39 min, m/z 453.1757) had a relatively high content in Guan Fangfeng and Kou Fangfeng, but a very small content in Chuan Fangfeng, because it was located in the corner of the first quadrant in the loading plot, which was the opposite to the third quadrant where Chuan Fangfeng placed. Compounds B–G were all detectable with high intensity in Guan Fangfeng samples, followed by Kou Fangfeng, and low intensity in Chuan Fangfeng samples. Therefore, the components that correlate to A–G could be regarded as potential chemical markers for the discrimination of Guan Fangfeng from others. Similarly, we found Compounds H (RT 15.51 min, m/z 187.0390), I (RT 14.28 min, m/z 247.0967) and J (RT 17.81 min, m/z 217.0496) in the loading plot, which might be significant in separating Chuan Fangfeng from others. According to their integrated ion intensity plots in Figure 3, the three compounds were detectable with high intensity in Chuan Fangfeng samples and undetectable in Guan Fangfeng and Kou Fangfeng. We regarded them as potential chemical markers of Chuan Fangfeng.

Univariate data analyses of the potential chemical markers

To further confirm the potential chemical markers, we used repeated measures of one-way ANOVA to compare Kou Fangfeng and Chuan Fangfeng samples against Guan Fangfeng, and the results showed that the levels in the three group samples were statistically significant ($p < 0.05$).

Identity assignment and confirmation of chemical markers

Based on accurate mass measurements within 5 ppm error for each molecular ion and subsequent fragment ions of TOFMS, as well as matching of empirical molecular formulae with those of published components in the in-house chemical library, a total of 28 compounds, including the 10 potential markers, were tentatively identified, four of which were unambiguously identified as 4′-*O*-glucosyl-5-*O*-methylvisamminol (Compound A), prim-*O*-glucosylcimifugin (Compound B), cimifugin (Compound C) and sec-*O*-D-glucosylhammaudol (Compound D) by the available reference standards. The results are shown in Table II. The fragmentation pathways of coumarins and chromones in RS have previously been investigated and summarized by Kang *et al.* (5). According to the results, Compound A at the retention time of 13.39 min was chosen as an example for the illustration of the identification approach.

Compound A generated a predominant $[M + H]^+$ ion at m/z 453.1763 at a low fragmentor voltage of 180 V. This enabled direct determination of its molecular formula of $C_{22}H_{28}O_{10}$, which matched that of 4′-*O*-glucosyl-5-*O*-methylvisamminol in the chemical library. With the fragmentor voltage increased to 350 V, it produced several structure-relevant fragment ions after in-source collision induced dissociation at m/z 291.1231, 273.1130, 243.0664 and 219.0659, as shown in Figure 4. The product ion at m/z 291 was obtained from the loss of a D-glucose residue (162Da), and then the aglycone product ion yielded the product ions at m/z 273.1130, 243.0664 and 219.0659 by the losses of H_2O (18Da), $H_2O + 2 \times CH_3$ (48Da) and a 72Da fragment, respectively. The cleavage of the C–2'–C–3' bond and the C–O bond at position 1'/2' of the dihydrofuran ring with a concomitant H-rearrangement led to the elimination of a 2,2-dimethyl-epoxyethane (72Da) moiety, which yielded a product ion at m/z 219.0659 (4). Thus, Compound A was identified as 4′-*O*-glucosyl-5-*O*-methylvisamminol.

Compound F gave an $[M + H]^+$ ion at m/z 291.1230 at a low fragmentor voltage of 180 V. In addition, it yielded the same product ions in the high fragment analysis as that of the aglycone product ion of Compound A. Hence, Compound F was characterized as 5-*O*-methylvisamminol. The proposed fragmentation mechanism of 5-*O*-methylvisamminol is displayed in Figure 5.

