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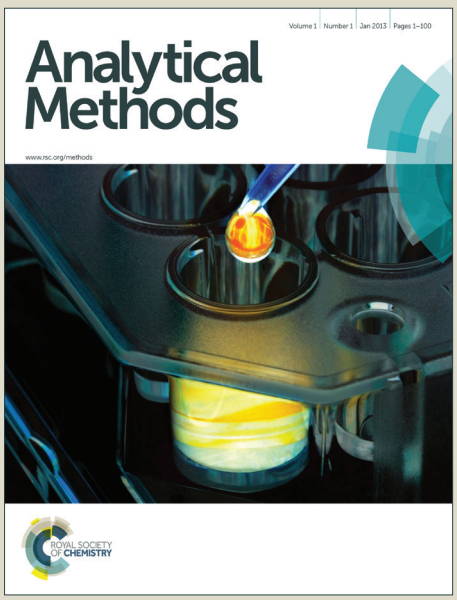





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# Analysis of six bioactive components in Semen Ziziphi Spinosae by UPLC-ELSD and UPLC-Q/TOF-MS

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## Abstract

A simple and sensitive method was firstly developed for the simultaneous determination of six bioactive compounds in Semen Ziziphi Spinosae, which is commonly used in traditional Chinese medicines by ultra-performance liquid chromatography coupled with evaporative light scattering detector (UPLC-ELSD). Furthermore, the main compounds were identified using UPLC coupled with electrospray ionisation and time-of-flight mass spectrometry (ESI–Q/TOF–MS). Separation of the compounds of interest was performed on a BEH C18 column with acetonitrile and water (0.1% aqueous formic acid) as mobile phase. Six analytes (spinosin, jujuboside A, jujuboside D, jujuboside B, jujuboside $B_1$, betulinic acid) demonstrated good linearity ($r^2$>0.9984) in a relatively wide concentration range. The method revealed high average recovery (range, 94.36-99.49%) and good precision with interday and intraday variations with RSD less than 4.72%. The limits of detection (LOD) ranged from 10.4~31.2 ng, while the limits of quantification (LOQ) were defined in the range of 21.9-84.0 ng. The validated method was successfully applied to quantitatively analyze 28 samples of different places from China. The results show there are great variations among the contents of the six ingredients. These results demonstrat that this approach has the potential for quality control of Semen Ziziphi Spinosae.


**Keywords:** Semen Ziziphi Spinosae; UPLC-ELSD; bioactive components; quality control

## 1 Introduction

Semen Ziziphi Spinosae (SZS), dried ripe seed of *Ziziphus jujube* Mill. Var. *spinosa* (Bunge) Hu ex H.F. Chou (Rhamnaceae), is commonly applied in traditional Chinese medicines for the treatment of insomnia and anxiety, [1] and also used as food in southeast of Asia.[2, 3] The broad usage of SZS has made it essential for the implement quality control of this herbal medicine. For this purpose, reported studies have shown three main species of active components are investigated including saponins, flavonoids and triterpenoids from the SZS. Phytochemical investigations revealed the presence of saponins and flavonoids,[4-8] which is partially responsible for hypnotic-sedative and anxiolytic activities of SZS.[9,10] Meanwhile, spinosin and jujuboside A are chosen as marker compounds to assess the quality of SZS in the Chinese pharmacopoeia (ChP). In addition, betulinic acid exhibits antitumor, anti-HIV, antiviral, anti-leukaemia, anti-inflammatory, antimicrobial and antihelmintic activities.[11-14] Therefore, a simple, effective and suitable targeted method for accurate analysis of these active ingredients is necessary requisite.

Although several analytical methods have been employed to simultaneously determine some of the major compounds based on HPLC, [4-7, 15-17] a rapid and validated multi-components analytical method is still required. Compared with the conventional HPLC, UPLC has the advantages of shorter running time, greater resolution, higher sensitivity and less solvent consumption.[18-20] UPLC-MS has also more frequently been applied in qualitative analysis on medicinal plants in recent years.[21-23] To the update of our knowledge, simultaneous analysis of flavonoids, saponins and triterpenoids of SZS using UPLC coupled with ELSD and MS is not available.

The objectives of the present study is to develop an analytical method for the rapid, simple and accurate determination of the six main bioactive components (spinosin, jujuboside A, B, $B_1$, D, and betulinic acid) in SZS by UPLC-ELSD and UPLC-Q/TOF-MS. The proposed method was validated by evaluating the linearity,

accuracy, precision and recovery, and results demonstrated that it can be successfully applied to the quality control of SZS.

## 2 Experimental

### 2.1 Materials and reagents

Semen Ziziphi Spinosae materials were collected from different origins in China. These samples were authenticated by professor Yulin Lin, Institute of Medicinal Plant Development, Peking Union Medical College, Chinese Academy of Medical Sciences.

Spinosin (11869-201203), jujubosides A (110734-200510), jujubosides B (110814-200607) and betulinic acid (111802-201001) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Jujubosides $B_1$ (20120527) and jujubosides D (20120605) were purchased from Dalian Melone Pharmaceutical Co. Ltd. (Dalian, China). The purity of all the compounds was more than 98.0%. The structures of the six compounds are shown in Figure 1. Acetonitrile (ACN) and methanol of HPLC-grade were obtained from Fisher Co. Ltd. (Emerson, IA, USA). Ethanol and formic acid of analytical grade were purchased from Beijing Chemical Works (Beijing, China). Water for UPLC analysis was purified by a Milli-Q academic water purification system (Milford, MA, USA). All the reagents and sample solutions were filtered through a 0.22 μm PTFE membrane (Agela Technology, Tianjin, China) prior to injection into the UPLC system.

### 2.2 Instruments and conditions

The analysis was performed on an Acquity$^{TM}$ UPLC H-Class system (Waters Corp., Milford, MA, USA) including quaternary solvent manager, sampler manager, column compartment and evaporative light scattering detector (ELSD), connected to Waters Empower 2 data station. An Acquity BEH C18 column (100 × 2.1 mm i.d., 1.7 μm; Waters Corp., Milford, MA, USA) was employed for the separation at 35℃. A gradient elution of A (0.1% aqueous formic acid) and B (acetonitrile) was used as

follows: 0–3 min, 20%–40% B; 3–6 min 40% B; 6–7 min, 40%–88% B; 7-15min, 88-100% B. The flow rate was 0.3 mL/min and the injection volume was 5.0 μL for UPLC analysis. The draft tube temperature of ELSD system was 50℃, pressure of nebulizer gas (nitrogen) was 25 psi, the nebulizer was heating mode (60% power level) and detector gain was 135.

