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# Qualitative and quantitative characterization of chemical constituents in Xin-Ke-Shu preparations by liquid chromatography coupled with a LTQ Orbitrap mass spectrometer

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ABSTRACT

Xin-Ke-Shu (XKS), a traditional Chinese medicine (TCM) preparation containing five herbal medicines, has been commonly used for the treatment of coronary heart disease in China. However, the chemical constituents in XKS have not been clarified yet. In order to quickly define the chemical profiles and control the quality of XKS preparations, liquid chromatography coupled with electrospray ionization hybrid linear trap quadrupole orbitrap (LC–LTQ-Orbitrap) mass spectrometry was applied for simultaneous identification and quantification of multi-constituent. A total of 51 compounds, including phenolic acids, isoflavone-*C*-glycosides, isoflavone-*O*-glycosides, flavonoids, and triterpenoid saponins, were identified or tentatively deduced on the base of their retention behaviors, MS and $MS^n$ data, or by comparing with reference substances and literatures. In addition, an optimized LC–ESI-MS method was established for quantitative determination of 15 marker compounds in XKS preparations from 7 independent pharmaceutical companies. The validation of the method, including spike recoveries, linearity, sensitivity (LOD and LOQ), precision, and repeatability, was carried out and demonstrated to be satisfied the requirements of quantitative analysis. This is the first report on the comprehensive determination of chemical constituents in XKS preparations by LC–LTQ-Orbitrap mass spectrometry. The results suggested that the established methods would be a powerful and reliable analytical tool for the characterization of multi-constituent in complex chemical system and quality control of TCM preparations.

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## 1. Introduction

Traditional Chinese medicines (TCMs) have been widely used in many oriental countries for thousands of years [1]. Nowadays, through of the changes in the types of disease, especially the prevalence of chronic and systematic diseases, and limitations of western medicines, the usage of TCMs has received widespread acceptance and attention due to their reliable therapeutic efficacy with low side effects [2]. However, because of the complexity of the chemical compositions and unclear mechanisms of action, it is difficult to guarantee the consistency of quality and therapeutic efficacy of TCMs. Thus, comprehensive analytical methods for the characterization of their chemical constituents and quality evaluation of a complex chemical system are urgently required for better address the inherent holistic nature of TCMs.

Xin-Ke-Shu (XKS), a traditional Chinese medicine preparation composed of five commonly used Chinese herbs: *Salviae miltiorrhizae* Bge. (Dan-Shen), *Pueraria lobata* (Willd.) Ohwi. (Ge-Gen), *Panax notoginseng* (Burk.) F.H. Chen. (San-Qi), *Crataegus pinnatifide* Bge. (Shan-Zha), and *Aucklandia lappa* Decne. (Mu-Xiang), has been widely used for the treatment of coronary heart disease in China for decades [3]. Pharmacological studies have revealed that XKS can effectively regulate the abnormal changes of blood lipid and lipid peroxide, and has protective effect on myocardial ischemia and reperfusion injury in animal models [4,5]. Major compounds in those individual herbs in XKS preparations have been well studied, including phenolic acids, isoflavonoids, flavonoids, triterpenoid saponins, and volatile oil [6–10]. Unfortunately, the integrated chemical identification of the XKS preparations is still lacking. In addition, XKS preparations are produced by independent pharmaceutical companies using raw herbs from different areas, and three dosage forms of preparations, tablet, capsule and pill, are commercially available in China. However, up to now, those medicines are still evaluated by quantitative determination of one or two major compounds using traditional TLC or HPLC methods [11]. Obviously,

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**Table 1**
A summary of the tested samples.

| Sample no. | Preparation form | Origins | Batch no. |
|---|---|---|---|
| C1 | Capsule | Chongqing Xieran Pharmaceutical Co., Ltd, China | 090901 |
| C2 | Capsule | Chongqing Xieran Pharmaceutical Co., Ltd, China | 100301 |
| D1 | Capsule | Shandong Wohua Pharmaceutical Technology Co., Ltd, China | 100104 |
| D2 | Capsule | Shandong Wohua Pharmaceutical Technology Co., Ltd, China | 100307 |
| S1 | Tablet | Shandong Wohua Pharmaceutical Technology Co., Ltd, China | 100425 |
| S2 | Tablet | Shandong Wohua Pharmaceutical Technology Co., Ltd, China | 100207 |
| H1 | Capsule | Hebei Guojin Pharmaceutical Co., Ltd, China | 1001051 |
| H2 | Capsule | Hebei Guojin Pharmaceutical Co., Ltd, China | 0904051 |
| L1 | Capsule | Sichuan Longren Pharmaceutical Co., Ltd, China | 100301 |
| L2 | Capsule | Sichuan Longren Pharmaceutical Co., Ltd, China | 090804 |
| X | Capsule | Xian Tianyi Pharmaceutical Co., Ltd, China | 100404-029 |
| Y | Capsule | Deyuantang Pharmacy Group, China | 90003004-076 |
| J | Pill | Jilin Jichun Pharmaceutical Co., Ltd, China | 100101 |

this procedure could definitely not provide a complete chemical profile for its quality control.

In the last decades, hyphenated techniques, such as LC–ELSD [12], LC–DAD [13], LC–MS [14], GC–MS [15], and LC–NMR/MS [16], have been widely applied for the analysis of the constituents in the botanic extracts and TCMs. Among them, LC–MS becomes more popular for direct identification of multi-component and quality control of TCMs, due to its wide suitability, sensitivity, and sufficient structural information [17]. Recently, the combination of orbitrap technology with a linear ion trap, known as LTQ-Orbitrap mass spectrometer were introduced, which could provide all the traditional MS and $MS^n$ scan functions using a linear IT and high mass accuracy measurements (error within a few ppm) [18]. Our previous study indicated that this analytical technique has the potential capability of simple, sensitive and reliable detection and identification of complex samples such as TCM formula [19].

In the present study, a sensitive LC–ESI-$MS^n$ method was established for rapid separating, reliable identifying and quantifying the multiple components in XKS preparations, by using a hybrid LTQ-Orbitrap mass spectrometer coupled with HPLC system. The qualitative analysis was carried out both in negative and positive ionization modes to acquire accurate mass data in full scan mode and MS/MS in a data dependent product ion spectrum. Further, 15 reference compounds were quantitatively determined in negative ionization mode and 13 commercial samples of XKS preparations were analyzed for assessment of quality consistence.

## 2. Experimental

### 2.1. Chemicals and materials

HPLC grade acetonitrile and methanol were purchased from Fisher (New Jersey, USA). Formic acid of HPLC grade was from Fluka (Steinheim, Germany). Ultra pure water was prepared using a Millipore Milli-Q purification system (Bedford, MA, USA).

Reference substances of danshensu, protocatechuicalhydryde, salvianolic acid B, chlorogenic acid, puerarin, 3′-methoxypuerarin, daidzin, daidzein, genistein, notoginsenoside R1, ginsenosides Rg1, Rg2, Re, Rb1, and Rd, were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All of the purities were above 98% by HPLC analysis.

13 batches of XKS preparations were collected from different pharmaceutical companies in China (Table 1), and voucher samples were deposited in our laboratory of the Institute of Medicinal Plant Development (IMPLAD), Chinese Academy of Medical Sciences and Peking Union Medical College.

### 2.2. Standard solutions and sample preparation

Stock solutions of 15 reference substances were prepared in the concentration ranging from 1.91 to 3.47 mg/mL in 80% methanol and stored at 4 °C until use. A standard working solution of the mixtures was obtained by diluting stock solutions to desired concentrations. Aliquots of this solution were further diluted with initial mobile phase to a series of concentrations for quantification.

XKS preparations were pulverized into fine powder. The powder (0.5 g) was accurately weighed, and immersed in 30 mL of 80% MeOH (v/v) for 1 h at room temperature, then extracted in an ultrasonic water bath for 30 min. After recruitment the weight, the extraction was filtrated through filter paper. Aliquots of 500 μL continued filtrate was transferred into a 5 mL volumetric flask which was made up to its volume with initial mobile phase. The obtained solution was filtered through a 0.45 μm syringe filter before use, and 10 μL was injected into the LC instrument for LC–MS analysis.