Using the same method, we finally determined 10 chemical markers, including Guan Fangfeng markers A, 4′-*O*-glucosyl-5-*O*-methylvisamminol (RT 13.39 min, $C_{22}H_{28}O_{10}$); B, prim-*O*-glucosylcimifugin (RT 10.29 min, $C_{22}H_{28}O_{11}$); C, cimifugin (RT 12.17 min, $C_{16}H_{18}O_6$); D, sec-*O*-D-glucosylhammaudol (RT 17.18 min, $C_{21}H_{26}O_{10}$); E, 3′-*O*-angeloylhammaudol (RT 28.11 min, $C_{20}H_{22}O_6$); F, 5-*O*-methylvisamminol (RT 16.12 min, $C_{16}H_{18}O_5$); G, deltoin (RT 23.43 min, $C_{19}H_{20}O_5$); and Chuan Fangfeng markers H,

Table II
Characterization of Constituents in RS

No	RT (min)	[M + H] *m/z*			Formula	Compound	Structure-relevant fragment ions with high fragmentor voltage (300 V)
		Detected	Expected	Error (ppm)			
1	10.29	469.1718	469.1711	1.5	$C_{22}H_{28}O_{11}$	prim-*O*-glucosylcimifugin	307.1196 $[M + H\text{-glu}]^+$ 289.1095 $[M + H\text{-glu-H2O}]^+$ 259.0627 $[M + H\text{-glu-H2O-2} \times CH3]^+$ 235.0621 $[M + H\text{-glu-2,2-dimethyl-epoxyethane}]^+$
2	10.32	223.0612	223.0608	1.8	$C_{11}H_{10}O_5$	fraxidin/isofraxidin	208.0377 $[M + H\text{-CH3}]^+$ 193.0143 $[M + H\text{-2} \times CH3]^+$
3	10.43	321.0974	321.0965	2.8	$C_{16}H_{16}O_7$	divaricatacid	303.0874 $[M + H\text{-H2O}]^+$ 273.0401 $[M + H\text{-H2O-2} \times CH3]^+$ 249.0395 $[M + H\text{-2,2-dimethyl-epoxyethane}]^+$
4	11.09	223.0611	223.0609	0.9	$C_{11}H_{10}O_5$	fraxidin/isofraxidin	208.0376 $[M + H\text{-CH3}]^+$ 193.0141 $[M + H\text{-2} \times CH3]^+$
5	12.17	307.1198	307.1190	2.6	$C_{16}H_{18}O_6$	cimifugin	289.1095 $[M + H\text{-H2O}]^+$ 259.0628 $[M + H\text{-H2O-2} \times CH3]^+$ 235.0623 $[M + H\text{-2,2-dimethyl-epoxyethane}]^+$
6	12.77	455.1549	455.1546	0.7	$C_{21}H_{26}O_{11}$	11-hydroxy-sec-*O*-β-D-glucosyl-hammaudol	293.1021 $[M + H\text{-glu}]^+$ 275.0921 $[M + H\text{-glu-H2O}]^+$
7	13.39	453.1763	453.1757	1.3	$C_{22}H_{28}O_{10}$	4′-*O*-glucosyl-5-*O*-methylvisamminol	291.1231 $[M + H\text{-glu}]^+$ 273.1130 $[M + H\text{-glu-H2O}]^+$ 243.0664 $[M + H\text{-glu-H2O-2} \times CH3]^+$ 219.0659 $[M + H\text{-glu-2,2-dimethyl-epoxyethane}]^+$
8	14.28	247.0982	247.0975	2.8	$C_{14}H_{14}O_4$	nodakenetin	229.0882 $[M + H\text{-H2O}]^+$ 175.0407 $[M + H\text{-C4H8O}]^+$
9	15.44	217.0509	217.0506	1.4	$C_{12}H_8O4$	Xanthotoxin/8-methoxypsoralen	202.0274 $[M + H\text{-CH3}]^+$ 174.0321 $[M + H\text{-CH3-CO}]^+$
10	15.51	187.0391	187.0396	-2.7	$C_{11}H_6O_3$	psoralen	159.0443 $[M + H\text{-CO}]^+$ 143.0493 $[M + H\text{-CO2}]^+$
11	15.53	293.1035	293.1028	2.4	$C_{15}H_{16}O_6$	(3S)-2,2-dimethyl-3,5-dihydroxy-8-hydroxymethyl-3, 4-dihydro-2H,6H-benzo[1,2-b:5,4-b′]dipyran-6-one	275.0929 $[M + H\text{-H2O}]^+$ 257.0823 $[M + H\text{-2} \times H2O]^+$
