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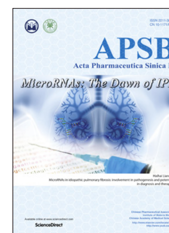

ORIGINAL ARTICLE

Identification and differentiation of *Panax ginseng*, *Panax quinquefolium*, and *Panax notoginseng* by monitoring multiple diagnostic chemical markers

Wenzhi Yang[a,b], Xue Qiao[a], Kai Li[a], Jingran Fan[a], Tao Bo[c], De-an Guo[b,*], Min Ye[a,]**

[a]*State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China*
[b]*Shanghai Research Center for Modernization of Traditional Chinese Medicine, National Engineering Laboratory for TCM Standardization Technology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China*
[c]*Agilent Technologies, Beijing 100102, China*

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Panax species;
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Abstract To differentiate traditional Chinese medicines (TCM) derived from congeneric species in TCM compound preparations is usually challenging. The roots of *Panax ginseng* (PG), *Panax quinquefolium* (PQ) and *Panax notoginseng* (PN) are used as popular TCM. They contain similar triterpenoid saponins (ginsenosides) as the major bioactive constituents. Thus far, only a few chemical markers have been discovered to differentiate these three species. Herein we present a multiple marker detection approach to effectively differentiate the three *Panax* species, and to identify them in compound preparations. Firstly, 85 batches of crude drug samples (including 32 PG, 30 PQ, and 23 PN) were analyzed by monitoring 40 major ginsenosides in the extracted ion chromatograms (EICs) using a validated LC–MS fingerprinting method. Secondly, the samples were clustered into different groups by pattern recognition chemometric approaches using PLS-DA and OPLS-DA models, and 17 diagnostic chemical markers were discovered. Aside from the previously known Rf and p-F_{11}, ginsenoside Rs_1 could be a new marker to differentiate PG from PQ. Finally, the above multiple chemical markers were used to identify the *Panax* species in 60 batches of TCM compound preparations.

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*Corresponding author. Tel.: +86 21 2023 1000x2221; fax: +86 21 50272789.
**Corresponding author. Tel./fax: +86 10 8280 2024.
E-mail addresses: daguo@simm.ac.cn (De-an Guo), yemin@bjmu.edu.cn (Min Ye).
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1. Introduction

The roots of *Panax ginseng* (PG), *Panax quinquefolium* (PQ), and *Panax notoginseng* (PN) are used as the popular traditional Chinese medicine (TCM) Ren-Shen, Xiyang-Shen, and San-Qi, respectively[1]. Chemical compositions of the three species are very similar. Nonetheless, PG, PQ and PN are considered to possess different properties in TCM theory and thus exhibit different therapeutic functions. PG has the "warm" property and is a good invigorator; PQ is "cool" and is thus capable of heat-clearing and refreshing;[2] PN is mainly used to dispel stasis and stop bleeding. These functional varieties may originate from the difference in chemical composition, particularly in the bioactive triterpenoid saponins, popularly known as ginsenosides[1]. However, chemical difference among the three *Panax* species has not been fully clarified thus far. In addition, the market prices differ remarkably among the *Panax* species (for instance, between PG and PQ), and among the same species of different production areas (for instance, PQ cultivated in China and North America). Taken together, there is great demand to establish a reliable analytical method to differentiate the *Panax* species, and to identify their raw materials in TCM compound preparations.

Many analytical approaches have been used to identify *Panax* species, including DNA barcoding[3], Raman or infrared spectrophotometry[4–6], NMR spectroscopy[7,8], and LC–MS[9–11]. Among these approaches, LC–MS appears to be the most promising one. Wang et al.[10] reported the potential significance of two pairs of ginsenosides (Rg_1/Rf and Rc/Rb_2) in the differentiation between PG and PQ by LC/MS/MS analysis. Chan et al.[11] later reported the chemical markers ginsenoside Rf and 24(*R*)-pseudoginsenoside F_{11} together with the intensity ratio of ginsenosides Rg_1/Re for species differentiation of PG and PQ. However, a limited number of markers may not be able to fully depict the chemical differences between the three species. The results could be more definitive by monitoring multiple markers.

