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Chemical investigation on Sijunzi decoction and its two major herbs *Panax ginseng* and *Glycyrrhiza uralensis* by LC/MS/MS

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Abstract

Sijunzi decoction consists of *Panax ginseng*, *Poria cocos*, *Atractylodes macrocephala* and *Glycyrrhiza uralensis*. High performance liquid chromatography coupled with tandem mass spectrometry (LC/MS^n) was applied to identify and characterize three types of active components, ginsenoside (from *P. ginseng*), flavonoid and triterpenoid (from *G. uralensis*) in Sijunzi decoction. Spectra of MS and MS/MS from $[M+\text{Na}]^+$ ions of ginsenosides were acquired and interpreted for their identification. Fragmentations with losing masses of 194 or 176 Da were the characteristic ions of triterpenoids in the MS/MS analysis. A characteristic fragment ion of the aglycon moiety at *m*/*z* 257 from source collision-induced dissociation was observed for flavonoid. LC/MS was also applied for the comparison of relative peak area of major active components between Sijunzi decoction and the single herb extracts. The concentration ratios of major active components detected in the individual herbs of *P. ginseng* and *G. uralensis* were found different from those in Sijunzi decoction. The experimental data indicated that the decocting process could result in the difference in the amount of active components.
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Keywords: Traditional Chinese medicine; Sijunzi decoction; LC/MS/MS

1. Introduction

Traditional Chinese medicine (TCM) has been widely used because of its high effectiveness against many diseases with low toxicity. TCM prescription is a formula of several single herbs combined at an intrinsic mass ratio. Combining the herbs together and boiled in water makes the decoction. Each herb has its own bioactivities, but when multiple herbs are combined and decocted, there may be chemical changes of active components, resulting in new bioactivities for new clinical indications. Qualitative evaluation of TCM prescription is often challenging because the active compounds may be originally from single herbs and also be resulted from the decocting process. Sijunzi decoction is one of the most famous traditional prescriptions consisting of four common herbs, namely *Panax ginseng*, *Poria cocos*, *Atractylodes macrocephala* and *Glycyrrhiza uralensis*. The decoction has been used either alone to replenish or invigorate the intestine and stomach function [1,2], or as a complement to the west drugs in treatment of liver and esophagus cancer [3,4]. Effects on cell apoptosis and related gene expression in human gastric cancer grafted onto nude mice have also been reported [5]. To investigate the effectiveness of Sijunzi decoction, characterization of active components in the individual herbs and in the prescription are crucial. Ginsenoside [6], flavonoid [7] and triterpenoid [8,9] have been regarded as the active compounds in *P. ginseng* and *G. uralensis*, respectively. Different methods have been developed for the determination of the active components in the individual crude herb [6,10–12]. However, the simultaneous determination of multiple constituents in Sijunzi decoction has not been reported. LC/MS has been extensively applied for the analysis of active components in botanical medicines and dietary supplements [13–16]. In this article, we used LC/MS^n to identify the active components in the prescription and compare the differences in concentration ratio of the chemical components between single herbs and prescription during the decocting process. The investigation focuses on the change of major components ginsenoside, flavonoid and triterpenoid during the

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decocting process in Sijunzi decoction and single herbs by using LC/MS/MS. To the best of our knowledge, this is the first report on the analysis of complicated constituents in Sijunzi decoction.

2. Experimental

2.1. Materials

Ginsenosides Rb_1 and Re were purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Rc was obtained from Sigma Chemicals (St. Louis, MO). Rg_1, R_0 and Rb_2 were provided by Dr. Z.H. Jiang, School of Chinese Medicine, Hong Kong Baptist University, Hong Kong SAR, China. Liquiritigenin-7,4′-*O*-diglucoside, liquiritigenin-4′-*O*-apiosyl-(1 → 2) glucoside and glycyrrhizic acid were obtained from Dr. H.X. Liu, Peking Union Medical College, Chinese Academy of Medical Sciences, Institute of Medicinal Plant Development, Beijing, China. *P. ginseng*, *P. cocos*, *A. macrocephala* and *G. uralensis* were purchased from Chinese Traditional Medicine Pharmacy Tong Ren Tang, Hong Kong SAR, China. Acetonitrile (ACN) was HPLC grade. Other reagents were analytical grade. Ultra-pure water was collected from a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Sample preparation of single herbs and Sijunzi decoction