### 2.3. HPLC–ESI-MS/MS system

The HPLC system consisted of a Finnigan Surveyor LC system (Thermo-Fisher Scientific, Bremen, Germany) equipped with a quaternary, an autosampler, and a built-in degasser. Chromatographic separation was performed on a Waters SunFire$^{TM}$ ODS $C_{18}$ column (150 mm × 2.1 mm, i.d., 5 μm) at a column temperature of 35 °C. The mobile phase was composed of acetonitrile (A) and water containing 0.1% formic acid (B). The line gradient program was carries out as follows: 5–10% A at 0–10 min; 11–35% A at 11–45 min; 36–88% A at 45–75 min at a flow rate of 0.2 mL/min. Re-equilibration duration was 18 min between individual runs.

The above HPLC system was connected with a LTQ-Orbitrap mass spectrometry system (Thermo-Fisher Scientific, Bremen, Germany) via an ESI interface. High purity nitrogen ($N_2$) was used as the sheath gas and helium (He) as the auxiliary gas with a flow rate of 35 and 8 arbitrary units, respectively.

### 2.4. Qualitative characteristic of chemical constituents

Identification of chemical constituents in XKS preparations was performed by LC–ESI-$MS^n$ analysis. The ESI-MS spectra of samples and reference compounds were acquired in both positive and negative ionization modes. For positive ESI analysis, the parameters were as follows: capillary temperature at 275 °C, capillary voltage at 40 V, ion spray voltage at 4.5 kV, tube lens voltage at 100 V. For negative ESI analysis, the parameters were as follows: capillary temperature at 275 °C, capillary voltage at −36 V, ion spray voltage at −4.0 kV, and tube lens voltage at −100 V.

For full scan MS analysis, the spectra were recorded in the range of $m/z$ 100–1200 with a resolution of 15000. Data-dependant acqui-

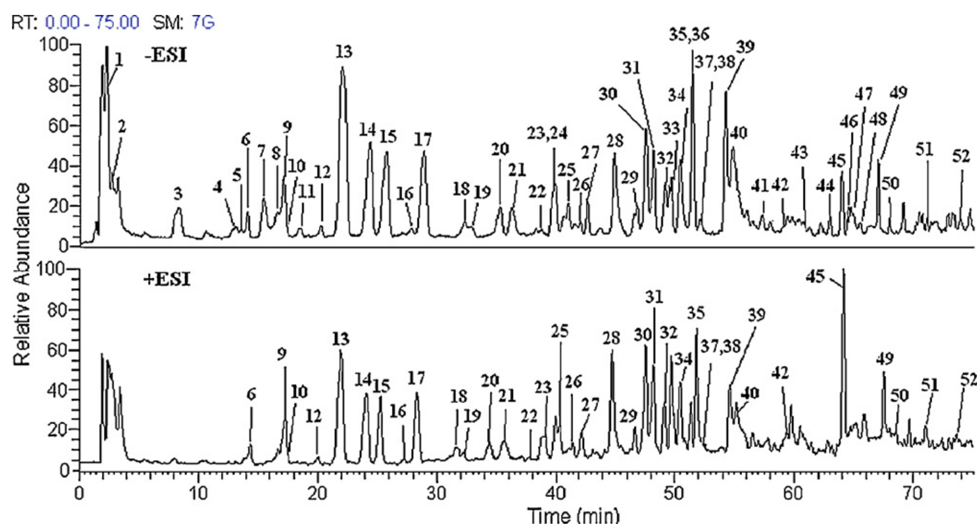


**Fig. 1.** TIC chromatograms of XKS extract in negative and positive ionization modes.

sition was applied and the most intense ions detected in each MS scan were selected for $MS^n$ data records with a resolution of 7500. The activation time was 30 ms and the collision energy was adjusted to 35%. Data were processed by Xcalibur software (Thermo-Fisher Scientific, Bremen, Germany). An external calibration for mass accuracy was carried out the day before the analysis according to the manufacturer's guidelines.

### *2.5. Validation of the quantitative analysis*

The stock solution containing 15 reference compounds was prepared and diluted to six-point calibration levels for the construction of calibration curves. Each concentration of the mixed standard solution was injected in triplicate. Calibration curves were established by plotting the peak area versus concentration of each analyte.

The limitation of detection (LOD) and quantification (LOQ) for each standard were defined at signal-to-noise ratio (S/N) of 3 and 10, respectively.

Intra- and inter-day variations were utilized to assess the precision of the method. The intra-day variation was determined by analyzing five replicates within 1 day and the inter-day variation was examined in 3 consecutive days. Recovery was used to evaluate the accuracy of the method. A certain amount of XKS sample was spiked with the mixed standard solution. The mixture was processed and analyzed using the method mentioned above (see Section 2.2), and three replicates were performed for the analysis. To confirm the repeatability, five replicates of the same sample were extracted and analyzed. Variations were expressed by relative standard deviation (RSD) in all three tests above.

## 3. Results and discussion

### *3.1. Optimization of LC and MS conditions*

The chromatographic conditions of the mobile phase systems (methanol-aqueous, acetonitrile-aqueous, and acetonotrile-aqueous with 0.1% formic acid), gradient program and the column temperature (25 °C, 30 °C, and 35 °C) were optimized in order to obtain overall constituents of XKS preparations with good resolution within a short analysis. Subsequently, the MS conditions including the sheath gas flow rate (30, 35, and 40 arbitrary units), the auxiliary gas flow rate (5 and 8 arbitrary units), spray voltage (4.5, 4.0, and 3.5 kV in positive ion mode; −4.5, −4.0, and −3.5 kV in negative ion mode), and tube lens voltage (80, 100, and 120 V in positive ion mode; −80, −100, and −120 V in negative ion mode) were optimized in order to acquire better detection of multi-compound in XKS preparations. The total peak area was taken as criteria for optimization. As a result, the optimum conditions were decided as described in Sections 2.3 and 2.4.

The total ion chromatogram (TIC) of the mixture of standards solution was analyzed by collecting their MS spectra in full-scan mode. The optimized LC–MS conditions showed that the negative ion mode was more sensitive and suitable for screening analysis of XKS than the positive ion mode. Therefore, negative ionization was selected for the quantitative analysis and $[M-H]^-$ or $[M+HCOOH-H]^-$ ions of individual compounds were selected as quantitation ions. For the extracted target ions in full scan mode, an accurate mass limit of 5 ppm accurate mass filter limit was used to characterize "real" compounds signals from the background peaks, as well as to increase the signal-to-noise ratio for each analyte.

### *3.2. Identification of chemical constituents in XKS preparations*

The reference substances and XKS sample were analyzed by using the optimized LC–ESI-$MS^n$ method. The TIC chromatograms of XKS sample in positive and negative ESI modes were shown in Fig. 1, and 52 peaks were observed in XKS sample. The MS data showed high precision with all the mass accuracy within 5 ppm. For most of the constituents, $[M-H]^-$, $[M+H]^+$, and/or adducted ions $[M+Na]^+$ were observed. Due to the use of formic acid in mobile phase, there were adducted ions of $[M+46-H]^-$ corresponding to $[M+HCOOH-H]^-$ in negative ion mode. These results provided valuable information for confirming accurate molecular weight and composition of the constituents.

51 compounds were tentatively identified on the basis of their retention behaviors, accurate molecular weight and $MS^n$ fragment data, or by comparison with reference substances or literature data (chemical structures shown in Fig. 2). Corresponding quasi-molecular ions and their fragment ions in the $MS^n$ spectra were listed in Table 2. The identified compounds can be classified into five classes including phenolic acids, isoflavone-*C*-glycosides, isoflavone-*O*-glycosides, flavonoids, and triterpenoid saponins. A total of 10 phenolic acids (peaks **3**, **4**, **10**, **24**, **28**, **30**, **39**, **40**, **43**, and **52**) were identified, most of which produced fragments of the loss