LC–MS-based fingerprinting followed by chemometric analysis has been increasingly used for TCM analysis, which enables species differentiation of congeneric plant species[12]. Direct infusion mass spectrometry combined with chemometric analysis has been reported to differentiate *Panax* species[13,14]. Our previous study has revealed the potential taxonomic significance of certain ginsenosides (oleanolic acid type, octillol type, malonylated, and peroxidized ginsenosides) in differentiating PG, PQ, and PN[15]. In this work, we present a new approach which integrates LC–MS based fingerprinting and pattern recognition chemometrics to discover more marker ginsenosides to differentiate these three species. These markers were further used in the identification of PG, PQ, and PN in 60 batches of TCM compound preparations.

2. Materials and methods

2.1. Chemical reagents and reference standards

Ginsenosides Ro, Ra_2, Ra_3, Rb_1, Rb_2, Rc, Rd, Re, Rg_1, Rg_2, Rf, 20-*O*-glc-Rf, and notoginsenosides R_1, R_2, R_4 were isolated from the roots of PG by the authors. Their structures were fully identified by NMR analysis[15]. 20(*S*)-Ginsenosides Rg_3, Rb_3, and 24(*R*)-pseudoginsenoside F_{11} were purchased from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China). Their structures are shown in Fig. 1. The purities were >95% by LC–MS analysis. HPLC grade ammonium acetate (Fluka, Sigma–Aldrich, Netherland), formic acid, methanol, acetonitrile (J.T. Baker, Phillipsburg, NJ, USA) and ultra-pure water prepared using a Milli-Q water purification system (Millipore, MA, USA) were used for HPLC analysis. Analytical grade methanol and *n*-butanol were purchased from Damao Chemical Reagent Factory (Tianjin, China). The OASIS HLB Cartridge SPE columns were from Waters Corporation (Milford, MA, USA).

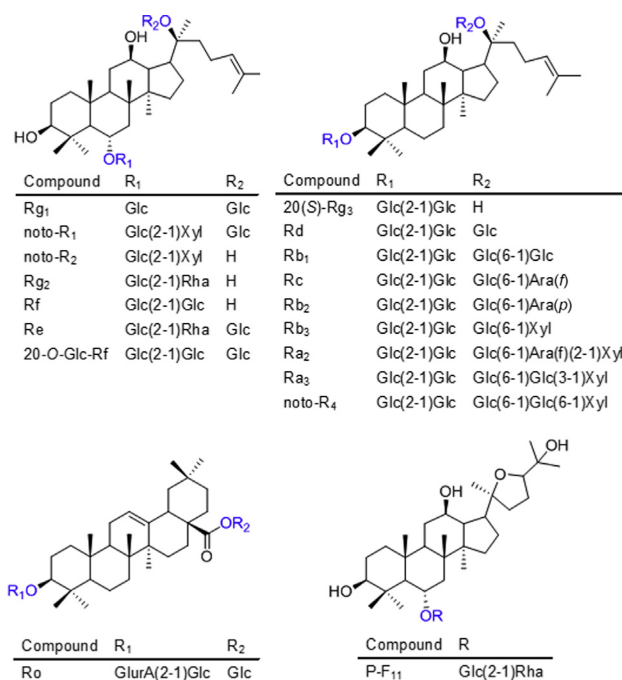

Figure 1 Structures for 18 ginsenoside reference compounds. Glc, *β*-D-glucopyranosyl; Rha, *α*-L-rhamnopyranosyl; Xyl, *β*-D-xylopyranosyl; Ara (*f*), *α*-L-arabinofuranosyl; Ara (*p*), *α*-L-arabinopyranosyl, GlurA, *β*-D-glucuronopyranosyl.