### 2.3 Mass spectrometry identification

The extract was identification using the same UPLC condition as mentioned above on the Waters Acquity UPLC system (Waters, USA) coupled with a quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Waters Q/TOF Premier $^{TM}$). The MS analysis was carried out by the ESI source in both positive and negative ion mode. The ESI source was operated with the capillary voltage and cone voltage set at 3000 and 40 V, respectively. The temperature of the source and desolvation was set at 120 and 450°C, respectively. The nitrogen flows were adjusted to 30 L/h for the cone gas and 450 L/h for the desolvation gas. The scan time was set at 1s, the interscan delay at 0.02 s. All analyses were acquired using the lock spray to ensure accuracy and reproducibility; leucine-enkephalin was used as the lock mass *m/z* 556.2771 in positive and *m/z* 554.2615 in negative mode. Data were acquired in centroid mode from 100～1500 mass-to-charge radio (*m/z*) in MS scanning. Centroided and integrated MS data from UPLC-ESI-Q/TOF-MS were processed to generate a multivariate data matrix using MassLynx (Waters Crop.).

### 2.4 Preparation of standard solutions

Standard stock solutions of six analytes accurately weighted reference compounds were directly prepared in mobile phase. Working standard solutions containing each of the six compounds were prepared by diluting the stock solution with mobile phase to a series of concentrations. All the solutions were stored in the refrigerator at 4°C for analysis.

### 2.5 Samples preparation

All the samples were previously dried to constant weight at 60°C and milled into

powder before determination. Under the optimized extraction conditions, accurately weighted the samples powers of 1.0g (40 mesh) were extracted with 5 mL 70% ethanol aqueous by ultrasonication at room temperature for 80 min. The solution was cooled to room temperature and adjusted to the original weight with 70% ethanol aqueous. The obtained solution was filtered through a membrane filter (0.22 μm pore size) prior to injection into UPLC system. All samples were determined in triplicate.

## 3 Results and discussion

### 3.1 Optimization of sample preparation

To achieve the optimal extraction condition, extraction method (refluxing and sonication), extraction solvent (50%, 70%, 80% ethanol and 50%, 70% methanol, v/v), solvent volume (5, 10, and 15 mL), and extraction time (30, 45, 60, and 80 min) were investigated from the sample collected from Linfen, China. The results (Figure 2) showed that extraction efficiency of sonication and refluxing has no significant differences. Ultrasonic extraction is simple and fast. As for the extraction solvent, ethanol aqueous was found to be more suitable for the samples because it can provide the highest values in the contents of the six compounds, and has non-toxic side effects for the operator. The extraction efficiency has no significant increase with the concentration of ethanol above 70%. Furthermore, the volume of ethanol aqueous and the extraction time were also investigated to optimize the extraction procedure (Table S1). Finally, the procedures of sample preparation were decided as follows: sonication extraction with 70% ethanol, the volume of extraction solvent was 5 mL and the extraction time was 80 min.

### 3.2 Optimization of chromatographic conditions

In order to separate the compounds and improve the sensitivity, the chromatographic and detector conditions were systemically optimized. Firstly, three candidate columns with different particle sizes and lengths, including Waters Acquity UPLC HSS T3 column (50 × 2.1 mm, 1.8 μm), Waters Acquity BEH C18 column (100 × 2.1 mm, 1.7 μm) and Acquity UPLC BEH RP18 (100 ×2.1 mm, 1.7 μm), were compared to

achieve an ideal separation of the 6 target ingredients. The separation efficiency of BEH C18 column was obviously superior to the others. Then, the mobile phase was optimized by using different compositions of solvent and adjusting the gradient elution. The methanol or acetonitrile and 0.1% formic acid were usually used as the mobile phase in the previous reported methods.[17,18] Compared with methanol, acetonitrile possesses stronger elution ability, which can shorten the analysis time. It was found that the addition of formic acid could inhibit peak tailing and the application of 0.1% formic acid instead of pure water obtained good peak shape and well separated resolution. Therefore, the mobile phase consisted of acetonitrile and 0.1% formic acid. The other parameters, such as injection volume, column temperature and the flow rate of the mobile phase were optimized to obtain a reliable separation. The optimized values were: the column temperature was 35°C, flow rate was 0.3 mL/min, 5 μL as the injection volume.

Several factors influencing the S/N ratio of the ELSD, including the draft tube temperature, pressure of nebulizer gas and the mode of nebulizer, were also investigated. The two main parameters were optimized by the injection of standard solution at different draft tube temperatures (50, 60, 70 °C) and gas pressures (20, 25, 30, 35 psi) under the fixed chromatographic conditions. For the detection of analytes the selected drift tube temperature and gas pressure were optimized at 50 °C and 25 psi. The nebulizer was set at heating mode (60% power level) and the gain was 135. The results demonstrated that the above conditions were adequate and appropriate for the analysis. Typical chromatograms are shown in Figure 3 under these conditions.

### 3.3 Method Validation

#### 3.3.1 Calibration curves, limits of detection and quantification

External standard calibration lines were generated by three repeated injections of standard solutions at seven concentration levels. The reference calibrations were constructed by plotting the logarithm of average peak areas vs the logarithm of concentrations of each compound. All the compounds showed good linearity ($r^2$>0.9984) in the linear ranges. The limits of detection (LOD) and quantification

(LOQ) under the above chromatographic conditions were determinated at a signal-to-noise (S/N) ratio of 3 and 10, respectively. The reference calibration curves, linear range, $r^2$, LOD and LOQ were listed in Table 1.

### 3.3.2 Precision, repeatability and stability

The intra- and inter-day precisions were investigated by analyzing a mixed standard solution in five replicates during a single day and by duplicating the experiments on three successive days. To further confirm the repeatability of the developed assay, SZS was analyzed in five replicates with the above method. The relative standard deviation (RSD) was calculated as a measurement of precision and repeatability. The results were shown in Table 1. Stability was analyzed with sample 17 at room temperature and analyzed at 0, 4, 8, 12, 24 48, and 72 h. The results (RSD<5.0%) showed that the sample solutions were stable within 3 days at room temperature.

### 3.3.3 Accuracy

Recoveries were tested to investigate the accuracy of the method by adding three different concentration levels (high, middle and low) of the mixed standard solutions to known amounts of SZS samples. The fortified samples were then extracted and determined (n=3) with the proposed method. The ratio of determined and add amount were used to calculate the recovery. The results were shown in Table 2, and the recovery of the method was between 94.35% and 99.49%, with RSD less than 2.43%.

## 3.4 Comparison of the proposed method with others

To assess the performance of method, its analytical parameters were compared with those of the other methods used in the analysis of target ingredients in SZS. Table 3 summarizes the methods, volume of organics, LOQ, analysis time, mobile phase and sample size of some analytical methods along with the developed method. It can be seen that ultrasonic and refluxing were main extraction methods for quantitative analysis of SZS based on HPLC with different detectors. Since the ultrasound extraction has the advantages of simple, economical, and easy to scale up to the industrial level. Therefore, this paper also chose the ultrasonic extraction. The proposed method shows good linear range, repeatability and precision along with the

mentioned method. The limits of quantification (LOQ) achieved by the method are better than or comparable with the others which used ELSD detector. However, it should be noted that in some of the techniques mentioned, high sensitive detection systems such as mass spectrometry or photodiode array detector were used which are inherently more sensitive than ELSD. Also, analysis time of the method is very short due to the UPLC using a column packed with 1.7 um stationary phase and higher operating pressures. In addition, it consumes less organic solvents. Based on the above results, it can be concluded that the proposed method is a fast, simple and sensitive technique that can be used for quantification of components in SZS.