12	16.12	291.1230	291.1227	1.0	$C_{16}H_{18}O_5$	5-*O*-methylvisamminol	273.1132 $[M + H\text{-H2O}]^+$ 243.0661 $[M + H\text{-H2O-2} \times CH3]^+$ 219.0658 $[M + H\text{-2,2-dimethyl-epoxyethane}]^+$
13	17.07	247.0605	247.0607	-0.8	$C_{13}H_{10}O_5$	isopimpinellin	232.0371 $[M + H\text{-CH3}]^+$ 217.0135 $[M + H\text{-2} \times CH3]^+$
14	17.18	439.1612	439.1609	0.7	$C_{21}H_{26}O_{10}$	sec-O-β-D-glucosylhammauol	277.1084 $[M + H\text{-glu}]^+$ 259.0985 $[M + H\text{-glu-H2O}]^+$ 205.0509 $[M + H\text{-glu-CHOCH(CH3)2}]^+$
15	17.81	217.0509	217.0507	0.9	$C_{12}H_8O_4$	bergapten	202.0274 $[M + H\text{-CH3}]^+$ 174.0327 $[M + H\text{-CH3-CO}]^+$
16	19.31	335.1133	335.1129	1.2	$C_{17}H_{18}O_7$	divaricatol	275.0922 $[M + H\text{-C2H4O2}]^+$ 257.0823 $[M + H\text{-C2H4O2-H2O}]^+$
17	19.99	277.1068	277.1067	0.4	$C_{15}H_{16}O_5$	hamaudol	259.0962 $[M + H\text{-H2O}]^+$ 205.0493 $[M + H\text{-C4H8O}]^+$
18	21.21	345.1337	345.1332	1.4	$C_{19}H_2OO_6$	3′-s-hydroxy deltoin	327.1231 $[M + H\text{-H2O}]^+$ 227.0706 $[M + H\text{-H2O-C5H8O2}]^+$
19	21.43	285.0766	285.0762	1.4	$C_{16}H_{12}O_5$	wogonin	270.0532 $[M + H\text{-CH3}]^+$
20	21.76	271.0970	271.0965	1.8	$C_{16}H_{14}O_4$	imperatorin	203.0339 $[M + H\text{-C5H8}]^+$
21	22.72	301.1085	301.1081	1.3	$C_{17}H_{16}O_5$	phellopterin	232.0381 $[M + H\text{-isopentenyl}]^+$ 217.0145 $[M + H\text{-isopentenyl-CH3}]^+$
22	23.36	319.1189	319.1185	1.3	$C_{17}H_{18}O_6$	3′-*O*-acetylhamaudol	259.0978 $[M + H\text{-C2H4O2}]^+$ 241.0878 $[M + H\text{-C2H4O2-H2O}]^+$
23	23.43	329.1392	329.1386	1.8	$C_{19}H_2OO_5$	deltoin	229.0868 $[M + H\text{-C5H8O2}]^+$ 214.0634 $[M + H\text{-C5H8O2-CH3}]^+$ 211.0767 $[M + H\text{-C5H8O2-H2O}]^+$
24	24.46	271.0978	271.0971	2.6	$C_{16}H_{14}O_4$	alloimperatorin/Isoimperatorin	229.0508 $[M + H\text{-propylene}]^+$ 211.0412 $[M + H\text{-propylene-H2O}]^+$
25	24.68	375.1443	375.1437	1.6	$C_{20}H_{22}O_7$	3′-*O*-(2″,3″-epoxy-2″-methylbutyryl)hammaudol	259.0970 $[M + H\text{-C5H8O3}]^+$ 241.0869 $[M + H\text{-C5H8O3-H2O}]^+$
26	26.09	427.1767	427.1760	1.6	$C_{24}H_{26}O_7$	anomalin	345.1346 $[M + H\text{-C5H6O}]^+$
27	26.77	347.1501	347.1496	1.4	$C_{19}H_{22}O_6$	3′-*O*-i-butyrylhammaudol	259.0973 $[M + H\text{-C4H8O2}]^+$ 241.0868 $[M + H\text{- C4H8O2-H2O}]^+$
28	28.11	359.1493	359.1487	1.7	$C_{20}H_{22}O_6$	3′-*O*-angeloylhamaudol	259.0969 $[M + H\text{-C5H8O2}]^+$ 241.0864 $[M + H\text{-C5H8O2-H2O}]^+$ 231.1023 $[M + H\text{-C5H8O2-CO}]^+$ 217.0506 $[M + H\text{-C5H8O2-propylene}]^+$