The powdered sample of *P. ginseng* (1 g) was immersed in 100 ml deionized water for 1 h and then decocted to boil keeping for 30 min. The water extract was concentrated by rotary evaporator to approximate 5 ml. Half millilitre of the extract was then added with 1 ml ethanol to precipitate the polysaccharide and protein. The sample was centrifuged for 5 min and the supernatant was blown to dryness under a stream of nitrogen at 80 °C in a water bath. The extract was reconstituted with 0.5 ml water and filtered through a 0.2 μm filter (Millipore) before introduced to the HPLC analysis. The *G. uralensis* sample was prepared using the same way as that for *P. ginseng*. The powdered samples of *P. ginseng* (0.4 g), *P. cocos* (0.4 g), *A. macrocephala* (0.4 g), and *G. uralensis* (0.2 g) were immersed in 140 ml deionized water for 1 h and then decocted to boil keeping for 30 min. The sample was prepared with the same procedure as that for *P. ginseng*. During sample preparation and analysis, great care was attended to prevent cross-contamination. Under the same operation, three portions of Sijunzi decoction, three portions of *P. ginseng* extract and three portions of *G. uralensis* extract were prepared for the reproducibility and precision analysis. All sample solutions were stored at 4 °C and used at room temperature.

2.3. Liquid chromatography–mass spectrometry analysis

A Zorbax 300SB-C3 column (150 mm × 2.1 mm i.d., 5 μm) (Agilent, Palo Alto, CA, USA) was used with an injection volume of 5 μl for the HPLC separation on a HP 1100 system (Agilent Technologies, Palo Alto, CA, USA). The mobile phases consisted of (A) water-5 mM ammonium acetate-0.8% acetic acid (pH* 3.8) and (B) ACN at a flow rate of 200 μl/min (0 min 10%B, 20 min 40%B, 30 min 60%B, 30–35 min 60%B). Bruker Esquire-4000 ion-trap mass spectrometer (Bruker-Fransen, Bremen, Germany) equipped with electrospray ionization source was used for the sample analysis in positive ion mode. Selections of the target mass 1000, compound stability 100%, trap drive level 100%, collision energy 1 V, dry temperature 350 °C, dry gas 8 l/min, and nebulizer gas 30 psi were made by examination of the full scan intensity, stability, and the product ions spectra.

3. Results and discussion

The Sijunzi decoction and single herb extracts were analyzed by using LC/MS/MS under the same conditions. Major components in Sijunzi decoction as well as in *P. ginseng* and *G. uralensis* were determined. Almost 30 peaks from Sijunzi decoction were detected under the current LC/MS condition within a run of 25 min. According to the retention times and *m*/*z* values of the molecular ions, ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, R_0, liquiritigenin-7,4′-*O*-diglucoside, liquiritigenin-4′-*O*-apiosyl-(1 → 2) glucoside, and glycyrrhizic acid (Fig. 1) were identified through the comparison with the standard compounds. MS/MS was used for the structural confirmation of the above components in Sijunzi decoction.

Intensive peaks of $[M+Na]^+$ ions were observed for the ginsenosides. Ginsenosides Rg_1, Re, R_0, Rb_1, Rc and Rb_2 were identified by the chromatographic and MS spectrum comparisons with the authentic standards, while others by the interpretation of MS/MS fragmentation when the standards were not available. The aglycon ions at *m*/*z* 407 for the protopanaxdiol type ginsenosides and *m*/*z* 405 for protopanaxtriol ginsenosides were determined from the source collision-induced dissociation (CID) of the protonated molecules [17]. Furthermore, the glycosidic linkage and the attached sugar(s) were analyzed by CID from $[M+Na]^+$ ions of ginsenosides [6,17]. Usually the loss of the intact sugar unit at O–C (20) preferentially occurred during the MS/MS experiment followed by the loss of the sugar units substituted at O–C (3) or O–C (6). It was worthwhile to mention that the fragmentation of oligosaccharide at O–C (3) or O–C (6) might not only result in the cleavage of intact sugar from O–C glucosidic bond but also in the terminus sugar (Fig. 2). The above described patterns were applied for the identification of two major ginsenosides Rf and Rd. Rf at the retention time of 19.0 min (peak 4, Fig. 3(A)) was detected with its $[M+Na]^+$ ion at *m*/*z* 823 and the characteristic fragment ion at *m*/*z* 405, while Rd (23.8 min, peak 11 on Fig. 3(A)) was identified with the detection of the corresponding ions at *m*/*z* 969 and 407 [17]. In addition, two fragment ions at *m*/*z* 365 (base peak) and *m*/*z* 643 from CID of the molecular ion of *m*/*z* 823 were detected for Rf (Fig. 3(B)), indicating that there was a glu–glu unit substituted at O–C (6) or O–C (3) in the molecule. Only one intensive fragment ion at *m*/*z* 789 was observed from the CID of *m*/*z* 969 for Rd (Fig. 3(C)) resulted from liberating a glucose unit with charge on aglycon with a disaccharide substituted, which indicated a glucose group substituted at O–C (20). The obtained information of the molecular and fragment ions, combined with the general pattern of ginsenoside retention times [6], allowed