### 3.5 Application of the development UPLC method

The established analytical method was utilized for the determination of the six active ingredients in 28 samples of SZS form different regions of China. Target ingredients were identified by comparing their retention times with those obtained by injecting standard solution under the same condition. Representative chromatograms were shown in Figure 3, and the mean contents of the six compounds in 28 samples of SZS were presented in Table 4.

As shown in Table 4, it was found that the contents of the six compounds in different regional samples were significantly different. Among the 28 samples, the spinosin and betulinic acid were all detected with the concentrations from 0.86~2.19 mg/g and 0.93~3.07 mg/g, which were 2.54- and 3.30-fold variation, respectively. The contents of spinosin and jujuboside A were not less than 0.03% and 0.08% according to the ChP, respectively. And in this study, the contents of spinosin in all samples were above this level. Obviously variations could also found in saponins. The jujuboside A in three batches (Nanjing, Chengdu and Yulin) were not detected and one batch (No.7: Anguo, 0.07mg/g) did not met this standard (ChP). The analogous result was reported in the literature.[6] The jujuboside $B_1$ content of only four samples (Yanan, Xi'an, Linfen and Shengyan) were higher than LOD, with the range of 0.08~0.12 mg/g. The total amount arrangement of four saponins varied from 0 (not detected) to 1.97 mg/g in 28 samples, which was a large variation. The highest content (1.97 mg/g) of total

saponins was found in the sample from the city of Xingtai, Hebei Province, while the other three batches (Nanjing, Chengdu and Yulin) were not detected. As for the sample from the city of Xingtai, Hebei Province, which was traditionally regarded as the indigenous SZS, the content of spinosin was 2.08 mg/g (2.6 times of the standard of ChP). Lots of reasons may contribute to the difference in the level of the 6 compounds among various samples, such as geographical, environment conditions, genetic variation, drying process and storage conditions.

This newly developed UPLC method provided much higher specificity, precision and accuracy. The results mentioned above showed that the proposed UPLC-ELSD method could be applied to the quality evaluation of SZS.

### 3.6 UPLC-ESI-Q/TOF-MS identification

The developed chromatographic and MS methods were applied for identification of SZS. The identification and MS data of 17 peaks detected in positive and negative ESI modes were shown in Table 5 and Figure S1, respectively. The compounds including 5 flavonoid glycosides, 9 triterpene saponins and 3 triterpenic acids were tentatively identified. Spinosin, jujuboside A, B, $B_1$, D, and betulinic acid were identified by comparing their retention times and accurate mass with those of reference standards. MS spectra of these six ingredients in sample 17 were shown in Figure 4. For other compounds whose standards were unavailable, their structures were presumedly identified mainly based on accurate mass and frangment ions. In this study, the molecular formula were established by the protonated molecule $[M + H]^+$, sodiated adduct $[M + Na]^+$, and deprotonated molecule $[M - H]^-$ within mass error of 5.0 ppm. In addition, some compounds are isomers with the same molecular formulae, including vicenin Ⅱ (peak 1), isovitexin-2''-O-β-D-glucopyranoside (peak 2) and kaempferol-3- rutinoside (peak 4); protojujuboside B (peak 6), protojujuboside $B_1$ (peak 7) and jujuboside H (peak 8); ceanothic acid (peak 15) and epiceanothic acid (peak 16). The mass information of those constituents as follow.

Compounds **1**, **2**, **4** yielded deprotonated molecule $[M - H]^-$ at *m/z* 593.1 in negative mode, but only **1** obtained protonated molecule $[M + H]^+$ at *m/z* 595.1 in

positive mode, which suggested that molecular weight of the three ingredients was 594. Compound **1** generated consecutive fragment ions at *m/z* 473.0 $[M-H-120]^-$, 383.0 $[M-H-120-90]^-$, 353.0 $[M-H-120-120]^-$, and 296.0 $[353.0-2CO]^-$ in negative mode. These ion fragments complied with the fragmentation patters of vicenin Ⅱ.[24-26] In negative mode, compound 2 yielded fragment ions at *m/z* 413.0 $[M - H- Glu - H_2O]^-$, 353.0 $[M - H- Glu - H_2O - 60]^-$, 323.0 $[M - H- Glu - H_2O – 90]^-$ and 293.0 $[M - H- Glu - H_2O -120]^-$. And its ion fragments were in line with isovitexin-2"-O-β-D-glucopyranoside in literature reported.[26] Obviously, the two compounds regularly removed 120 and 90 Da. According to the structural characteristics of flavonoids in SZS and Wang *et al* (2009), compounds **1** and **2** were identified as vicenin Ⅱ and isovitexin-2"-O-β-D- glucopyranoside, respectively. On the other hand, compound **4** yielded fragment ions at *m/z* 285.0 $[M-H-rutinosyl]^-$ and 255.0 $[M-H-rutinosyl-CO]^-$ in negative mode, which is complied with fragmentation pathway of kaempferol-3-rutinoside by ESI mass spectrometry.[29] Therefore, compound **4** was identified as kaempferol-3-rutinoside based on above analysis results and reported literature.[25,26]

The peaks at $t_R$ 2.482, 2.645 and 2.930 min showed deprotonated molecule at *m/z* 1223.6 $[M – H]^-$ in negative mode and sodiated adduct at *m/z* 1247.6 $[M + Na]^+$ in positive mode, respectively, which corresponded to $C_{58}H_{96}O_{27}$ of protojujuboside B, protojujuboside $B_1$ and Jujuboside H. In negative ESI mode, both peaks 6 and 7 yielded characteristic ions at *m/z* 919.3 $[M – H – C_{14}H_{24}O_7$ (304 Da)$]^-$, while peak 8 produced characteristic ion at *m/z* 1081.3 $[M – H – C_8H_{14}O_2$ (142Da)$]^-$. The characteristic ion (*m/z* 1081.3) existed only in peak 8 but not in peaks 6 and 7, and the ion at *m/z* 919.3 existed only in peaks 6 and 7 but not in peak 8.[22] Therefore, peak 8 was unambiguously discriminated from peaks 6 and 7. Consequently, peak 8 was identified as jujuboside H.[22] In positive ESI mode, peaks 6 and 7 produced similar characteristic ions such as at *m/z* 943.4 $[M + Na – C_{14}H_{24}O_7]^+$, 811.4 and 327.0, suggested that two peaks underwent similar fragmentation pathways in which McLafferty rearrangement occurred.[22] While, peak 6 yield ion at *m/z* 1045.5 which may be $[M + Na]^+$ loss of 23-glucosyl moiety and one $H_2O$. And successive ions at

*m/z* 895.5, 733.4, and 455.3 were observed, which were product ions of elimination reaction occurred at sugar moiety.[22] Based on the above analysis and reported literature,[22,27] peaks 6 and 7 were deduced as protojujuboside B and protojujuboside $B_1$, respectively.