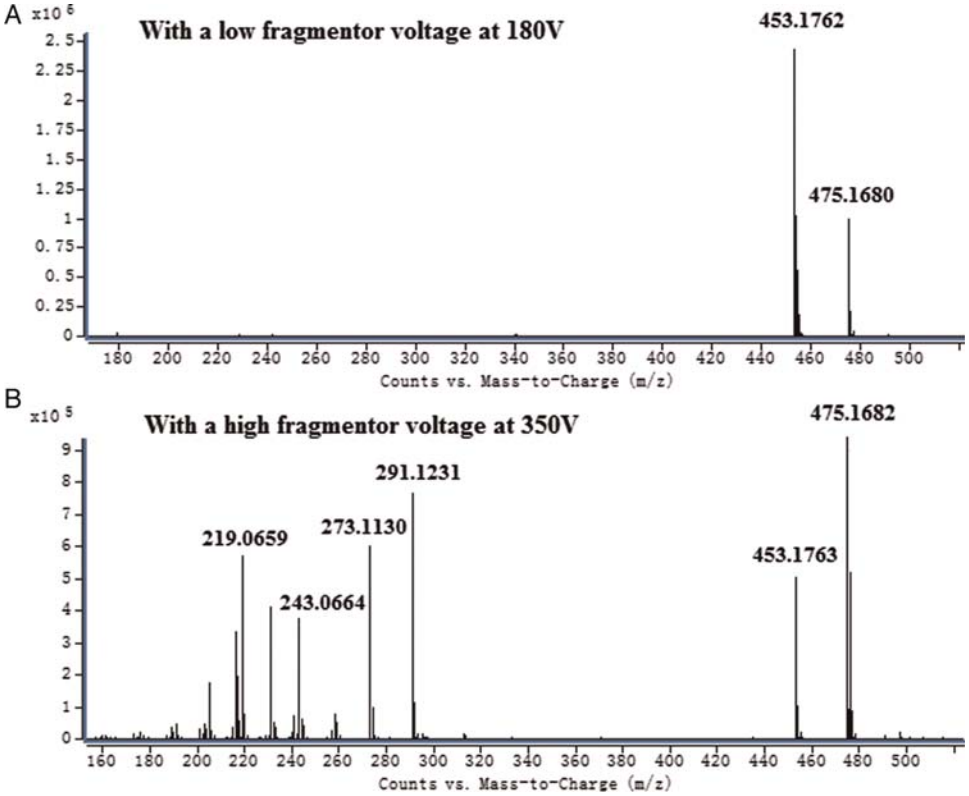

Figure 4. TOFMS spectra of 4′-*O*-glucosyl-5-*O*-methylvisamminol at low (180 V) and high (350 V) fragmentors.