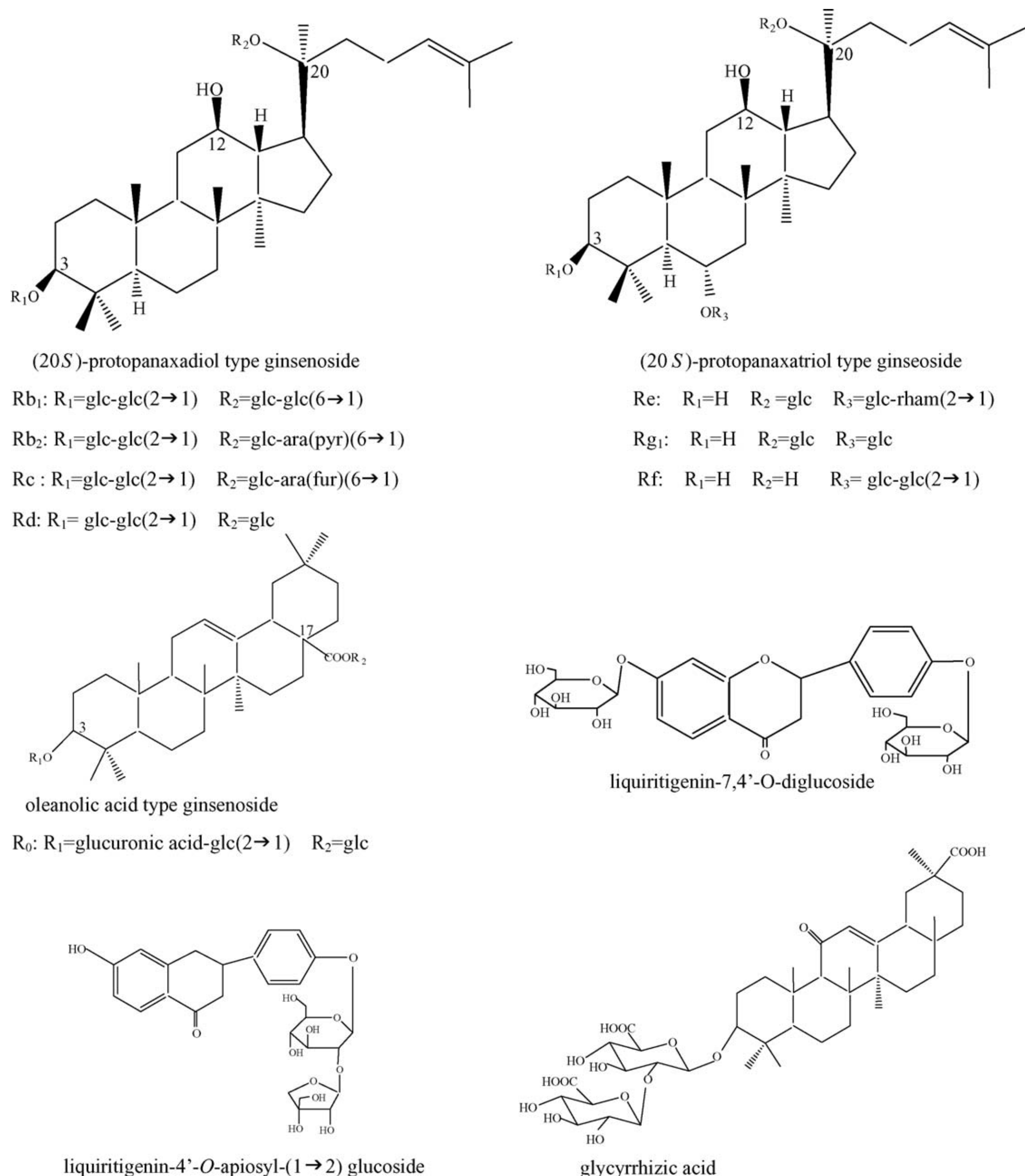

Fig. 1. Structures of the major active components in herbs *P. ginseng* and *G. uralensis* composed of the Sijunzi decoction.

the identification of the ginsenosides at *m/z* 823 and 969 as Rf and Rd, respectively.

Both $[M+H]^+$ and $[M+Na]^+$ of triterpenoids were observed in the positive ESI-MS analysis (Fig. 4). However, the use of MS/MS analysis of the protonated precursor ion provided good structural information. Fragmentations with losing masses of 194 Da (glucuronic acid) or 176 Da (deoxy-glucuronic acid) were the characteristic ions of triterpenoids from *G. uralensis*. For glycyrrhizic acid, $[M+H]^+$ ion at *m/z* 823 and $[M+Na]^+$ ion at *m/z* 845 were detected with the molecular weight of 822 Da. The MS/MS analysis of the protonated molecule produced fragment ions at *m/z* 647 and 453, demonstrating the characteristic neutral losses of 176 and 194 Da for the glucuronic acid-substituted triterpenoids. The triterpenoids with the substitution of glucuronic acid do not exist in *P. cocos*, although lanostane and secolanostane triterpenoids have been reported in *P. cocos* [18].

The LC/MS analysis of flavonoid showed that its $[M+Na]^+$ ion was much more intense than $[M+H]^+$ ion. Fig. 5(A) and (B) show the chromatograms of the detected flavonoids. Peaks 1 and 4 were identified as liquiritigenin-7,4′-*O*-diglucoside and liquiritigenin-4′-*O*-apiosyl-(1 → 2) glucoside from the chromatographic and mass spectrometric comparisons with the authentic standards. Peaks 2, 3, and 5 were identified with the detection of the $[M+Na]^+$ ions at *m/z* 735, 603 and 573, respectively. The characteristic ion of the aglycon at *m/z* 257 was observed from the source CID fragmentation of flavonoids liquiritigenin-4′-*O*-apiosyl-(1 → 2) glucoside and liquiritigenin-7,4′-*O*-diglucoside (Fig. 5(C) and (D)). Collision-induced dissociation of the $[M+Na]^+$ ion led to the cleavage of