Ceanothic acid and epiceanothic acid were detected at $t_R$ 7.851 and 8.328 min respectively. They had the same dirtribution of precursor ($[M - H]^-$ at *m/z* 485.3) and fragment ions ($[M - COOH - H_2O]^-$ at *m/z* 423.2). In combination with the literature,[15,25] they were identified to be ceanothic acid and epiceanothic acid, respectively.

## 4 Conclusion

The method of ultrasound extraction with UPLC-ELSD was applied to the extraction and determination of 6 characteristic compounds in SZS. Ultrasound extraction was optimized to obtain the maximum extraction efficiency of the different ingredients. This sample pretreatment technique provided high extraction efficiency and allowed the determination of the 6 analytes in SZS with a simple and inexpensive instrument. UPLC-ELSD exhibited a reliable and powerful method for rapid quantitative analysis of 6 compounds with three different types of structures in SZS collected from different origins. UPLC offered better chromatographic resolution, shorter run time, fewer solvent and higher S/N ratio, which highly improved the analytical performance. In addition, UPLC-MS greatly facilitates the identification of compounds. The method using UPLC-ELSD in conjunction with UPLC-MS for characterization of bioactive constituents should be applied in quality control of SZS.

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## References

1 The Pharmacopoeia Commission of People's Republic of China, *Part Ⅰ:* The Pharmacopoeia of PRC, China Medical Science Press, 2010.

2 C. H. Shou, J. Wang, X. X. Zheng and D. W. Guo, *Acta. Pharmacol. Sin.*, 2001, 22, 986.

3 C. Wang, Z. L. You, Q. Xia, T. Xiong, Y. Xia, and D. Z. Yao, *Acta. Pharmacol. Sin.*, 2007, 28, 334.

4 J. Zhao, S. P. Li, F. Q. Yang, P. Li and Y. T. Wang, *J. Chromatogr. A*, 2006, 1108, 188.

5 M. Liu, Y. G. Li, G. X. Chou, X. M. Cheng, M. Zhang and Z. T. Wang, *J. Chromatogr. A*, 2007, 1157, 51.

6 M. C. Zhang, Y. Q. Zhang and J. B. Xie, *J. Pharm. Biomed. Anal.*, 2008, 48, 1467.

7 K. D. Bao, P. Li, H. J. Li and L. W. Qi, Chin. *J. Nat. Med.*, 2009, 7, 47.

8 Z. L. You, Q. Xia, F. R. Liang, Y. J. Tang, C. L. Xu, J. Huang, L. Zhao, W. Z. Zhang and J. J. He, *J. Ethnopharmacol*, 2010, 128, 419.

9 J. G. Jiang, X. J. Huang, J. Chen and Q. S. Lin, *Nat. Prod. Res.*, 2007, 21, 310.

10 L. E. Wang, X. Y. Cui, S. Y. Cui, J. X. Cao, J. Zhang, Y. H. Zhang, Q. Y. Zhang, Y. J. Bai and Y. Y. Zhao, *Phytomedicine*, 2010, 17, 404.

11 D. Z. L. Bastos, I. C. Pimentel, D. A. de Jesus and B. H. de Oliveira, *Phytochemistry*, 2007, 68, 834.

12 S. Fulda, Mol. Nutr. *Food Res.*, 2009, 53, 140.

13 F. B. Mullauer, J. H. Kessler and J. P. Medema, *Anticancer Drugs*, 2010, 21: 215.

14 Y. Feng, M. Li, J. Liu, T. Y. Xu, R. S. Fang, Q. H. Chen and G. Q. He, *Food Chem*, 2013, 136, 73.

15 S. Guo, J. A. Duan, Y. P. Tang, N. Y. Yang, D. W. Qian, S. L. Su and E. X. Shang, *J. Agric. Food Chem.*, 2010, 58, 6285.

16 S. Guo, J. A. Duan, Y. P. Tang, Y. F. Qian, J. L. Zhao, D. W. Qian , S. L. Su and E. X. Shang, *J. Pharm. Biomed. Anal.*, 2011, 56, 264.

17 L. J. Liao, T. H. Won, S. S. Kang and J. H. Shin, *J. Pharm. Inves.*, 2012, 42, 21.

18 J. J. Liu, B. Chen and S. Yao, *Talanta*, 2007, 71, 668.

19 M. Gratacós-Cubarsí, A. Ribas-Agustí, J. A. García-Regueiro and M. Castellari, *Food Chem.*, 2010, 121, 257.

20 B. Avula, Y. H. Wang, Wang M, T. J. Smillie and I. A. Khan, *J. Pharm. Biomed.*

*Anal.*, 2012, 70, 53.

21 B. Yang, W. Dong, A. H. Zhang, H. Sun, F. F. Wu, P. Wang and X. J. Wang, *J. Sep. Sci.*, 2011, 34, 3208.

22 J. J. Ma, L. P. Kang, W. B. Zhou, H. S. Yu , P. Liu and B. P. Ma, *J. Med. Plants Res.*, 2011, 5, 6152.

23 A. Taamalli, I. Iswaldi, D. Arráez-Román, A. Segura-Carretero, A. Fernández-Gutiérrezc and M. Zarrouk, *Phytochem. Anal.*, 2014, 25,89.

24 S. Sun, H. Liu, S. J. Xu, Y. Z. Yan and P. S. Xie. *J. Pharm. Anal.,* 2014.

25 Y. Bao, H. S. Yang, F. Chen, Y. L. Hua and Y. M. Jiang. *Analyst.,* 2013, 138,6881.

26 W. J. Wang, J. G. Luo and L. Y. Kong. *Chin. J. Chin. Mater. Med.,*2009, 21, 2768.

27 H. Matsuda, T. Murakami, A. Ikebata, J. Yamahara and M. Yoshikawa. *Chem. Pharm. Bull.,*1999, 12, 1744.

28 J. Z. Wang and J. S. Yang. *Chin. J. Organ. Chem.*, 2008, 28, 69.

29 M. Tomczyk, J. Gudej, M. Sochacki, *Z. Naturforsch*, 2002, 57c, 440.

**Figure captions:**

**Figure 1.** Chemical structures of the six compounds.

**Figure 2.** Effects of extraction method, solvent type, extraction time and solvent volume of the extraction efficiency of the six analytes in the Semen Ziziphi Spinosae collected from Linfen, China (Sample 17).

**Figure 3.** Typical UPLC-ELSD chromatograms of mixed standards and samples. (A) Mixed standards, (B) Sample 17 (Linfen, Shanxi ).