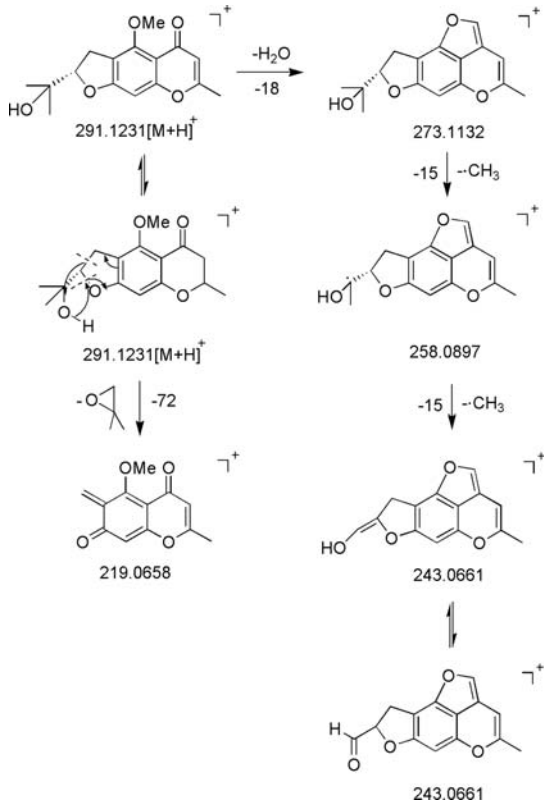

Figure 5. Proposed fragmentation of 5-*O*-methylvisamminol.

psoralen (RT 15.51 min, $C_{11}H_6O_3$); I, nodakenetin (RT 14.28 min, $C_{14}H_{14}O_4$); J, bergapten (RT 17.81 min, $C_{12}H_8O_4$). Among them, 4′-*O*-glucosyl-5-*O*-methylvisamminol, cimifugin, prim-*O*-glucosylcimifugin and sec-*O*-D-glucosylhammaudol were identified by comparing the mass spectra and retention time with those of reference compounds.

Conclusion

This work has developed a convenient and reliable HPLC–ESI-TOFMS method to determine the fingerprints of RS. With multivariate statistical analysis, RS from different geographical locations could be distinguished, and the marker compounds, which are important to the classification of RS, were discovered. It provides an effective approach to identify and evaluate RS from different sources. Moreover, we can conduct quantity determination of corresponding marker compounds to optimize the quality control of RS, making it more effective and comprehensive. Ultimately, this work also provides a novel and effective method for the quality evaluation of TCM.

References

1. Okuyama, E., Hasegawa, T., Matsushita, T., Fujimoto, H., Ishibashi, M., Yamazaki, M.; Analgesic components of saposhnikovia root (*Saposhnikovia divaricata*); *Chemical and Pharmaceutical Bulletin (Tokyo)*, (2001); 49: 154–160.
2. Wang, F.R., Xu, Q.P., Li, P; Comparative studies on the febrifugal analgesic and anticonvulsive activities of water extracts from