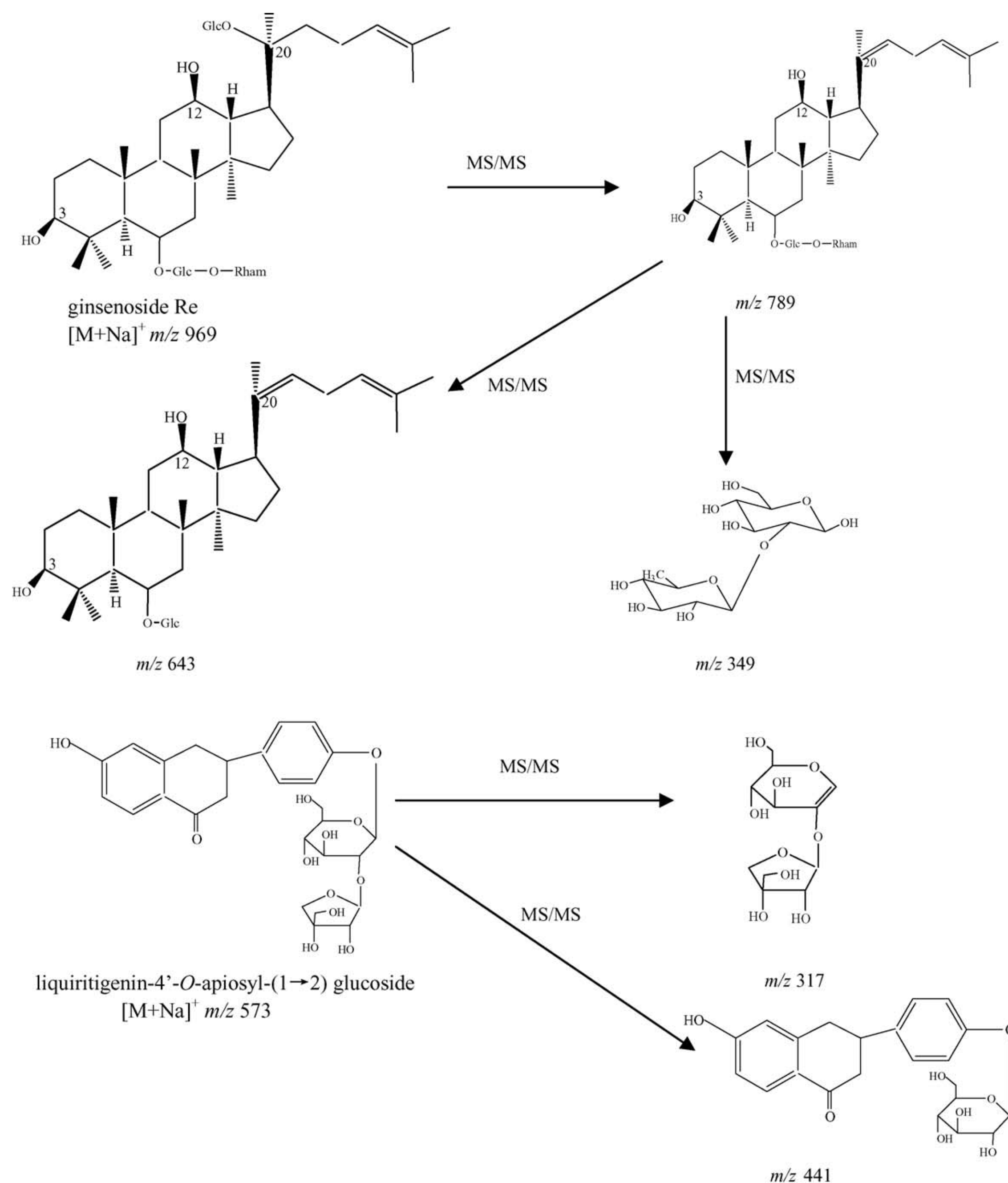

Fig. 2. Fragmentation pathway of ginsenoside Re and liquiritigenin-4′-*O*-apiosyl-(1 → 2) glucoside from their $[M+Na]^+$ ions.

glycosidic bond. Two flavonoids were conclusively identified by the comparison with the corresponding authentic standards, other three were identified with the characteristic fragmentation patterns. For liquiritigenin-4′-*O*-apiosyl-(1 → 2) glucoside, fragment ion at *m*/*z* 317 representing the sodiated ion of the deoxy-api-glu was detected in the MS/MS spectrum (Fig. 2). Another minor peak at *m*/*z* 441 resulted from the loss of the terminus deoxy-glucose was also observed.

LC/MS was also applied for the comparison of concentration ratios of the major active components in the sample extracts of single herb and Sijunzi decoction. The results from the comparison of relative peak area ratios were obtained from the analysis of three samples, each of which was injected three times for the LC/MS determination ($n = 9$). The concentration ratios of some ginsenosides, triterpenoids and flavonoids in the individual herbs and Sijunzi decoction varied significantly, indicating that the effectiveness of the decoction treatment of TCM might change the levels of active components. Fig. 3 showed the major ginsenosides detected in Sijunzi decoction. The concentration ratios of Rg_1/Rf, Re/Rf, and Rb_1/Rf were 0.77 (R.S.D. 5.7%), 0.40 (R.S.D. 4.6%) and 0.42 (R.S.D. 4.8%) in *P. ginseng*, while in Sijunzi decoction the ratios changed to 1.63 (R.S.D. 5.5%), 0.74 (R.S.D. 6.6%) and 0.72 (R.S.D. 3.5%), respectively. It is well known that *P. ginseng* and *Panax quinquefolius* (American ginseng) have different properties and medicine values, which is generally attributed to the different levels of ginsenosides in the