**Table 2**
Identification of the chemical constituents of XKS preparation by LC–ESI-MS$^n$ analysis.

| Peak No. | Identification | Rt[a] (min) | Formula | Negative ion ($m/z$) | | | Positive ion ($m/z$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Quasi-molecular (Error: ppm) | MS$^2$ ions ($m/z$) | MS$^3$ ions ($m/z$) | Quasi-molecular (Error: ppm) | MS$^2$ ions ($m/z$) | MS$^3$ ions ($m/z$) |
| **1** | Quinic acid | 2.44 | $C_7H_{12}O_6$ | 191.0559 (4.84) [M−H]$^-$ | – | – | – | – | – |
| **2** | Malic acid | 2.63 | $C_4H_6O_5$ | 133.0137 (4.51) [M−H]$^-$ | 115.0039 | 71.0142 | – | – | – |
| **3** | Danshensu[b] | 8.25 | $C_9H_{10}O_5$ | 197.0453 (4.26) [M−H]$^-$ | 179.0354 | 135.0453 | – | – | – |
| **4** | Protocatechualdehyde[b] | 13.06 | $C_7H_6O_3$ | 137.0239 (3.65) [M−H]$^-$ | 109.0290 | – | – | – | – |
| **5** | 3′-Hydroxypuerarin 4′-*O*-glucoside | 13.49 | $C_{27}H_{30}O_{15}$ | 593.1493 (−1.36) [M−H]$^-$ | 473.1092; 431.0988 | 445.1137; 353.0671 | 595.1633 (−4.16) [M+H]$^+$ | 577.1544; 433.1122 | 415.1015; 397.0916; 367.0803 |
| **6** | Puerarin 7-*O*-glucoside | 14.03 | $C_{27}H_{30}O_{14}$ | 577.1543 (−1.53) [M−H]$^-$ | 457.1138 | 429.1192; 267.0662 | 579.1685 (−4.10) [M+H]$^+$ | 417.1178 | 399.1068; 381.0960; 351.0855 |
| **7** | Unidentified | 15.39 | $C_7H_{12}O_4$ | 205.0715 (4.31) [M+HCOOH−H]$^-$ | 115.0769; 143.0717 | 73.0299; 85.0664 | – | – | – |
| **8** | Daidzin 4′-*O*-glucoside | 16.43 | $C_{29}H_{32}O_{18}$ | 623.1600 (−1.01) [M+HCOOH−H]$^-$ | 415.1037 | 253.0505 | 579.1687 (−3.68)[M+H]$^+$ | 417.1174 | 255.0647 |
| **9** | 3′-Hydroxypuerarin | 17.01 | $C_{21}H_{20}O_{10}$ | 431.0974 (0.18) [M−H]$^-$ | 311.0568 | 283.0616 | 433.1112 (−3.95)[M+H]$^+$ | 415.1018; 313.0706 | 397.0908; 367.0800; 337.0699 |
| **10** | Chlorogenic acid[b] | 17.49 | $C_{16}H_{18}O_9$ | 353.0871 (1.02) [M−H]$^-$ | 191.0563 | 173.0454; 127.0403 | 377.0825 (4.51) [M+Na]$^+$ | – | – |
| **11** | 3′-Methoxydaidzin 4′-*O*-glucoside | 18.29 | $C_{28}H_{32}O_{15}$ | 653.1702 (−1.52) [M+HCOOH−H]$^-$ | – | – | 609.1796 (−2.99)[M+H]$^+$ | 447.1280 | 285.0751; 429.1170 |
| **12** | 3′-Hydroxypuerarin xyloside | 22.04 | $C_{26}H_{28}O_{14}$ | 563.1390 (−0.88) [M−H]$^-$ | 311.0566 | 283.0612 | 565.1530 (−3.83) [M+H]$^+$ | 433.1124 | 415.1014; 367.0805; 313.0577 |
| **13** | Puerarin[b] | 22.01 | $C_{21}H_{20}O_9$ | 415.1030 (1.40) [M−H]$^-$ | 295.0617 | 267.0661 | 417.1169 (−2.77) [M+H]$^+$ | 399.1079; 297.0758 | 381.0956; 351.0854; 321.0749 |
| **14** | 3′-Methoxypuerarin[b] | 24.18 | $C_{22}H_{22}O_{10}$ | 445.1133 (0.94) [M−H]$^-$ | 325.0719 | 297.0777; 310.0490; 282.0540 | 447.1274 (−2.60) [M+H]$^+$ | 429.1175 | 411.1070; 381.0962; 351.0857 |
| **15** | Mirificin | 25.76 | $C_{26}H_{28}O_{13}$ | 547.1445 (−0.27) [M−H]$^-$ | 295.0619 | 267.0662 | 549.1588 (−2.76) [M+H]$^+$ | 417.1175 | 399.1068; 351.0855; 297.0753 |
| **16** | 3′-Methoxy puerarin 6″-*O*-β-Apionoside | 27.74 | $C_{27}H_{30}O_{14}$ | 577.1547 (−0.90) [M−H]$^-$ | 325.0724 | 297.0775; 282.0537 | 579.1691 (−2.84) [M+H]$^+$ | 447.1284 | 429.1173; 411.1072; 381.0961 |
| **17** | Daidzin[b] | 28.88 | $C_{21}H_{20}O_9$ | 461.1081 (−0.62) [M+HCOOH−H]$^-$ | 415.1028; 253.0510 | 224.0481; 209.0609 | 417.1170 (−2.25) [M+H]$^+$ | 255.0647 | 227.0703; 199.0753; 137.0231 |
| **18** | 3′-Methoxy daidzin | 32.26 | $C_{22}H_{22}O_{10}$ | 491.1181 (−0.67) [M+HCOOH−H]$^-$ | 445.1141; 283.0613 | 265.0505; 237.0558 | 469.1093 (−2.69) [M+Na]$^+$ | 285.0751 | 270.0522; 253.0493; 225.0543 |
| **19** | Genistein-8-*C*-glucoside | 32.61 | $C_{21}H_{20}O_{10}$ | 431.0974 (0.25) [M−H]$^-$ | 311.0568 | 283.0616 | 433.1116 (−3.05) [M+H]$^+$ | 415.1021; 397.0915 | 379.0807; 367.0805; 337.0703 |

Table 2 (*Continued*)

| Peak No. | Identification | Rt[a] (min) | Formula | Negative ion (*m*/*z*) | | | Positive ion (*m*/*z*) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Quasi-molecular (Error: ppm) | MS² ions (*m*/*z*) | MS³ ions (*m*/*z*) | Quasi-molecular (Error: ppm) | MS² ions (*m*/*z*) | MS³ ions (*m*/*z*) |
| **20** | Genistein-8-C-apiosyl(1-6)-glucoside | 35.16 | $C_{26}H_{28}O_{14}$ | 563.1390 (−0.87) [M−H]⁻ | 311.0566 | 283.0616 | 565.1538 (−2.43) [M+H]⁺ | 433.1122; 313.0705 | 415.1017; 397.0915; 367.0807 |
| **21** | Pueroside A | 36.40 | $C_{29}H_{34}O_{14}$ | 605.1862 (−0.43) [M−H]⁻ | 297.0773 | 253.0874 | 629.1825 (−2.50) [M+Na]⁺ | 483.1252; 321.0730 | – |
| **22** | Hyperoside | 38.79 | $C_{21}H_{20}O_{12}$ | 463.0870 (−0.20) [M−H]⁻ | 301.0358 | 273.0413; 151.0042 | 487.0836 (−2.97) [M+Na]⁺ | 303.0491 | 257.0445; 229.0495; 165.0178 |
| **23** | Apigenin-7-*O*-glucoside | 39.76 | $C_{21}H_{20}O_{10}$ | 477.1029 (0.25) [M+HCOOH−H]⁻ | 431.0980; 269.0372 | 268.0376 | 433.1119 (−2.69) [M+H]⁺ | 271.0594 | 243.0650; 215.0701; 153.0180 |
| **24** | Lithosperimic acid | 40.17 | $C_{27}H_{22}O_{12}$ | 537.1024 (−0.97) [M−H]⁻ | 493.1130 | 295.0609; 313.0716 | 561.0987 (−2.95) [M+Na]⁺ | – | – |
| **25** | Pueroside B | 40.78 | $C_{30}H_{36}O_{15}$ | 681.2017 (−1.18) [M+HCOOH−H]⁻ | 635.1982 | 473.1454 | 637.2109 (−2.57) [M+H]⁺ | 475.1584 | 313.1066 |
| **26** | 4′-Methoxypuerarin | 42.02 | $C_{22}H_{22}O_9$ | 429.1181 (0.21) [M−H]⁻ | 309.0777 | 281.0825; 266.0590 | 431.1324 (−2.94) [M+H]⁺ | 413.1228 | 395.1122; 365.1014; 335.0909 |
| **27** | Formononetin-8-C-glucoside-*O*-xyloside | 42.54 | $C_{27}H_{30}O_{13}$ | 561.1602 (−0.19) [M−H]⁻ | 309.0771 | 281.0825 | 563.1743 (−2.94) [M+H]⁺ | 431.1331 | 413.1221; 395.1117; 365.1011; 335.0905; 311.0908; 281.0789 |
| **28** | Salvianolic acid D | 45.18 | $C_{20}H_{18}O_{10}$ | 417.0816 (−0.05) [M−H]⁻ | 399.0924; 373.0932 | 197.0459 | 441.0777 (−4.21) [M+Na]⁺ | – | – |
| **29** | 6″-*O*-Acetyl daidzin | 46.78 | $C_{23}H_{22}O_{10}$ | 503.1185 (0.19) [M+HCOOH−H]⁻ | 457.1147; 253.0510 | 295.0612; 252.0427 | 459.1275 (−2.30) [M+H]⁺ | 255.0648 | 199.0755; 227.0705; 137.0232 |
| **30** | Rosmarinic acid | 47.59 | $C_{18}H_{16}O_8$ | 359.0765 (1.02) [M−H]⁻ | 197.0459; 161.0250 | 133.0298 | – | – | – |
| **31** | Sophoraside A | 48.18 | $C_{24}H_{26}O_{10}$ | 519.1497 (0.03) [M+HCOOH−H]⁻ | 473.1461 | 311.0925; 267.1006 | 475.1586 (−2.62) [M+H]⁺ | 313.1064 | 295.0965; 219.0651; 107.0488 |
| **32** | Ononin | 49.14 | $C_{22}H_{22}O_9$ | 475.1231 (−0.73) [M+HCOOH−H]⁻ | 429.1192 | 267.0662 | 431.1329 (−1.69) [M+H]⁺ | 269.0805 | 254.0573; 237.0546; 213.0911 |
| **33** | Notoginsenoside R1[b] | 49.60 | $C_{47}H_{80}O_{18}$ | 977.5311 (−0.45) [M+HCOOH−H]⁻ | 931.5253 | 799.4867; 637.4330 | 955.5219 (−1.92) [M+Na]⁺ | 775.4591 | 643.4168; 463.3495; 335.0944 |
| **34** | Daidzein[b] | 50.40 | $C_{15}H_{10}O_4$ | 253.0499 (1.44) [M−H]⁻ | 224.0480; 209.0607 | – | 255.0645 (−2.65) [M+H]⁺ | 199.0752; 137.0231 | 181.0645; 171.0803; 153.0696 |