**Figure 4.** MS spectra of the six analytes from sample 17.

**Table captions:**

**Table 1.** Calibration curves, test range, LOD, LOQ, precision and repeatability for the six analytes.

**Table 2.** Recoveries of the six analytes.

**Table 3.** Comparison of the proposed method with other methods used in the determination of the target analytes.

**Table 4.** Quantitative analytical results of various samples (n=3).

**Table 5.** The mass data of 17 components from SZS by UPLC-Q/TOF-MS.

**Table 1** Calibration curves, test range, LOD, LOQ, precision and repeatability for the six analytes

| Aanlytes | Calibration curves[a] | $r^2$ | Linear range (μg/mL) | LOD (ng) | LOQ (ng) | Precision (RSD, %) | | Repeatability (RSD,%, n=6) |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Intraday (n=6) | Interday (n=6) | |
| Spinosin | $Y$=1.7593$X$+2.1723 | 0.9984 | 16.80-420.0 | 31.2 | 84.0 | 1.21% | 2.60% | 2.32% |
| Jujuboside A | $Y$=1.5859$X$+2.7450 | 0.9998 | 6.80-340.0 | 15.6 | 34.0 | 0.75% | 2.50% | 3.14% |
| Jujuboside D | $Y$=1.5486$X$+2.8661 | 0.9990 | 5.84-292.0 | 18.7 | 29.2 | 0.82% | 4.03% | 2.16% |
| Jujuboside B | $Y$=1.4396$X$+2.8836 | 0.9993 | 12.20-244.0 | 21.8 | 61.0 | 1.04% | 4.72% | 1.29% |
| Jujuboside $B_1$ | $Y$=1.4598$X$+2.7565 | 0.9994 | 15.30-306.0 | 23.9 | 76.5 | 0.73% | 3.96% | 2.78% |
| Betulinic acid | $Y$=0.7156$X$+5.2843 | 0.9987 | 4.38-438.0 | 10.4 | 21.9 | 1.08% | 2.88% | 1.37% |

[a] $Y$ is the logarithmic value of peak area and $X$ is the logarithmic value of the reference compound's concentration (μg/mL)

**Table 2** Recoveries of the six analytes

| Analytes | Original mean (mg/g) | Spiked mean (mg) | Found mean (mg) | Recovery mean (%) | RSD (%) (n=3) |
|---|---|---|---|---|---|
| Spinosin | 1.71 | 2.05 | 3.72 | 98.10 | 1.37 |
| | | 1.70 | 3.37 | 97.51 | 1.42 |
| | | 1.35 | 3.03 | 97.89 | 1.64 |
| Jujuboside A | 1.03 | 1.20 | 2.20 | 97.74 | 2.12 |
| | | 1.00 | 2.01 | 97.93 | 1.61 |
| | | 0.80 | 1.82 | 98.69 | 2.20 |
| Jujuboside D | 0.14 | 0.17 | 0.31 | 98.59 | 1.10 |
| | | 0.14 | 0.28 | 97.70 | 1.92 |
| | | 0.11 | 0.25 | 96.18 | 1.98 |
| Jujuboside B | 0.38 | 0.45 | 0.83 | 99.23 | 2.67 |
| | | 0.38 | 0.75 | 97.22 | 0.84 |
| | | 0.30 | 0.67 | 96.93 | 2.22 |
| Jujuboside $B_1$ | 0.12 | 0.14 | 0.26 | 96.93 | 1.48 |
| | | 0.12 | 0.24 | 99.49 | 1.78 |
| | | 0.10 | 0.22 | 97.68 | 1.44 |
| Betulinic acid | 1.65 | 2.00 | 3.54 | 94.36 | 1.44 |
| | | 1.60 | 3.15 | 94.93 | 2.42 |
| | | 1.30 | 2.86 | 94.41 | 2.40 |

**Table 3** Comparison of the proposed method with other methods used in the determination of the target analytes.

| Analytes | Methods[a] | Volume of Organics (mL) | LOQ (ng) | AT[b] (min) | Mobile phase[c] | Sample size (g) | Ref. |
|---|---|---|---|---|---|---|---|
| Spinosin, Jujuboside A, B | SE-HPLC–DAD–ELSD–MS/MS | 10 | 25.2-27.1 | 55 | MeOH-$H_2O$ (0.1% FA) | 0.2 | 17 |
| Spinosin, Jujuboside A, D, B | RE-HPLC-ELSD | 60 | 186-370 | 45 | ACN-$H_2O$ (0.1% AA) | 1.0 | 7 |
| Betulinic acid | SE-HPLC–PDA–MS/ELSD | 20 | 107 | 80 | ACN-$H_2O$ (0.2% AA) | 1.0 | 16 |
| Betulinic acid | SE-HPLC-ELSD-MS | 110 | 68.5 | 50 | MeOH (0.3%AA)-$H_2O$ (0.15%TA) | 5.0 | 15 |
| Jujuboside A, B and Betulinic acid | SSE-HPLC-ELSD | 100 | 86.1-94.2 | 20 | ACN-$H_2O$ (0.1% AA) | 1.2 | 6 |
| Jujuboside A, B | PLE-HPLC-ELSD | 11 | 16.7-18.3 | 60 | MeOH (0.1% AA)-$H_2O$ (0.1% AA) | 0.5 | 4 |
| Spinosin, Jujuboside A, B | HPLC-PDA-MS | 20 | 2.5-100[d] | 65 | ACN-$H_2O$ (0.08%FA) | 1.0 | 5 |
| Spinosin, Jujuboside A, B, $B_1$, D and Betulinic acid | SE-UPLC-ELSD | 5 | 21.9-84.0 | 15 | ACN-$H_2O$ (0.1% FA) | 1.0 | This method |

[a] Method: Applied instrumental and extraction methods; SE: sonication extraction; RE: refluxing extraction; SSE: soxhlet extraction; PLE: Pressurized liquid extraction; [b] AT: analysis time; [c] FA: formic acid; AA: acetic acid; TA: triethylamine; [d] LOD (ng/mL)