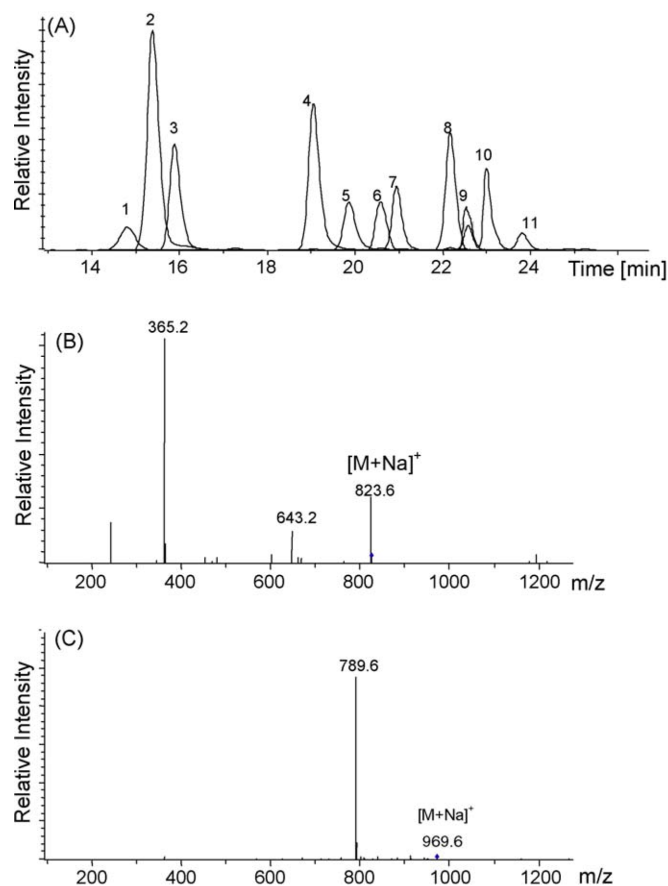

Fig. 3. Extracted ion chromatogram of ginsenosides in Sijunzi decoction (A) as well as the MS/MS spectra of Rf (B) and Rd (C). Peaks 2, 3, 4, 7, 8, 9, 10, 11 on (A) were identified as ginsenosides Rg_1, Re, Rf, R_0, Rb_1, Rc, Rb_2 and Rd, respectively. Peaks 1, 5, 6 were detected with the sodiated molecular ions at *m/z* 955, 793 and 807, respectively.

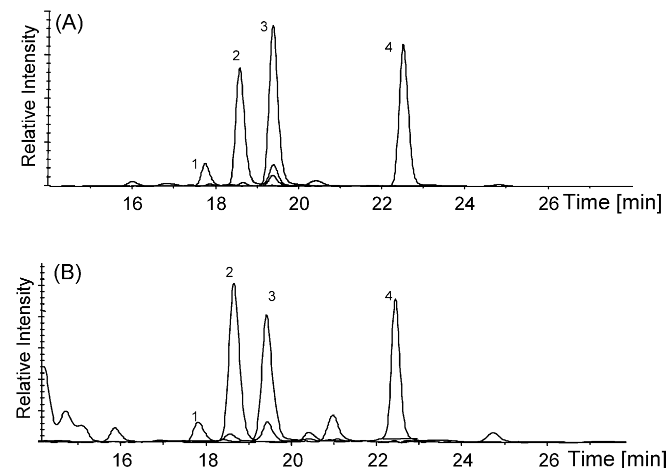

Fig. 4. Extracted ion chromatogram of triterpenoids detected in *G. uralensis* (A) and Sijunzi decoction (B). Peak 4 was identified as glycyrrhizic acid by the comparison of the authentic standard. Peaks 1, 2 and 3 were identified with the fragmentations from the protonated molecules at *m/z* 1001, 919 and 985, respectively.

ginseng products [17]. During the decocting process, chemical reactions might happen on the complicated chemical components in the four herbs. Flavonoids (with phenolic hydroxyl group) and triterpenoids (with carboxyl group) from *G. uralensis* may increase acidity of the decoction. Under acidic condition with high temperature, there may be a series of chemical reactions to cleave the glucosidic bonds in ginsenosides, resulting in different component quantities during the decoction process. Depending on the sugar numbers and attached positions, there are many ginsenosides. These ginsenosides may undergo structural changes by glycosidic bonds cleavage during the decocting process. This could explain the differences in ginsenoside concentration ratios in the single herbs versus the decoction.