2.2. Plant materials

Crude drug samples of PG were collected from Northeast China or local Tong-ren-tang drugstores (Beijing, China). PN samples were collected from Wenshan County, Yunnan Province, China. PQ samples were purchased from Xushi Yangshen Specialty Co,. Ltd. (Nanjing, China). Detailed information for the 85 batches of samples is given in Supplementary Table 1. In addition, 60 batches of TCM compound preparations which contain PG, PQ or PN were purchased from local drugstores. Their information is given in Supplementary Table 2. Voucher specimens are deposited at the author's laboratory, School of Pharmaceutical Sciences, Peking University (Beijing, China).

2.3. LC–MS conditions

The LC–MS fingerprints were recorded on a Surveyor HPLC instrument coupled with a TSQ triple-quadruple tandem mass spectrometer *via* ESI interface (Thermo Fisher, San Jose, CA, USA). The samples were separated on a YMC-Pack ODS-A column (250 mm × 4.6 mm, 5 μm) equipped with an Agilent Zorbax SB-C18 guard column (12.5 mm × 4.6 mm, 5 μm). The column temperature was maintained at 35 °C. A three-component mobile phase was used, composed of acetonitrile (A), methanol (B), and water containing 1 mmol/L ammonium acetate (C). The

following gradient elution program was applied: 0 min: 12% A, 35% B, 53% C; 9 min: 20% A, 30% B, 50% C; 22 min: 35% A, 15% B, 50% C; 35 min: 50% A, 50% C; 45 min: 60% A, 40% C; 50 min: 90% A, 10% C; 55 min: 90% A, 10% C; 58 min: 12% A, 35% B, 53% C. The flow rate was 1 mL/min. For MS detection, the ESI source was operated in the negative ion mode. The LC eluant was introduced into the mass spectrometer at a post-column splitting ratio of 5:1. Ultra-high purity helium (He) and high purity nitrogen (N_2) were used as the collision gas and nebulizing gas, respectively. The analyzer scanned over *m/z* 400–1500 in the full scan mode. An optimal source fragmentation voltage of 20 V was applied to suppress the adducted precursor ions. Using ginsenosides Re (protopanaxatriol type) and Rb_2 (protopanaxadiol type) as reference compounds, the capillary voltage and tube lens offset voltage were optimized as –22 and –60 V, respectively. Ionspray voltage, 4.5 kV; sheath gas (N_2), 45 arbitrary units; auxiliary gas (N_2), 10 units; capillary temperature, 320 °C. To further identify the peaks in the LC–MS fingerprints, the samples were analyzed by LC–ESI-MS^n and LC–qTOF-MS, as described in Supplementary Information Sections 1 and 2.

2.4. Sample preparation

Ultrasound-assisted extraction was used to prepare the herbal extract samples. An aliquot of 0.2 g finely ground dry powder was soaked in 10 mL of 50% aqueous methanol (*v*/*v*) for 30 min before extraction for 40 min at 40 °C. The extract was centrifuged at 4000 rpm for 15 min (Thermo Multifuge 1S-R, Thermo Fisher Scientific, MA, USA), and the supernatant was filtered through a 0.22-μm microporous membrane to obtain the test solution. An aliquot of 10 μL of the test solution was injected for analysis. The test solutions of TCM compound preparations were obtained in a similar manner, as described in Supplementary Information Section 3.

2.5. Multivariate data analysis

The peak areas for 40 major ginsenosides (marked in blue in Fig. 2) in the extracted ion chromatograms (EICs) were used as variables for multivariate data analysis. The peak areas were normalized to their sum values to minimize the deviation caused by system instability or different drug concentration. The normalized peak areas of 40 peaks in the 85 batches of crude drug samples were used to generate a 2D data lattice, which was subsequently imported into SIMCA-P 13.0 (Umetrics AB. Umeå, Sweden) for chemometric analysis. PLS-DA and OPLS-DA models were used for pattern recognition. The variables were *pareto*-scaled prior to automatic fitting. The variable importance in projection (VIP) plot, which directly reflects the contribution of each variable, together with a two-tailed *t*-test, were used to identify potential marker compounds. Characteristic markers were defined for those only detectable in one unique species, whereas the significantly differential markers should exhibit top-5 VIP values and statistical significance between two groups ($P < 0.05$).