Table 2 (*Continued*)

| Peak No. | Identification | Rt[a] (min) | Formula | Negative ion (*m/z*) | | | Positive ion (*m/z*) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Quasi-molecular (Error: ppm) | MS$^2$ ions (*m/z*) | MS$^3$ ions (*m/z*) | Quasi-molecular (Error: ppm) | MS$^2$ ions (*m/z*) | MS$^3$ ions (*m/z*) |
| **35** | Ginsenoside Rg1[b] | 51.47 | $C_{42}H_{72}O_{14}$ | 845.4894 (0.15) [M+HCOOH−H]$^-$ | 799.4839 | 637.4308; 475.3788 | 823.4793 (−2.57) [M+Na]$^+$ | 643.4166 | 463.3530 |
| **36** | Ginsenoside Re[b] | 51.62 | $C_{48}H_{82}O_{18}$ | 991.5464 (−0.84) [M+HCOOH−H]$^-$ | 945.5407 | 783.4885; 637.4332 | 969.5381 (−2.36) [M+Na]$^+$ | 789.4751 | 643.4165 |
| **37** | Biochanin A | 52.05 | $C_{16}H_{12}O_5$ | 283.0609 (2.86) [M−H]$^-$ | 268.0381 | 240.0430; 211.0405 | 285.0750 (−2.59)[M+H]$^+$ | 270.0522; 225.0544 | 253.0493; 214.0624; 137.0230 |
| **38** | 4′,6-Dimethoxyisoflavone-7-*O*-glucoside | 52.26 | $C_{23}H_{24}O_{10}$ | 505.1342 (0.39) [M+HCOOH−H]$^-$ | 459.1307; 297.0767 | 282.0532 | 461.1424 (3.89) [M+H]$^+$ | 299.0906; 284.0676 | 284.0677; 239.0701; 166.0260 |
| **39** | Salvianolic acid A | 54.13 | $C_{26}H_{22}O_{10}$ | 493.1131 (0.36) [M−H]$^-$ | 295.0617; 313.0724 | 277.0511; 109.0300 | 495.1274 (−2.41) [M+H]$^+$ | 297.0751 | 269.0805; 251.0701; 233.0596 |
| **40** | Salvianolic acid B[b] | 54.79 | $C_{36}H_{30}O_{16}$ | 717.1447 (−0.42) [M−H]$^-$ | 519.0933 | 321.0400; 339.0504 | 741.1401 (1.15) [M+Na]$^+$ | 543.0892; 561.0997 | 517.1088; 381.0570; 337.0678 |
| **41** | 5′-Hydroxyl oninin | 57.14 | $C_{22}H_{22}O_{10}$ | 491.1186 (0.38) [M+HCOOH−H]$^-$ | 445.1145; 283.0616 | 268.0378 | 447.1271 (−3.34) [M+H]$^+$ | 285.0748 | 270.0521; 253.0493; 229.0858 |
| **42** | Genistein[b] | 58.81 | $C_{15}H_{10}O_5$ | 269.0452 (2.90) [M−H]$^-$ | 225.0556; 201.0561 | 181.0666; 169.0655 | 271.0591 (−3.65) [M+H]$^+$ | 243.0651; 153.0180 | 215.0700 |
| **43** | Salvianolic acid C | 60.92 | $C_{26}H_{20}O_{10}$ | 491.0974 (0.16) [M−H]$^-$ | 293.0459; 311.0564 | 265.0505; 237.0558 | – | – | – |
| **44** | Notoginsenoside R2/isomer | 62.84 | $C_{41}H_{70}O_{13}$ | 815.4780 (−0.88) [M+HCOOH−H]$^-$ | 769.4746 | 637.4324; 475.3802 | – | – | – |
| **45** | Ginsenoside Rb1[b] | 63.86 | $C_{54}H_{92}O_{23}$ | 1153.6010 (0.87) [M+HCOOH−H]$^-$ | 945.5428 | 783.4897; 621.4387 | 1109.6085 (−1.53) [M+H]$^+$ | – | – |
| **46** | Ginsenoside Rg2[b] | 64.37 | $C_{42}H_{72}O_{13}$ | 829.4935 (−0.93) [M+HCOOH−H]$^-$ | 783.4895 | 637.4310; 475.3792 | 807.4852 (−2.34) [M+Na]$^+$ | | – |
| **47** | Ginsenoside Rh1/F1 | 64.64 | $C_{36}H_{62}O_9$ | 683.4357 (−1.29) [M+HCOOH−H]$^-$ | 637.4316 | 475.3789 | – | – | – |
| **48** | Formononetin | 65.58 | $C_{16}H_{12}O_4$ | 267.0657 (2.04) [M−H]$^-$ | 252.0429 | 223.0405; 208.0534 | 269.0803 (−2.14) [M+H]$^+$ | 254.0572; 213.0910 | 237.0545; 118.0410 |
| **49** | Ginsenoside Rd[b] | 66.90 | $C_{48}H_{82}O_{18}$ | 991.5462 (−1.03) [M+HCOOH−H]$^-$ | 945.5520 | 783.4903; 621.4354 | 969.5378 (−1.54) [M+Na]$^+$ | 789.47425 | 305.0840 |
| **50** | NotoginsenosideK/Gypenoside XVII | 67.92 | $C_{48}H_{82}O_{18}$ | 991.5461 (−1.09) [M+HCOOH−H]$^-$ | 945.5400 | 783.4914 | 969.5370 (−2.05) [M+Na]$^+$ | 365.1046 | 305.0840 |
| **51** | Ginsenoside Rg3 | 71.33 | $C_{42}H_{72}O_{13}$ | 829.4929 (−0.90) [M+HCOOH−H]$^-$ | 783.4901 | 621.4358; 459.3841 | 807.4848 (−2.12) [M+Na]$^+$ | 627.4214 | – |
| **52** | Salvianolic acid F | 74.66 | $C_{19}H_{22}O_4$ | 313.1442 (2.34) [M−H]$^-$ | 269.1548 | 213.0918; 241.1602 | 337.1404 (−1.75) [M+Na]$^+$ | 309.1121; 293.1516 | 281.1173 |

[a] Rt: retention time.

[b] Structurally confirmed by comparison with reference chemicals.