**Table 4** Quantitative analytical results of various samples (n=3).

| NO. | Source | Content（mg/g） | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | Total saponins |
| 1 | Beijing | 1.75 | 0.15 | ND[a] | <LOQ | ND | 1.86 | 0.15 |
| 2 | Beijing | 0.94 | 0.46 | 0.10 | 0.17 | ND | 1.70 | 0.73 |
| 3 | Beijing | 2.18 | 1.06 | 0.32 | 0.16 | ND | 2.47 | 1.54 |
| 4 | Xingtai, Heibei | 2.08 | 1.33 | 0.17 | 0.47 | <LOQ | 1.18 | 1.97 |
| 5 | Shijiazhuang, Hebei | 1.31 | 0.69 | 0.12 | 0.37 | ND | 1.13 | 1.18 |
| 6 | Tangshan, Hebei | 1.39 | 0.74 | 0.16 | 0.40 | <LOQ | 1.13 | 1.30 |
| 7 | Anguo, Hebei | 2.11 | 0.07 | ND | 0.11 | ND | 0.97 | 0.18 |
| 8 | Anguo, Hebei | 1.60 | 1.16 | 0.13 | 0.26 | ND | 2.45 | 1.55 |
| 9 | Anguo, Hebei | 1.30 | 0.65 | 0.11 | 0.30 | <LOQ | 1.20 | 1.06 |
| 10 | Yulin, Shanxi | 1.29 | 1.25 | 0.17 | 0.41 | ND | 1.36 | 1.82 |
| 11 | Yanan, Shanxi | 2.19 | 0.93 | <LOQ | 0.27 | 0.08 | 1.68 | 1.28 |
| 12 | xi'an, Shanxi | 0.86 | 0.57 | 0.09 | 0.21 | 0.08 | 1.66 | 0.96 |
| 13 | Xinxiang, Henan | 1.58 | 1.01 | 0.13 | 0.29 | <LOQ | 1.61 | 1.43 |
| 14 | Zhengzhou, Henan | 1.37 | 0.72 | 0.09 | 0.21 | ND | 2.05 | 1.02 |
| 15 | Puyang, Henan | 1.15 | 0.62 | 0.09 | 0.21 | ND | 2.55 | 0.91 |
| 16 | Lvliang, Shanxi | 1.04 | 0.50 | 0.08 | 0.15 | ND | 1.45 | 0.73 |
| 17 | Linfen, Shanxi | 1.71 | 1.03 | 0.14 | 0.38 | 0.12 | 1.65 | 1.67 |
| 18 | Jining, Shandong | 1.72 | 0.64 | <LOQ | 0.41 | ND | 2.14 | 1.05 |
| 19 | Zibo, Shandong | 1.60 | 0.82 | ND | ND | ND | 1.55 | 0.82 |
| 20 | Chifeng, Inner Mongolia | 0.90 | 0.56 | 0.06 | 0.14 | ND | 2.74 | 0.76 |
| 21 | Tongliao, Tnner Mongolia | 1.05 | 0.18 | ND | 0.30 | <LOQ | 1.55 | 0.48 |
| 22 | Shenyang, Liaoning | 1.31 | 1.13 | ND | 0.27 | 0.09 | 2.18 | 1.49 |
| 23 | Yinchuan, Ningxia | 2.07 | 0.14 | ND | <LOQ | <LOQ | 1.27 | 0.14 |
| 24 | Qingyang, Gansu | 1.89 | 0.73 | 0.25 | 0.58 | <LOQ | 3.07 | 1.56 |
| 25 | Bozhou, Anhui | 0.97 | 0.88 | <LOQ | <LOQ | ND | 2.08 | 0.88 |
| 26 | Nanjing, Jiangsu | 1.85 | <LOQ | ND | ND | ND | 0.93 | <LOQ |
| 27 | Chengdu, Sichuan | 2.02 | ND | ND | ND | ND | 2.73 | ND |
| 28 | Yulin, Guangxi | 1.80 | ND | ND | ND | ND | 1.41 | ND |

1: Spinosin; 2:Jujuboside A; 3:Jujuboside D; 4:Jujuboside B; 5:Jujuboside $B_1$; 6:Betulinic acid.

[a] Not detected.

**Table 5** The mass data of 17 components from SZS by UPLC-Q/TOF-MS

| No. | RT (min) | Molecular formula | $[M+Na]^+$/ $[M+H]^+$ | Error (ppm) | Frangment ions($ESI^+$, *m/z*) | $[M-H]^-$ | Error (ppm) | Fragment ions($ESI^-$,*m/z*) | Identification | Rf. |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.025 | $C_{27}H_{30}O_{15}$ | 595.1666 | 0.50 | 448.1,189.0 | 593.1505 | -0.16 | 473.0,383.0,353.0,296.0 | Vicenin Ⅱ | 24-26 |
| 2 | 1.300 | $C_{27}H_{30}O_{15}$ | - | - | - | 593.1503 | -0.50 | 413.0,353.0,323.0,293.0 | Isovitexin-2"-O-β-D-Glucopyranoside | 26 |
| 3 | 1.366 | $C_{28}H_{32}O_{15}$ | 631.1633 | -0.95 | 469.1,451.1,331.0 | 607.1669 | 0.98 | 427.0, 367.0,337.0 | Spinosin | 21,24-26 |
| 4 | 1.687 | $C_{27}H_{30}O_{15}$ | - | - | - | 593.1501 | -0.84 | 487.0,427.0,413.0,285.0,255.0 | Kaempferol-3-Rutinoside | 25,26 |
| 5 | 1.835 | $C_{38}H_{40}O_{18}$ | 785.2292 | 0.12 | 755.2,411.1,393.0,351.0, 327.0,297.0 | 783.2163 | 3.4 | 753.1,607.1,427.0,367.0,307.4 | 6-feruloylspinosin or 6-feruloulisospinosin | 21,24-26 |
| 6 | 2.482 | $C_{58}H_{96}O_{27}$ | 1247.6084 | 3.76 | 1045.3,943.4,811.4,327.0 | 1223.6049 | -0.98 | 919.3,787.3,625.3,479.2 | Protojujuboside B | 22,27 |
| 7 | 2.645 | $C_{58}H_{96}O_{27}$ | 1247.6018 | -1.52 | 1105.4,943.4,327.0 | 1223.6049 | -0.98 | 919.3,787.3,625.3,479.2,427.0 | Protojujuboside $B_1$ | 22,27 |
| 8 | 2.930 | $C_{58}H_{96}O_{27}$ | 1247.6008 | -2.32 | 1085.5,747.2,327.0 | 1223.6031 | -2.45 | 1081.5,929.4,749.3,607.1 | Jujuboside H | 22 |
| 9 | 3.145 | $C_{52}H_{86}O_{22}$ | 1085.5557 | 4.51 | 943.3,896.7,733.4,569.2,327.0 | 1061.5567 | 3.29 | 919.3,787.3,625.3,607.1,479.2 | Jujuboside G | 22,28 |
| 10 | 4.091 | $C_{58}H_{94}O_{26}$ | 1229.5923 | -0.33 | 1083.5,625.1 | 1205.5962 | 0.58 | 1073.4,911.4,749.4,603.3,455.1,323.0 | Jujuboside A | 21,22,26 |
| 11 | 4.331 | $C_{58}H_{94}O_{26}$ | 1229.5956 | -0.33 | 1097.5,1083.5 | 1205.5962 | 0.58 | 1073.49,925.4,749.4,603.3,455.1,323.0 | Jujuboside D | 26 |
| 12 | 4.831 | $C_{52}H_{84}O_{21}$ | 1067.5453 | 0.17 | 935.4,789.4 | 1043.5451 | 2.29 | 911.4,749.4,603.3,567.3 | Jujuboside B | 21,22,26 |
| 13 | 5.242 | $C_{52}H_{84}O_{21}$ | 1067.5474 | 1.50 | 935.4 | 1043.5454 | 2.58 | 911.4,749.4,603.3,567.3 | Jujuboside $B_1$ | 22,26 |
| 14 | 6.347 | $C_{54}H_{86}O_{22}$ | 1109.5517 | 0.81 | 787.3,684.2 | 1085.5562 | 2.76 | 1043.4,893.4,749.3,603.3, | Acetyljujuboside B | 22,26 |
| 15 | 7.851 | $C_{30}H_{46}O_5$ | - | - | - | 485.3272 | 1.03 | 423.2 | Ceanothic acid | 15,25 |
| 16 | 8.328 | $C_{30}H_{46}O_5$ | - | - | - | 485.3273 | 1.23 | 423.2 | Epiceanothic acid | 15,25 |
| 17 | 8.933 | $C_{30}H_{48}O_3$ | - | - | - | 455.3513 | 2.64 | 411.1 | Betulinic acid | 25 |