3. Results and discussion

3.1. Optimization of LC–MS conditions

An LC–MS-based fingerprinting method was established to analyze the chemical constituents of PG, PQ, and PN. The sample preparation procedure, chromatographic conditions, and MS detection parameters were optimized to achieve baseline separation of similar ginsenosides and sensitive detection of minor compounds. The method was validated in terms of inter-day and intra-day variation and reproducibility. The details were described in Supplementary Information Sections 4 and 5.

3.2. Identification of chromatographic peaks

Based on the fragmentation pathways of 18 reference ginsenosides derived from an ion-trap mass spectrometer, a total of 87 chromatographic peaks were identified or putatively characterized. The fragmentation pathways were described in Supplementary Information Section 6, and the fragments for representative 20(*S*)-protopanaxadiol (PPD) type (Ra_3), 20(*S*)-protopanaxatriol (PPT) type (20-*O*-glucosyl-Rf), and oleanolic acid (OA) type (Ro) ginsenosides were illustrated in Supplementary Fig. 1. Detailed information of identified ginsenosides is given in Supplementary

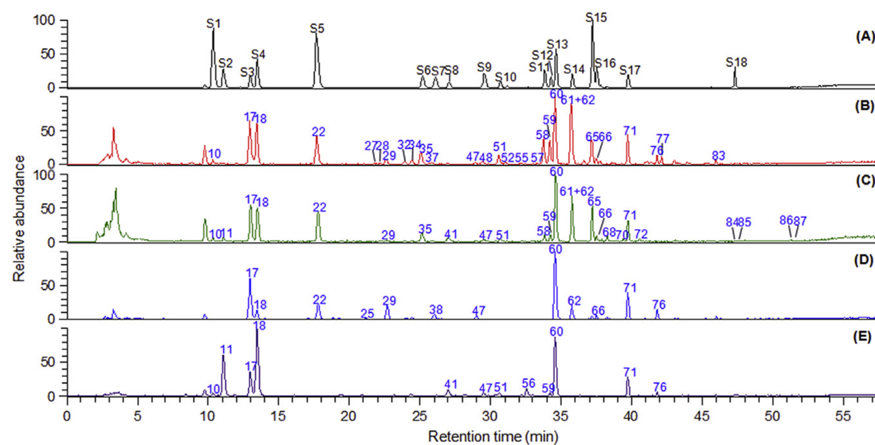

Figure 2 The LC–(–)ESI-MS TIC chromatograms for reference standards (A), *P. ginseng* (B), steamed *P. ginseng* (red ginseng, C), *P. quinquefolium* (D), and *P. notoginseng* (E). The peak areas for 40 ginsenosides (10, 11, 17, 18, 22, 25, 27–29, 32, 34, 35, 37, 38, 41, 47, 48, 51, 52, 55, 56–62, 65, 66, 68, 70–72, 76, 77, 83–87) were used as variables for multivariate data analysis. S1: 20-*O*-glc-Rf (10), S2: noto-R_1 (11), S3: Re (17), S4: Rg_1 (18), S5: Ro (22), S6: Rf (35), S7: p-F_{11} (38), S8: noto-R_2 (41), S9: Rg_2 (48), S10: noto-R_4 (51), S11: Ra_2 (58), S12: Ra_3 (59), S13: Rb_1 (60), S14: Rc (62), S15: Rb_2 (65), S16: Rb_3 (66), S17: Rd (71), S18: 20(*S*)-Rg_3 (84).

Table 3. Among the 87 chromatographic peaks, peaks 10, 11, 17, 18, 22, 35, 38, 41, 48, 51, 58, 59, 60, 62, 65, 66, 71, and 84 were unambiguously identified by comparing the retention time (t_R), MS and MS/MS product ions with those obtained by reference compounds. Here we take peaks 25, 68, 70 and 72 as examples to clarify the identification process. Other ginsenosides were identified following the same manner (Supplementary Table 3).