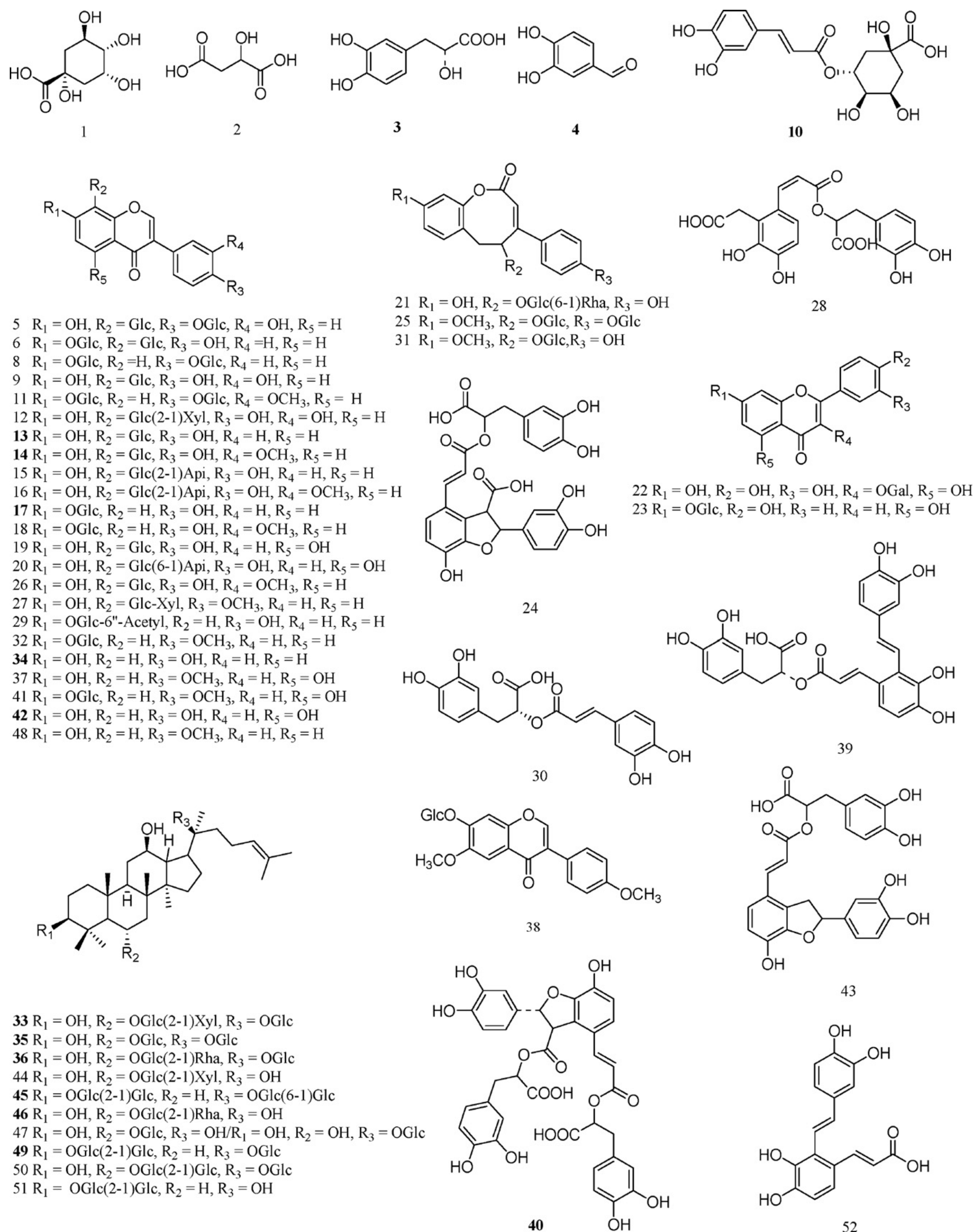


**Fig. 2.** The chemical structures of identified compounds in XKS preparation.

of caffeic acid (180 Da) or danshensu (198 Da), as previous reported [20,21]. While the fragment ion corresponding to the loss of $CO_2$ (44 Da) and $H_2O$ (18 Da) indicated the existence of "–COOH" and "–OH" groups in the structure, respectively. Among the identified constituents, 12 isoflavone *C*-glycosides (peaks **5**, **6**, **9**, **12**, **13**, **14**, **15**, **16**, **19**, **20**, **26**, and **27**), 8 isoflavone *O*-glycosides (peaks **8**, **11**, **17**, **18**, **29**, **32**, **38**, and **41**), 4 isoflavones (peaks **34**, **37**, **42**, and **48**), and 2 flavonones (peaks **22** and **23**) were characterized by $MS^n$ analy-

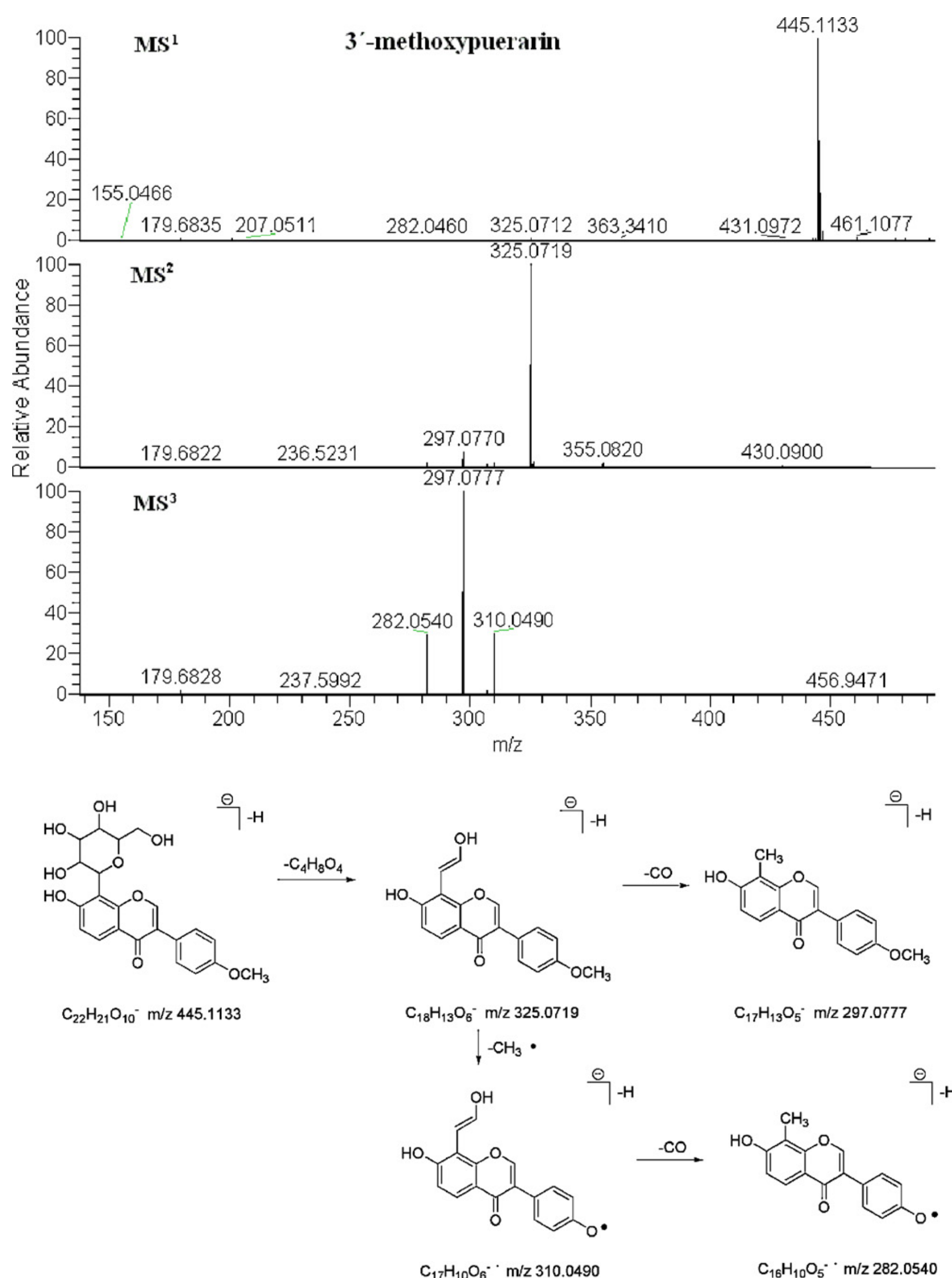


**Fig. 3.** Spectra of ion fragments in MS$^n$ analysis, and the proposed fragmentation pathway of 3′-methoxypuerarin (**14**) in negative ESI.