20

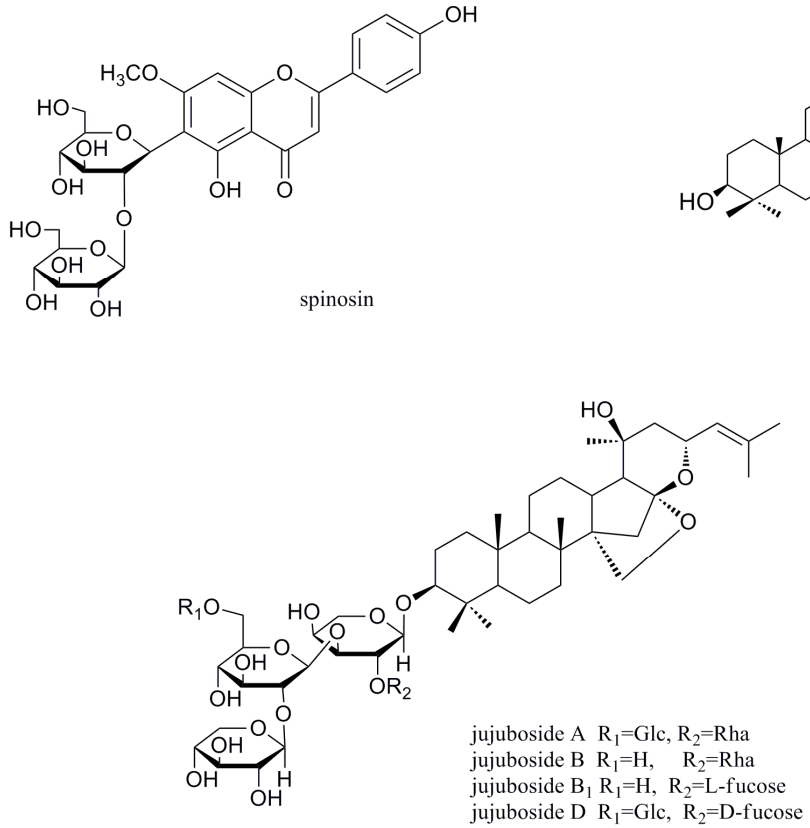



**Figure 1.** Chemical structures of six compounds.

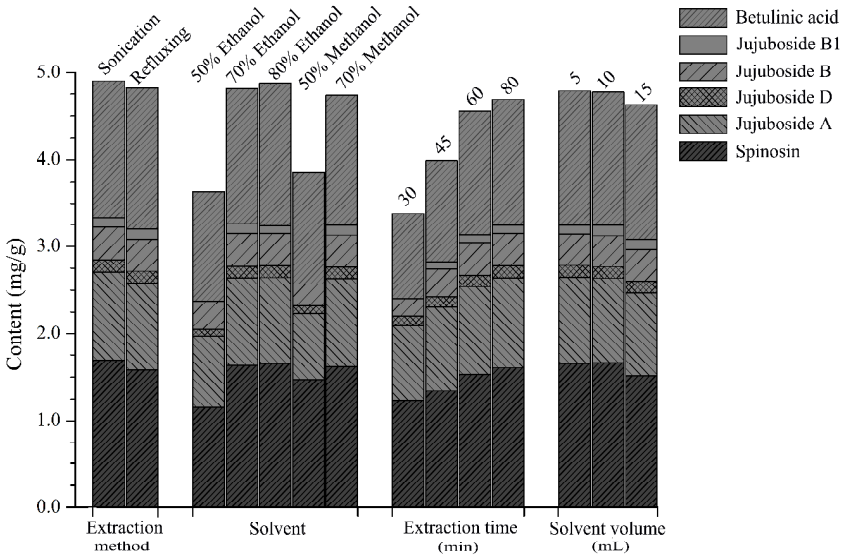



**Figure 2.** Effects of extraction method, solvent type, extraction time and solvent volume on extraction efficiency of the six analytes in the Semen Ziziphi Spinosae collected from Linfen, China (Sample 17).

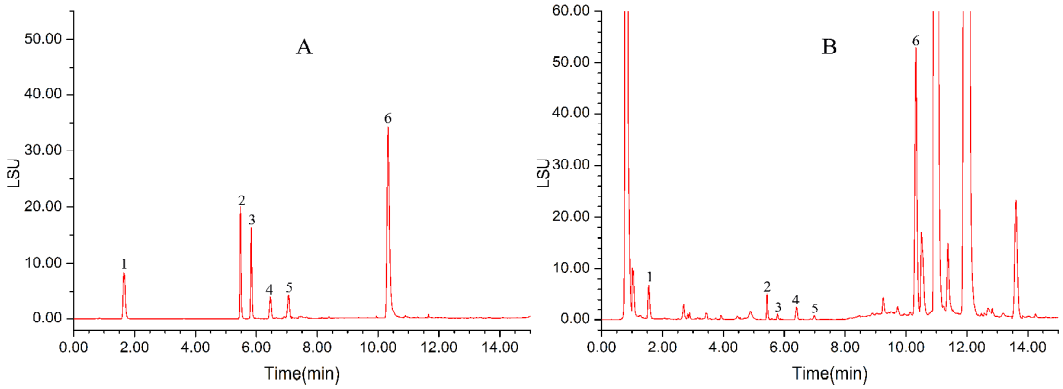



**Figure 3.** Typical UPLC-ELSD chromatograms of mixed standards and samples. (A) Mixed standards, (B) Sample 17 (Linfen, Shanxi ). 1, Spinosin; 2, Jujuboside A; 3, Jujuboside D; 4, Jujuboside B; 5, Jujuboside $B_1$; 6, Betulinic acid.