Peak 25 (t_R 21.37 min), a common peak for PG, PQ and PN, had a molecular formula of $C_{47}H_{74}O_{18}$ (*m/z* 925.4803 for $[C_{47}H_{73}O_{18}]^-$, mass error 0.11 ppm). The deprotonated precursor ion (*m/z* 925) fragmented into *m/z* 613 ($[M–H–Glc–Xyl–H_2O]^-$) and 569 ($[M–H–Glc–Xyl–H_2O–CO_2]^-$) (Fig. 3). The product ion *m/z* 455 ($[OA–H]^-$), dissociated from *m/z* 569, suggested a possible OA sapogenin. In the positive ion mode, CID of the sodium-adduct precursor *m/z* 949 generated the same $Y_{0\beta}^+$ (*m/z* 641 for $[M+Na–Xyl–GlurA]^+$) and $Z_{0\beta}^+$ (*m/z* 623 for $[M+Na–Xyl–GlurA–H_2O]^+$) product ions as those of Ro (Supplementary Fig. 1), which indicated the presence of a 3-GlurA-Xyl disaccharide chain. The structure difference between peak 25 and Ro was a pentose instead of a hexose (Glc) at the terminal of 3-sugar chain. Moreover, peak 25 was eluted later than Ro (t_R 21.37 *versus* 18.05 min), in agreement with its relatively lower polarity than Ro. These data supported the identification of 25 as chikusetsusaponin IV (OA-28-Glc-3-GlurA-Xyl)[16].

Among the three acetyl-substituted ginsenosides (68, 70, and 72), peak 68 (t_R 37.97 min) was characterized as PPD-20-GlcGlc-3-GlcGlc-acetyl, while peaks 70 (t_R 39.14 min) and 72 (t_R 40.30 min) were both PPD-20-GlcXyl-3-GlcGlc-acetyl. In the extracted ion chromatograms, both the elution order and relative abundance of these three peaks were in agreement with those for Rb_1, Rc, and Rb_2 (Supplementary Fig. 2). Therefore, peaks 68, 70, and 72 were characterized as Ac-Rb_1 (quinquenoside R_1), Ac-Rc (ginsenoside Rs_2), and Ac-Rb_2 (ginsenoside Rs_1), respectively[17,18]. For all the ginsenosides characterized in PG, PQ and PN, the acetyl, malonyl or butenoyl group was substituted at 3-OH sugar chain for PPD type compounds or 6-OH sugar chain for PPT type, except for peak 2.

3.3. *Discovery of marker compounds to differentiate among PG, PQ, and PN*

3.3.1. *Differentiation between PG and PQ*

Several studies have compared the chemical composition of PG and PQ. The two major marker compounds that could discriminate the two species were ginsenoside Rf and 24(*R*)-pseudoginsenoside-F_{11} (p-F_{11}).[19,20] Our results were consistent with these studies, where Rf (35) and p-F_{11} (38) were only detected in PG and PQ, respectively. In addition, a potential new marker compound, tentatively identified as ginsenoside Rs_1 (72, Ac-Rb_2), was found characteristic for PG. Fig. 4 exhibits the remarkably differential content of Rs_1 between PG and PQ. Therefore, Rf and Rs_1 were considered as the characteristic markers for PG, whereas p-F_{11} was characteristic for PQ.

Chemometric analysis was then applied to identify ginsenosides capable of differentiating PG and PQ. The established OPLS-DA model, with good fitness (R^2X 0.575, R^2Y 0.983) and predictability (Q^2 0.979), enabled good separation of PG and PQ groups (Fig. 5A). Two outliers (W11 and W12) were observed, but still segregated from the PQ group. The VIP plot could directly reflect the contribution level of the variables to group classification[21,22]. A cutoff VIP value of 1.3 identified nine major potential marker ginsenosides: p-F_{11} (38), chikusetsusaponin IV (25), 20-*O*-glc-Rf (10), Rf (35), Re (17), Rb_2 (65), Rb_1 (60), Rg_2 (48), and m-Rd (47) (Fig. 5B). The fact that Rs_1 was not ranked among top-9 variables could be due to its low content in both PG and PQ. All the nine variables showed statistical significance between PG and PQ by a two-tailed *t*-test (type 3). Finally, we identified three characteristic markers (Rf, p-F_{11}, and Rs_1) and five significantly differential markers (chikusetsusaponin IV, 20-*O*-glc-Rf, Re, Rb_2, and Rb_1) to differentiate PG and PQ.