**Table 3**

Summary of quantitation ion, calibration curves, linear range, LOD, LOQ and repeatability for 15 analytes analyzed with the LC–MS system.

| Analyte | Quantitation ion ($m/z$) | Linear range (μg/mL) | Calibration curve ($n$ = 6) | $r^2$ ($n$ = 6) | LOD (μg/mL) | LOQ (μg/mL) | Repeatability RSD (%) |
|---|---|---|---|---|---|---|---|
| Danshensu (**3**) | 197.0444 | 1.041–41.640 | $Y = 7.25 \times 10^5 + 7.78 \times 10^5 X$ | 0.9991 | 0.0297 | 0.104 | 0.66 |
| Protocatechualdehyde (**4**) | 137.0233 | 0.137–5.488 | $Y = 4.72 \times 10^5 + 5.64 \times 10^6 X$ | 0.9994 | 0.0261 | 0.0914 | 4.01 |
| Chlorogenic acid (**10**) | 353.0867 | 0.0422–1.688 | $Y = -7.77 \times 10^4 + 2.49 \times 10^6 X$ | 0.9997 | 0.00603 | 0.0211 | 3.02 |
| Puerarin (**13**) | 415.1024 | 0.672–26.88 | $Y = 2.28 \times 10^6 + 3.86 \times 10^6 X$ | 0.9993 | 0.00192 | 0.00672 | 2.34 |
| 3′-Methoxypuerarin (**14**) | 445.1129 | 0.456–18.24 | $Y = 1.42 \times 10^5 + 4.44 \times 10^6 X$ | 0.9994 | 0.00101 | 0.00304 | 4.18 |
| Daidzin (**17**) | 461.1056 | 0.425–16.992 | $Y = 7.29 \times 10^5 + 3.10 \times 10^6 X$ | 0.9993 | 0.00121 | 0.00425 | 3.21 |
| Notoginsenoside R1 (**33**) | 977.5257 | 0.0222–0.888 | $Y = 5.68 \times 10^4 + 6.76 \times 10^6 X$ | 0.9990 | 0.00106 | 0.00317 | 4.71 |
| Daidzein (**34**) | 253.0495 | 0.0852–3.408 | $Y = 7.10 \times 10^5 + 1.11 \times 10^7 X$ | 0.9983 | 0.00122 | 0.00426 | 4.07 |
| Ginsenoside Rg1 (**35**) | 845.4893 | 0.0562–2.248 | $Y = 2.16 \times 10^4 + 7.86 \times 10^6 X$ | 0.9993 | 0.00141 | 0.00464 | 3.59 |
| Ginsenoside Re (**36**) | 991.5472 | 0.0146–0.586 | $Y = 4.62 \times 10^3 + 6.09 \times 10^6 X$ | 0.9990 | 0.00110 | 0.00362 | 4.18 |
| Salvianolic acid B (**40**) | 717.1415 | 1.545–61.800 | $Y = -6.00 \times 10^6 + 2.55 \times 10^6 X$ | 0.9990 | 0.00905 | 0.0317 | 1.00 |
| Genistein (**42**) | 269.0444 | 0.0114–0.458 | $Y = -5.66 \times 10^4 + 1.99 \times 10^7 X$ | 0.9998 | 0.00347 | 0.0114 | 1.41 |
| Ginsenoside Rb1 (**45**) | 1153.6000 | 0.110–4.420 | $Y = 4.64 \times 10^4 + 2.96 \times 10^6 X$ | 0.9992 | 0.00154 | 0.00507 | 3.19 |
| Ginsenoside Rg2 (**46**) | 829.4944 | 0.0154–0.614 | $Y = 3.13 \times 10^4 + 8.61 \times 10^6 X$ | 0.9997 | 0.00674 | 0.0222 | 2.71 |
| Ginsenoside Rd (**49**) | 991.5472 | 0.0210–0.840 | $Y = 1.08 \times 10^4 + 9.57 \times 10^6 X$ | 0.9998 | 0.00525 | 0.0184 | 4.92 |

$Y$ and $X$ stand for the peak area and the concentration (μg/mL) of each analyte, respectively.

**Table 4**
Precisions and recoveries of 15 analytes.

| Analyte | Intra-day ($n=5$) (μg/mL) | | Inter-day ($n=3$) (μg/mL) | | Recoveries ($n=3$) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Means ± SD | RSD (%) | Means ± SD | RSD (%) | Initial (μg) | Added (μg) | Detected (μg) | Recovery (%) | RSD (%) |
| Danshensu (**3**) | 1.032 ± 0.044 | 4.32 | 1.015 ± 0.076 | 4.76 | 2340.26 | 1664.00 | 3996.53 | 99.54 | 1.45 |
| | 6.013 ± 0.067 | 1.12 | 5.980 ± 0.085 | 1.43 | | 2080.00 | 4496.70 | 103.67 | 0.85 |
| | 19.250 ± 0.630 | 3.27 | 19.050 ± 0.674 | 3.54 | | 2496.00 | 4906.25 | 102.80 | 2.75 |
| Protocatechualdehyde (**4**) | 0.134 ± 0.002 | 2.01 | 0.134 ± 0.002 | 2.00 | 255.63 | 217.60 | 481.84 | 94.60 | 3.43 |
| | 0.738 ± 0.021 | 2.79 | 0.722 ± 0.034 | 4.77 | | 272.00 | 556.53 | 100.67 | 2.03 |
| | 2.644 ± 0.064 | 2.43 | 2.654 ± 0.060 | 2.26 | | 326.40 | 607.46 | 97.01 | 2.05 |
| Chlorogenic acid (**10**) | 0.0540 ± 0.0013 | 2.37 | 0.0558 ± 0.0013 | 2.29 | 87.93 | 70.89 | 157.85 | 98.62 | 3.43 |
| | 0.202 ± 0.004 | 2.02 | 0.201 ± 0.003 | 1.77 | | 88.62 | 186.26 | 100.97 | 2.03 |
| | 0.858 ± 0.002 | 0.29 | 0.858 ± 0.003 | 0.36 | | 106.34 | 210.09 | 103.38 | 2.05 |
| Puerarin (**13**) | 0.659 ± 0.011 | 1.68 | 0.659 ± 0.016 | 2.42 | 3803.92 | 2695.20 | 6528.28 | 101.08 | 2.05 |
| | 3.588 ± 0.068 | 1.92 | 3.616 ± 0.057 | 1.59 | | 3369.00 | 7394.00 | 96.97 | 1.60 |
| | 13.496 ± 0.290 | 2.15 | 13.709 ± 0.342 | 2.49 | | 4042.80 | 8108.91 | 95.84 | 1.12 |
| 3′-Methoxypuerarin (**14**) | 0.462 ± 0.018 | 3.98 | 0.464 ± 0.015 | 3.22 | 1249.11 | 963.33 | 2198.29 | 98.53 | 2.61 |
| | 2.458 ± 0.021 | 0.86 | 2.476 ± 0.040 | 1.63 | | 1273.33 | 2616.38 | 97.71 | 3.27 |
| | 9.209 ± 0.199 | 2.16 | 9.213 ± 0.173 | 1.88 | | 1456.67 | 2814.46 | 96.71 | 1.18 |
| Daidzin (**17**) | 0.426 ± 0.008 | 1.90 | 0.444 ± 0.013 | 3.13 | 1030.50 | 777.60 | 1766.65 | 94.67 | 2.85 |
| | 2.263 ± 0.036 | 1.58 | 2.280 ± 0.044 | 1.95 | | 972.00 | 2064.23 | 96.78 | 1.27 |
| | 8.679 ± 0.167 | 1.93 | 8.830 ± 0.160 | 1.81 | | 1166.40 | 2289.05 | 97.11 | 2.48 |
| Notoginsenoside R1 (**33**) | 0.0223 ± 0.0007 | 3.18 | 0.0228 ± 0.0008 | 3.66 | 82.46 | 63.94 | 147.15 | 101.18 | 2.09 |
| | 0.110 ± 0.0006 | 0.59 | 0.110 ± 0.0007 | 0.66 | | 79.92 | 168.54 | 98.01 | 2.06 |
| | 0.436 ± 0.008 | 1.83 | 0.443 ± 0.012 | 2.91 | | 95.90 | 191.69 | 102.50 | 4.77 |
| Daidzein (**34**) | 0.0854 ± 0.0019 | 2.32 | 0.0854 ± 0.0014 | 1.68 | 204.22 | 154.40 | 365.98 | 104.76 | 2.76 |
| | 0.471 ± 0.003 | 0.74 | 0.471 ± 0.002 | 0.61 | | 193.00 | 429.50 | 106.22 | 1.85 |
| | 1.698 ± 0.036 | 2.16 | 1.701 ± 0.039 | 2.29 | | 231.60 | 452.00 | 96.29 | 3.32 |
| Ginsenoside Rg1 (**35**) | 0.0522 ± 0.0014 | 2.69 | 0.0533 ± 0.0024 | 4.60 | 423.93 | 351.20 | 774.80 | 99.91 | 1.67 |
| | 0.286 ± 0.002 | 0.95 | 0.286 ± 0.003 | 1.16 | | 439.00 | 891.96 | 97.02 | 1.15 |
| | 1.177 ± 0.003 | 3.10 | 1.195 ± 0.041 | 3.50 | | 526.80 | 989.36 | 96.60 | 4.90 |
| Ginsenoside Re (**36**) | 0.0134 ± 0.0002 | 1.99 | 0.0137 ± 0.0005 | 3.76 | 74.22 | 60.51 | 136.22 | 102.46 | 2.48 |
| | 0.0777 ± 0.0011 | 1.44 | 0.0778 ± 0.0014 | 1.75 | | 75.64 | 156.42 | 98.89 | 2.45 |
| | 0.313 ± 0.011 | 3.77 | 0.316 ± 0.011 | 3.59 | | 90.77 | 173.07 | 98.02 | 1.43 |
| Salvianolic acid B (**40**) | 0.632 ± 0.030 | 4.71 | 0.620 ± 0.030 | 4.80 | 2901.77 | 2440.80 | 5467.96 | 105.13 | 2.28 |
| | 3.052 ± 0.012 | 0.39 | 3.081 ± 0.013 | 0.44 | | 3051.00 | 6428.54 | 105.19 | 4.70 |
| | 10.615 ± 0.225 | 2.12 | 10.680 ± 0.243 | 2.28 | | 3661.20 | 7034.31 | 103.84 | 3.75 |
| Genistein (**42**) | 0.0122 ± 0.0004 | 3.74 | 0.0123 ± 0.0004 | 3.68 | 8.3859 | 6.5952 | 14.95 | 99.52 | 0.10 |
| | 0.0564 ± 0.0005 | 0.89 | 0.0564 ± 0.0006 | 1.09 | | 8.2440 | 17.29 | 98.30 | 1.21 |
| | 0.208 ± 0.003 | 1.67 | 0.212 ± 0.007 | 3.52 | | 9.8928 | 18.93 | 95.94 | 4.23 |
| Ginsenoside Rb1 (**45**) | 0.111 ± 0.001 | 1.22 | 0.110 ± 0.002 | 2.61 | 377.24 | 316.00 | 698.45 | 101.65 | 0.61 |
| | 0.555 ± 0.009 | 1.63 | 0.555 ± 0.011 | 1.99 | | 395.00 | 805.19 | 98.59 | 0.68 |
| | 2.134 ± 0.083 | 3.91 | 2.203 ± 0.089 | 4.05 | | 474.00 | 922.62 | 103.55 | 2.95 |
| Ginsenoside Rg2 (**46**) | 0.0125 ± 0.0002 | 1.82 | 0.0121 ± 0.0002 | 2.24 | 20.70 | 16.70 | 37.71 | 101.88 | 3.44 |
| | 0.0819 ± 0.0024 | 2.96 | 0.0828 ± 0.0021 | 2.55 | | 20.88 | 43.34 | 98.70 | 1.64 |
| | 0.305 ± 0.009 | 2.99 | 0.312 ± 0.012 | 3.88 | | 25.05 | 48.15 | 98.63 | 2.17 |
| Ginsenoside Rd (**49**) | 0.0194 ± 0.0004 | 2.34 | 0.0188 ± 0.0005 | 2.63 | 72.79 | 58.80 | 129.65 | 96.69 | 4.96 |
| | 0.105 ± 0.001 | 1.52 | 0.103 ± 0.003 | 3.19 | | 73.50 | 151.78 | 97.79 | 2.33 |
| | 0.429 ± 0.013 | 3.03 | 0.437 ± 0.015 | 3.52 | | 88.20 | 167.30 | 96.44 | 1.80 |