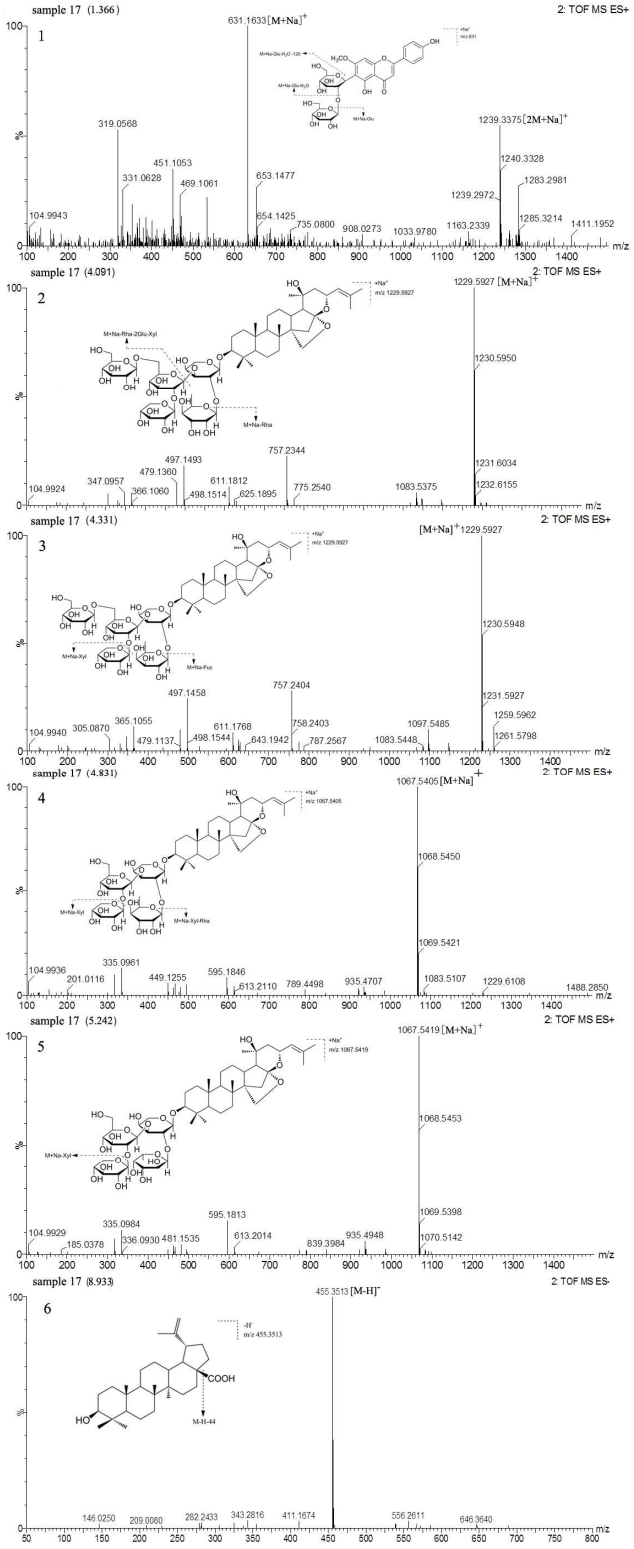



**Figure 4.** MS spectra of the six analytes from sample 17. Spinosin (1), Jujuboside A (2), Jujuboside D (3), Jujuboside B (4), Jujuboside $B_1$ (5), Betulinic acid (6).

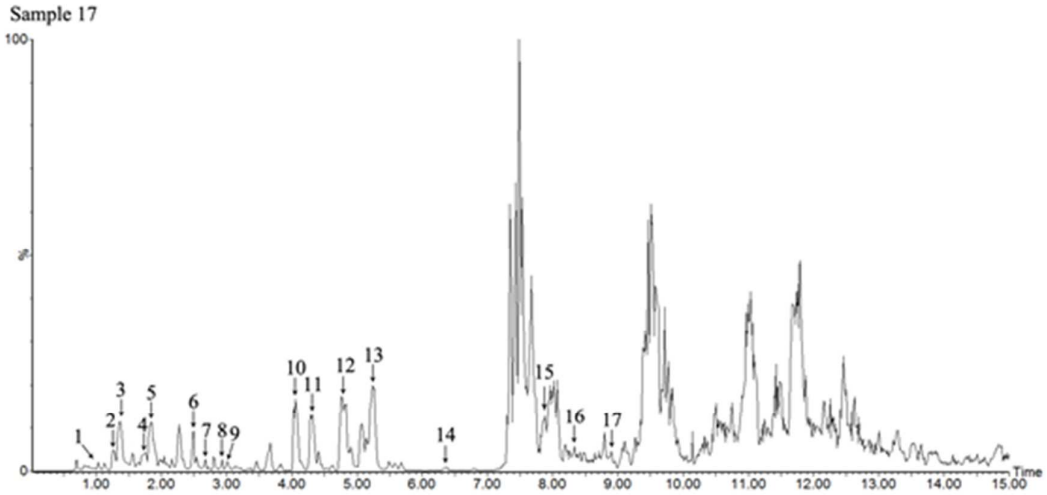
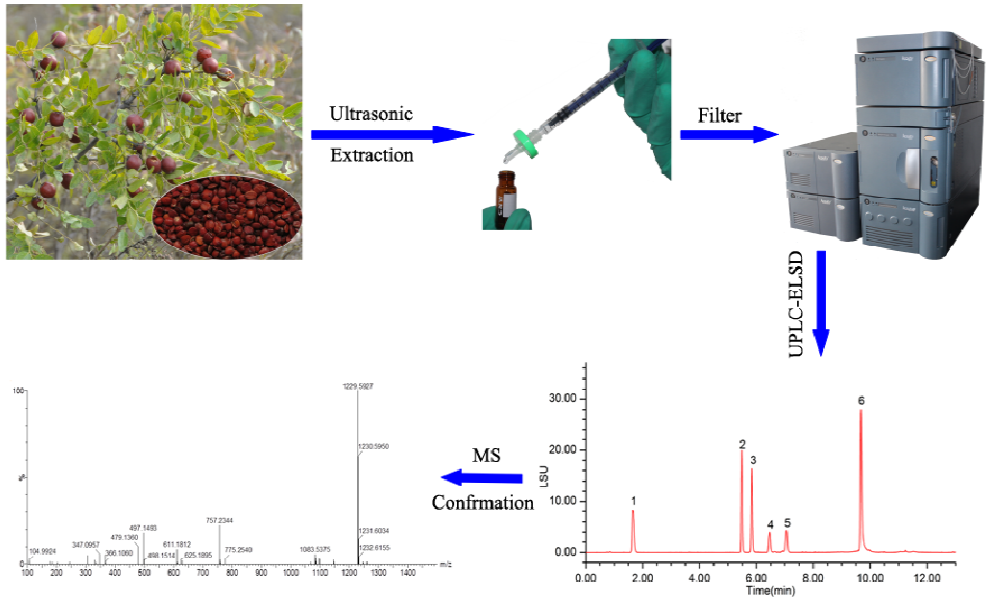



**Figure S1**. BPI chromatograms (negative modes) of the extract from SZS (sample 17) analyzed by UPLC-Q/TOF-MS.

**Table S1**. Effect of solvent volume on extraction efficiency

| Solvent volume (mL) | Content (mg/g) | | | | | |
|---|---|---|---|---|---|---|
| | Spinosin | Jujuboside A | Jujuboside D | Jujuboside B | Jujuboside $B_1$ | Betulinic acid |
| 5 | 1.71 | 1.03 | 0.14 | 0.38 | 0.12 | 1.65 |
| 10 | 1.72 | 1.05 | 0.12 | 0.34 | 0.11 | 1.63 |
| 15 | 1.68 | 0.99 | 0.13 | 0.37 | 0.11 | 1.65 |




A fast and validated multi-components analytical method base on ultrasonic extraction, detected by UPLC-ELSD and confirmed by UPLC-Q/TOF-MS was developed.