cultivated and wild *Saposhnikovia divaricata*; *Zhong Xi Yi Jie He Za Zhi*, (1991); 11: 730–732, 710.
3. Tang, R.J., Min, Z.H., Xu, C.Y.; Pharmacologic studies on the root of *Saposhnikovia divaricata* (Turcz.) Schischk; *Zhongguo Zhong Yao Za Zhi*, (1988); 13: p44–46, 64.
4. Zheng, Z.G., Wang, R.S., Cheng, H.Q., Duan, T.T., He, B., Tang, D., *et al.*; Isolated perfused lung extraction and HPLC-ESI-MS(n) analysis for predicting bioactive components of *Saposhnikoviae Radix*; *Journal of Pharmaceutical and Biomedical Analysis*, (2011); 54: 614–618.
5. Kang, J., Sun, J.H., Zhou, L., Ye, M., Han, J., Wang, B.R., *et al.*; Characterization of compounds from the roots of *Saposhnikovia divaricata* by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry; *Rapid Communications in Mass Spectrometry*, (2008); 22: 1899–1911.
6. Xiao, Y.Q., Li, L., Yang, B., Huang, L.Q.; Studies on chemical constituents from root of *Saposhnikovia divaricata* (Turcz.) Schischk; *Zhongguo Zhong Yao Za Zhi*, (2001); 26: 117–119.
7. Jiang, Y.Y., Liu, B., Shi, R.B., Tu, G.Z.; Isolation and structure identification of chemical constituents from *Saposhnikovia divaricata* (Turcz.) *Schischk; Acta Pharmaceutica Sinica*, (2007); 42: 505–510.
8. Shan, J.Y., Liu, J.; Identification of wild and cultural *Saposhnikovia divaricata* (Turcz.) Schischk. and their trace element determination; *Heilongjiang Yi Yao Ke Xue*, (2008); 31: 36–37.
9. Meng, X.C., Sun, H., Sun, X.L., Wang, X.J.; A comparative analysis on chromone components contents in three stem and root of *Saposhnikovia divaricata*; *Zhongguo Zhong Yao Za Zhi*, (2008); 33: 1344–1346.
10. Sun, H., Cao, L., Wang, X.J.; Study on the cumulative regular rule of effective components content in *Saposhnikovia Divaricata* (Turcz) Schischk sampled in different seasons; *Zhongguo Zhong Yi Yao Ke Ji*, (2003); 10: 355–356.
11. Meng, X.C., Lou, Z.H., Cao, L.; The effect on quantity of effective constituents of *Saposhnikova divaricata* by transplanting and direct seeding; *Te Chan Yan Jiu*, (2004); 26: 29–30.
12. Sun, H., Sun, X.L., Meng, X.C., Wang, X.J.; Bolt effect on quality and yield of *Saposhnikovia divaricata* roots; *Shi Jie Ke Xue Ji Shu—Zhong Yao Xian Dai Hua*, (2008); 10: 101–104, 108.
13. Wang, N.H., Yuan, C.Q.; Determination of cultivated and wild *Saposhnikova Divaricata* by HPLC; *Zhong Yao Cai*, (1990); 13: 9–10.
14. Li, W., Wang, Z., Sun, Y.S., Chen, L., Han, L.K., Zheng, Y.N.; Application of response surface methodology to optimise ultrasonic-assisted extraction of four chromones in *Radix Saposhnikoviae*; *Phytochemical Analysis*, (2011); 22: 313–321.
15. Sun, A.L., Feng, L., Liu, R.M.; Preparative isolation and purification of prim-O-glucosyl-cinmifugin and 4′-O-beta-D-glucosyl-5-O-methylvisamminol from *Radix Saposhnikoviae* by high speed counter-current chromatography; *Journal of Liquid Chromatography& Related Technologies*, (2006); 29: 751–759.
16. Liu, R., Wu, S., Sun, A.; Separation and purification of four chromones from *Radix Saposhnikoviae* by high-speed counter-current chromatography; *Phytochemical Analysis*, (2008); 19: 206–2011.
17. Yu, L.F., Li, X.R., Liu, S.Y., Xu, G.W., Liang, Y.Z.; Comparative analysis of essential components between the herbal pair *Radix Saposhnikoviae-Rhizoma seu Radix Notopterygii* and its single herbs by GC-MS combined with a chemometric resolution method; *Analytical Methods*, (2009); 1: 45–51.
18. Kim, M.K., Yang, D.H., Jung, M., Jung, E.H., Eom, H.Y., Suh, J.H., *et al.*; Simultaneous determination of chromones and coumarins in *Radix Saposhnikoviae* by high performance liquid chromatography with diode array and tandem mass detectors; *Journal of Chromatography A*, (2011); 1218: 6319–6330.