Triterpenoid has been proven to have antiviral activity [19–21] and induces apoptosis in cancer cells [22]. Four triterpenoids were detected in the sample extracts of *G. uralensis* and Sijunzi decoction with $[M+H]^+$ ions at *m/z* 1001, 919, 985, and 823, respectively (Fig. 4). The concentration ratio of peak 2 (*m/z* 919) to peak 3 (*m/z* 985) increased from 0.7 (R.S.D. 9.8%) in *G. uralensis* to 1.2 (R.S.D. 8.3%) in Sijunzi decoction.

Another major type of active constituent in Sijunzi decoction is flavonoid, which can inhibit cell proliferation and induces apoptosis in human hepatoma cells [23]. Significant changes in concentration ratios of some flavonoids were also observed

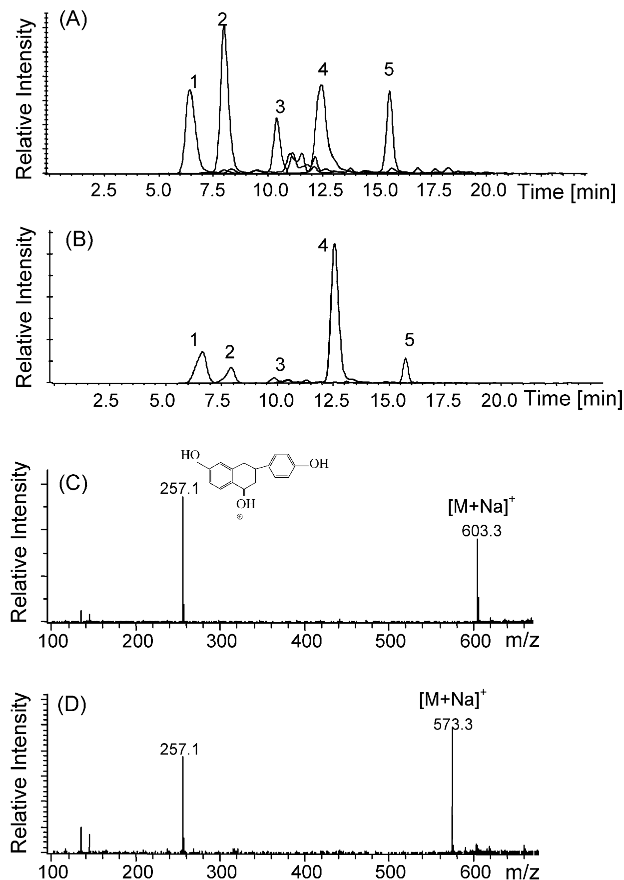

Fig. 5. Extracted ion chromatogram of flavonoids detected in *G. uralensis* (A) and in Sijunzi decoction (B) as well as the characteristic fragmentation of liquiritigenin-7,4′-*O*-diglucoside (C) and liquiritigenin-4′-*O*-apisol-(1 → 2) glucoside (D).

with the decocting process (Fig. 5). The concentration ratio of liquiritigenin-4′-*O*-apiosyl-(1 → 2) glucoside compared to the flavonoid peak 5 was found to change from 2.1 (R.S.D. 10.7%) in *G. uralensis* to 10.1 (R.S.D. 15.7%) in Sijunzi decoction. The change of the concentration ratio between the detected flavonoid with the $[M+\mathrm{Na}]^{+}$ ion at *m/z* 735 (peak 2, Fig. 5) to liquiritigenin-4′-*O*-apiosyl-(1 → 2) glucoside was more significant, from 1.1 (R.S.D. 7.4%) in *G. uralensis* to 0.17 (R.S.D. 8.3%) in Sijunzi decoction.

4. Conclusion

Three types of active components, namely ginsenoside, flavonoid and triterpenoid were identified in Sijunzi decoction by using LC/MS/MS. The concentration ratios of these components changed in the two single herbs, namely *P. ginseng* and *G. uralensis*, compared to the prescribed Sijunzi medicine. LC/MS/MS was proven a powerful technology to analyze TCM prescription that has a very complicated chemical system containing many types of compounds. Different components could de determined by their specific molecular ions and fragment ions, along with their chromatographic retention times. The differences in concentration ratios of major components between Sijunzi decoction and single herbs indicated that the decocting process may change the concentrations of constituents which may contribute to the different clinical indications.

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