sis due to their fragmentation pathways, as well as by comparing with standards and previous studies [8,9,22,23]. The neutral loss of 120 Da was attributed to the characteristic ion $[M-H-C_4H_8O_4]^-$ or $[M+H-C_4H_8O_4]^+$ of *C*-glycoside in negative or positive ion mode, respectively [23]. Interestingly, the isoflavone-*O*-glycosides compounds were more easy to be detected the $[M+46-H]^-$ adduct ions than $[M-H]^-$ ions in negative ion mode, this may be an easy way to distinct *O*-glycosides from *C*-glycosides. In addition, peaks **21**, **25**, and **31** were identified as pueroside A, pueroside B, and sophoraside A by comparing with literature [24]. Moreover, 10 triterpenoid saponins (peaks **33**, **35**, **36**, **44**, **45**, **46**, **47**, **49**, **50**, and **51**) were verified by careful studies of the MS and $MS^n$ spectra, and by comparison with available standards and reference [7,25,26]. Distinct $[M+46-H]^-$ adduct ions in negative ion mode and $[M+Na]^+$ adduct ions in positive ion mode were observed for the saponins, respectively. Most of them exhibited fragmentation patterns corresponding to the loss of sugar units (162 Da, 146 Da, or 132 Da) and $H_2O$ (18 Da).

Here, peak **14** at the retention time of 45.18 min was chosen as an example for the illustration of the identification approach. The base peak of its $[M-H]^-$ ion at $m/z$ 445.1133 (calcd. 445.1129), as well as its $[M+H]^+$ at $m/z$ 447.1274 (calcd. 447.1286), which deduced the most possible elemental composition of this compound as $C_{22}H_{22}O_{10}$. Additionally, in the $MS^2$ spectra in negative ESI, the neutral loss of 120 Da was attributed to the characteristic ion $[M-H-C_4H_8O_4]^-$ which indicated peak **14** was an isoflavone-*C*-glycosides. In the $MS^3$ spectra, the loss of 15 Da indicated the existence of "$-CH_3$" group. Additionally, the fragmentation in positive ion mode was initiated by the direct loss of series $H_2O$ and $CH_2O$ groups which evidenced the results in negative ion mode. Thus, peak **14** was tentatively identified as 3′-methoxypuerarin, further confirmed by comparing with reference compound. Spectra

**Table 5**
The contents (μg/g) of 15 analytes in XKS preparations ($n = 3$).

| Samples No. | 3 | 4 | 10 | 13 | 14 | 17 | 33 | 34 | 35 | 36 | 40 | 42 | 45 | 46 | 49 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C1 | 913.74[a] | 1277.51 | 70.74 | 4401.45 | 1131.62 | 3083.80 | –[b] | 787.64 | 3.48 | – | 2816.31 | 34.50 | 7.89 | 17.15 | – |
| C2 | 778.75 | 1206.58 | 63.40 | 4127.40 | 1046.45 | 2472.72 | – | 751.77 | 2.47 | – | 2715.18 | 35.43 | 4.17 | 13.50 | – |
| D1 | 4817.31 | 1239.73 | 224.53 | 5547.31 | 1503.33 | 3284.55 | 130.89 | 435.90 | 415.19 | 74.62 | 6991.73 | 22.87 | 650.42 | 38.27 | 197.56 |
| D2 | 5392.91 | 1353.75 | 224.75 | 5818.70 | 1615.46 | 3941.07 | 139.90 | 503.08 | 518.61 | 90.34 | 8399.92 | 27.85 | 795.15 | 49.01 | 209.84 |
| S1 | 5123.96 | 2063.20 | 225.19 | 6052.49 | 1771.11 | 4352.41 | 138.56 | 456.47 | 538.65 | 89.27 | 7388.19 | 25.30 | 806.25 | 38.55 | 246.85 |
| S2 | 3156.35 | 1204.23 | 130.59 | 5975.96 | 1719.08 | 3928.48 | 116.35 | 512.73 | 498.38 | 74.04 | 5184.26 | 24.82 | 743.31 | 30.87 | 228.51 |
| H1 | 7615.42 | 795.98 | 20.97 | 5242.35 | 1504.62 | 3133.06 | – | 931.57 | 83.41 | 197.52 | 2623.29 | 38.29 | 87.34 | 53.47 | 169.12 |
| H2 | 4304.99 | 1107.02 | 69.68 | 4551.93 | 1346.85 | 2163.81 | 143.96 | 608.59 | 505.16 | 61.76 | 723.86 | 14.44 | 747.01 | 33.57 | 354.02 |
| L1 | 1313.22 | 478.38 | 32.46 | 831.99 | 223.70 | 285.45 | 108.88 | 79.09 | 345.64 | 55.11 | 2535.48 | 1.38 | 568.36 | 19.35 | 147.01 |
| L2 | 560.04 | 77.46 | 55.90 | 2185.91 | 589.06 | 1408.85 | 102.80 | 247.98 | 402.99 | 67.81 | 4055.72 | 7.17 | 620.23 | 21.82 | 147.31 |
| X | 5413.71 | 1414.89 | 41.99 | 5172.65 | 1505.40 | 2805.93 | 19.53 | 776.99 | 80.16 | 10.85 | 2506.60 | 27.88 | 192.37 | 3.46 | 42.01 |
| Y | 5240.92 | 1152.19 | 77.64 | 3718.18 | 807.47 | 1378.44 | 6.13 | 435.66 | 36.77 | 4.61 | 2242.38 | 9.59 | 57.97 | 22.97 | 10.42 |
| J | 5720.50 | 753.69 | 116.17 | 3827.14 | 898.58 | 1552.40 | 195.20 | 748.54 | 645.55 | 76.32 | 1023.37 | 28.29 | 906.82 | 64.80 | 309.55 |
| Range[c] | 0.06–6.99 | 0.52–5.84 | 1.44–6.08 | 0.78–5.77 | 1.71–5.26 | 0.68–5.74 | 1.31–5.55 | 0.37–4.70 | 0.32–5.84 | 0.51–5.57 | 2.59–5.83 | 1.09–5.51 | 0.77–5.84 | 0.20–4.89 | 0.79–5.16 |