3.3.2. *Differentiation between PG and PN*

The same procedure was employed to discover potential markers to discriminate PG from PN. Ginsenosides Ro (22), m-Rc (32) and m-Rb_2 (37) were detected as common peaks for all PG samples, but not detected in PN. However, no characteristic peak was found for PN. Using the 40 ginsenosides as variables for multivariate data analysis, p-F_{11} was not detected in either PG or PN samples, and was thus excluded from the dataset. Here we applied PLS-DA for model fitting since over-fitting was observed when using the OPLS-DA model (Supplementary Fig. 3). The PLS-DA model was efficient to separate the samples into two different groups with good fits to the underlying models (R^2X 0.606; R^2Y 0.984) and excellent predictability (Q^2 0.982) (Fig. 5C). However, W11 and W12 were also outliers, which was consistent with PG and PQ. Ten variables displayed VIP values of higher than 1.3 in the VIP plot (Fig. 5D), including noto-R_1 (11), Rc (62), Rg_1 (18), Rd (71), Rf (35), Rb_2 (65), Ro (22), Rb_1 (60), Rb_3 (66), and noto-K (76). All these ten variables showed significant difference between PG and PN (P < 0.001). The characteristic components, Ro, m-Rc, and m-Rb_2, and five most significantly differential components (noto-R_1, Rc, Rg_1, Rd, and Rf) were selected as markers to discriminate PG and PN.

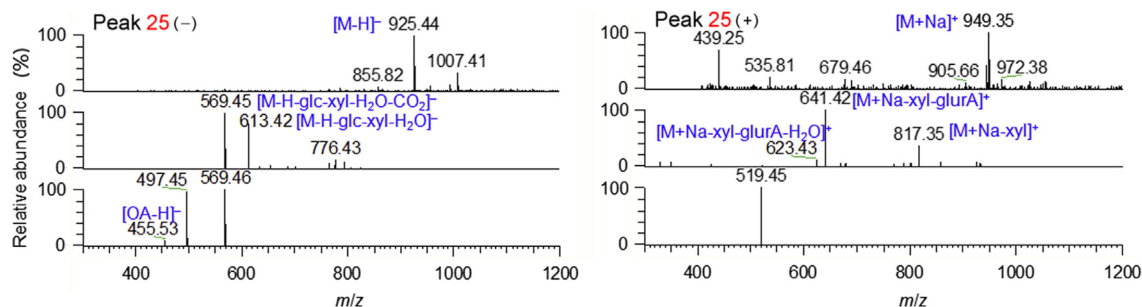

Figure 3 ESI-MS^n spectra in negative and positive ion modes for chikusetsusaponin IV (OA-28-Glc-3-GlurA-Xyl, 25).