19. Li, W., Wang, Z., Chen, L., Zhang, J., Han, L.K., Hou, J.G., *et al.*; Pressurized liquid extraction followed by LC-ESI/MS for analysis of four chromones in *Radix Saposhnikoviae*; *Journal of Separation Science*, (2010); 33: 17–18.
20. Li, L., Liu, Y.Y., Geng, L.D., Xiao, Y.Q.; Determination of four components in root of *Saposhnikovia divaricata* by HPLC gradient elution; *Zhongguo Zhong Yao Za Zhi*, (2006); 31: 194–196.
21. Liu, S.L., Gao, Y.G., Zhang, C.H., Zhang, L.X., Liu, H.Y., Zhu, Y.Y.; Studies on quality evaluation for *Saposhnikovia divaricata* herbs; *Zhongguo Zhong Yao Za Zhi*, (2007); 32: 1462–1465.
22. Zhang, C., Xiao, Y.Q., Li, L., Pang, Z., Li, G.L.; Isolation-preparation and determination of chromones from *Saposhnikovia divaricata*; *Zhongguo Zhong Yao Za Zhi*, (2008); 33: 2761–2764.
23. Yang, L.L., *et al.*; Cimifugin preparation and quantitative analysis of *Saposhnikoviae radix* by HPLC; *Journal of Food And Drug Analysis*, (1999); 7: 191–197.
24. Xie, P.S., Chen, S.B., Liang, Y.Z., Wang, X.H., Tian, R.T., Upton, R.; Chromatographic fingerprint analysis–A rational approach for quality assessment of traditional Chinese herbal medicine. *Journal of Chromatography A*, (2006); 1112: 171–180.
25. Serkova, N.J., Standiford, T.J., Stringer, K.A.; The emerging field of quantitative blood metabolomics for biomarker discovery in critical illnesses; *American Journal of Respiratory and Critical Care Medicine*, (2011), 184: 647–55.
26. Allwood, J.W., De Vos, R.C., Moing, A, Deborde, C., Erban, A., Kopka, J., *et al.*; Plant metabolomics and its potential for systems biology research background concepts, technology, and methodology; *Methods in Enzymology*, (2011); 500: 299–336.
27. Tang, J.; Microbial metabolomics; *Current Genomics*, (2011);12(6): 391–403.
28. Fiehn, O., Garvey, W.T., Newman, J.W., Lok, K.H., Hoppel, C.L., Adams, S.H.; Plasma metabolomic profiles reflective of glucose homeostasis in non-diabetic and type 2 diabetic obese African-American women; *PLoS One*, (2010); 5(12): pe15234.
29. Sana, T.R., Fischer, S., Wohlgemuth, G., Katrekar, A., Jung, K.H., Ronald, P.C., *et al.*; Metabolomic and transcriptomic analysis of the rice response to the bacterial blight pathogen Xanthomonas oryzae pv. oryzae; *Metabolomics*, (2010); 6: 451–465.
30. Lin, L., Huang, Z.Z., Gao, Y., Yan, X.M., Xing, J.C., Hang, W., *et al.*; LC-MS based serum metabonomic analysis for renal cell carcinoma diagnosis, staging, and biomarker discovery; *Journal of Proteome Research*, (2011); 10: 1396–1405.
31. Sun, H., Ni, B., Zhang, A.H., Wang, M., Dong, H., Wang, X.J.; Metabolomics study on Fuzi and its processed products using ultra-performance liquid-chromatography/electrospray-ionization synapt high-definition mass spectrometry coupled with pattern recognition analysis; *Analyst*, (2012); 137: 170–185.
32. Xie, G.X., Plumb, R., Su, M.M., Xu, Z.H., Zhao, A.H., Qiu, M.F., *et al.*; Ultra-performance LC/TOF MS analysis of medicinal Panax herbs for metabolomic research; *Journal Of Separation Science*, (2008); 31: 1015–1026.
33. Tomita, M., Nishioka, T.; Metabolomics: The frontier of systems biology. Springer, Tokyo, Japan, (2005), pp. 25–52.
34. Cloarec, O., Dumas, M.E., Trygg, J., Craig, A., Barton, R.H., Lindon, J.C., *et al.*; Evaluation of the orthogonal projection on latent structure model limitations caused by chemical shift variability and improved visualization of biomarker changes in 1H NMR spectroscopic metabonomic studies; *Analytical Chemistry*, (2005); 77: 517–526.
35. Noda, I.J. Scaling techniques to enhance two-dimensional correlation spectra. *Journal of Molecular Structure*, (2008);p883-884: 216-227.
36. Woo, S.S., Song, J.S., Lee, J.Y., In, D.S., Chung, H.J., Liu, J.R., *et al.*; Selection of high ginsenoside producing ginseng hairy root lines using targeted metabolic analysis; *Phytochemistry*, (2004); 65: 2751–2761.