[a] The data was present as average of triplicates.
[b] Under the limit of detect.
[c] Range: the range of RSD% of each compound in all tested samples.

of ion fragments in $MS^n$ analysis and the proposed fragmentation pathway of 3′-methoxypuerarin in negative ion mode were shown in Fig. 3.

Some peaks, for example peaks **9** and **19**, were the isomeric compounds with the same quasi-molecular and similar fragment ions in $MS^n$ spectra, in which the consecutive neutral losses of 120 Da and 28 Da from $[M-H]^-$ of $m/z$ 431.0974 were observed. But they showed different retention behavior on column which was helpful for the identification. Comparing with previous study [8], peak **9** was identified as 3′-hydroxypuerarin and peak **19** was attributed to genistein-8-*C*-glucoside. Similarly, peaks **12** and **20** were identified as 3′-hydroxypuerarin xyloside and genistein-8-*C*-apiosyl (1-6)-glucoside. Though some position isomers were hardly discriminated using only their mass spectrometric data and several kinds of possibility were listed in Table 2.

Among the identified compounds, peaks **3**, **4**, **10**, **13**, **14**, **17**, **33**, **34**, **35**, **36**, **40**, **42**, **45**, **46**, and **49** were unambiguously attributed to danshensu (**3**), protocatechualdehyde (**4**), chlorogenic acid (**10**), puerarin (**13**), 3′-methoxypuerarin (**14**), daidzin (**17**), notoginsenoside R1 (**33**), daidzein (**34**), ginsenoside Rg1 (**35**), Re (**36**), salvianolic acid B (**40**), genistein (**42**), ginsenoside Rb1 (**45**), Rg2 (**46**), and Rd (**49**) by comparing individual peak retention times and mass spectral data with the reference substances. Further study was to establish a sensitive and reliable quantitative method for quantitative analysis of these compounds in XKS preparations from different companies.

### 3.3. Method validation of the quantitative analysis

The calibration curves, linear ranges, LOD, LOQ, and repeatability of 15 analytes were performed using the developed HPLC–MS method (Table 3). Reasonable correlation coefficient values ($r^2 > 0.9983$) indicated good correlations between investigated standards concentrations and their peak areas within the ranges tested. The ranges of LOD and LOQ for all the analytes were from 0.00101 to 0.0297 μg/mL, and 0.00304 to 0.104 μg/mL, respectively. The repeatability present as RSD ($n = 5$) was between 0.66% and 4.92% of the 15 compounds. The overall intra- and inter-day variations (RSD) of the 15 analytes were in the range from 0.29 to 4.71%, and 0.36 to 4.80% (Table 4), respectively. The developed method had good accuracy with the recoveries were between 94.60% and 106.22% (Table 4). Therefore, the results demonstrated that the LC–ESI-MS method was sensitive, precise, and accurate enough for quantitative evaluation of multi-compound in XKS preparations.

### 3.4. Quantitative determination of XKS preparations

Typical chromatograms for the quantitative determination of 15 marker compounds in XKS preparations are shown in supplementary data (Fig. S1). A total of 13 different batches of XKS preparations from 7 independent pharmaceutical companies were tested using the developed LC–ESI-MS method. The contents ($n = 3$) of 15 investigated compounds were summarized in Table 5. It was recognized that danshensu (**3**), protocatechualdehyde (**4**), puerarin (**13**), 3′-methoxypuerarin (**14**), daidzin (**17**), daidzein (**34**), and salvianolic acid B (**40**) were the dominant compound in all examined samples. However, the contents of each compound or the total content of certain type of constituents varied in different XKS preparation, even in different batches from the same pharmaceutical manufacturer. For example, danshensu (**3**) was abundant in the batches of D1, D2, S1, S2, H1, H2, X, Y, and J, but lower in the batches of C1, C2, L1, and L2. Moreover, the content of danshensu (**3**) in batch L1 is twice higher than that in batch L2, both of which produced from the same company. Triterpenoid saponins (peaks **33**, **35**, **36**, **45**, **46**, and **49**), the important compounds came from *Panax noto-*

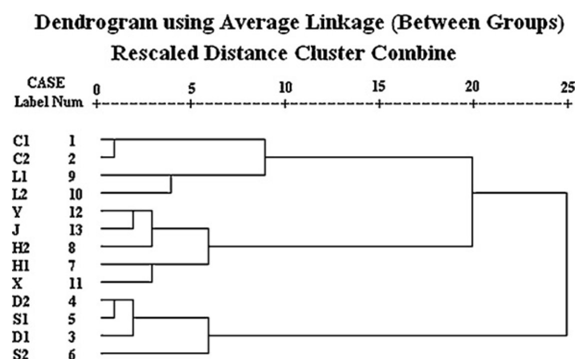


**Fig. 4.** Dendrogram of hierarchical cluster analysis for the 13 investigated batches of XKS preparations. The hierarchical cluster was done by SPSS software. Between-group linkage method was applied, and squared Euclidean distance was selected as measurement. Dengrogram resulted from the contents of 15 analytes in the investigated samples.

*ginseng* in the preparations, were hardly detected in the batches of C1 and C2, while the contents of those compounds were much higher in the batch of J. Additionally, the batches of H1 and H2 from the company of Hebei Guojin, as well as the batches of L1 and L2 from the company of Sichuan Longren, showed marked variations in almost all the analytes. In order to evaluate the variations in detail, hierarchical cluster analysis was performed based on the contents of 15 analytes of 13 investigated batches. Between-groups linkages method was applied, and Squared Euclidean distance was selected as measurement. Fig. 4 shows the results on the investigated batches of XKS preparations, which were divided into two main clusters. The results suggested that the contents of 15 analytes were relatively more stable and higher in the batches of D1, D2, S1, and S2 from the company of Shandong Wohua than in the batches from others.

## 4. Conclusion

An efficient and sensitive method employing liquid chromatography coupled with linear trap quadrupole and high resolution mass analyzer-orbitrap (LC–LTQ–Orbitrap) was developed for qualitative and quantitative analysis of chemical constituents of XKS preparations. 51 compounds including phenolic acids, isoflavone-*C*-glycosides, isoflavone-*O*-glycosides, flavonoids, and triterpenoid saponins, were characterized on the basis of retention behaviors, abundant MS and $MS^n$ data, or by comparing with reference substances and literatures. All compounds identified were found to be existed in individual traditional Chinese medicines of XKS preparation. However, the constituents from the roots of *Aucklandia lappa* (Mu-Xiang) were not detected. Further investigation focused on those lipophilic constituents in XKS preparation is required.

An optimized LC–ESI-MS method was then established for assay of the 15 marker compounds in XKS preparations from various pharmaceutical companies. The validation of the method, represent a good accuracy, sensitivity and repeatability. The quantification results indicated an obvious difference of marker compounds contents among various commercial samples.

This is the first report on the comprehensive determination of chemical constituents in XKS preparations by LC–ESI-$MS^n$. The results would provide the chemical support for the further pharmacokinetic studies and for the improvement of quality control of XKS preparations. The study also suggested that LC–LTQ-Orbitrap mass spectrometry would be a powerful and reliable analytical tool for the characterization of chemical profile in complex chemical system, such as TCM preparations.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.03.045.

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