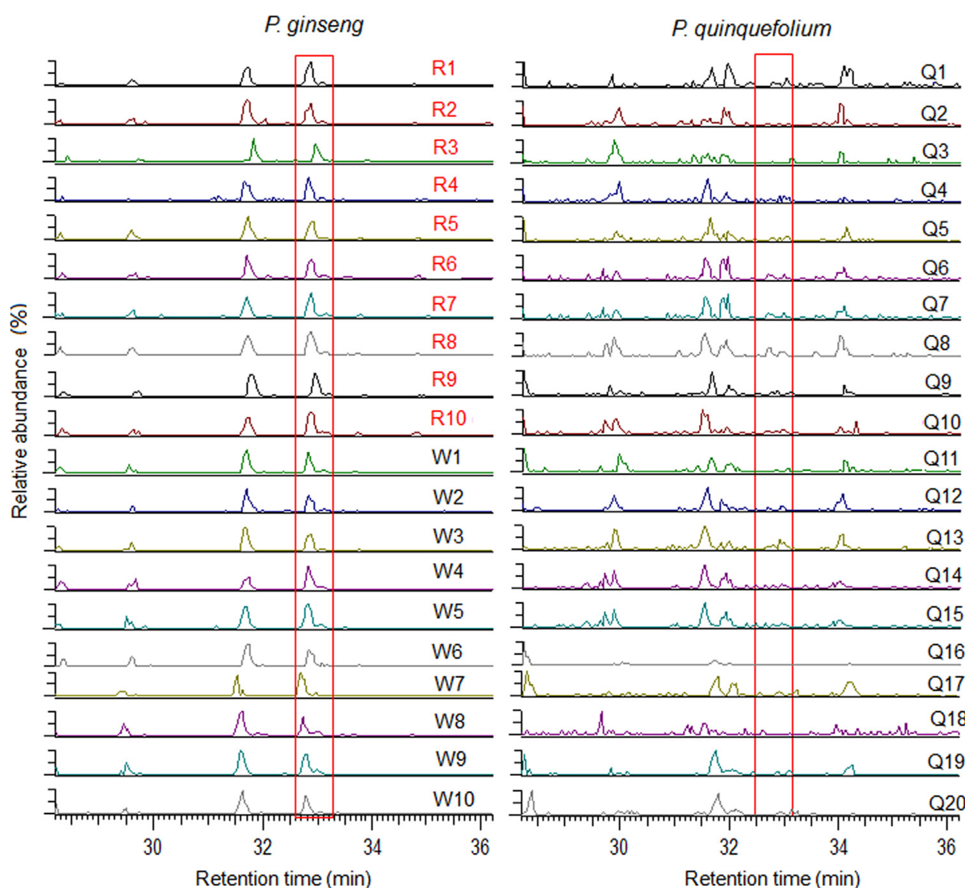

Figure 4 Extracted ion chromatograms for ginsenoside Rs_1 ($[M–H]^−$, *m/z* 1119.5), a potential characteristic marker to differentiate *P. ginseng* and *P. quinquefolium*. The chromatograms for 20 batches of *P. ginseng* and 20 batches of *P. quinquefolium* samples are shown. W and R refer to air-dried and steamed *P. ginseng* (white and red ginseng), respectively; Q refers to *P. quinquefolium*.

3.3.3. Differentiation between PQ and PN

Relatively obvious differences were observed between PQ and PN (Fig. 2). Based on the analysis of EIC for 30 PQ and 23 PN samples, we found that Ro (22) and p-F_{11} (38) were characteristic markers for PQ, whereas Rf (35) and Ra_3 (59) were characteristic for PN. Compounds m-Ra_2 (27), m-Ra_3 (28), m-Ra_1 (34), Rs_1 (72), Ra_2 (58), peaks 52, 57, and 77 were not present or very low in both PQ and PN. Thus, the other 32 compounds were analyzed as variables using the OPLS-DA model. As shown in Fig. 5E, two obvious groups were separated. When the VIP boundary was set as 1.2, seven compounds were discovered, corresponding to noto-R_1 (11), noto-R_2 (41), Rg_1 (18), 20-*O*-glc-Rf (10), p-F_{11} (38), Ro (22), and Re (17) (Fig. 5F). They all exhibited significant difference between PQ and PN. Four characteristic markers for PQ (Ro and p-F_{11}) and PN (Rf and Ra_3), together with five significantly differential markers (noto-R_1, noto-R_2, Rg_1, 20-*O*-glc-Rf, and Re), were finally identified to differentiate PQ and PN.

Based on the above analyses, we were able to summarize the following points to rapidly differentiate the three *Panax* species (Fig. 6, Supplementary Table 3): (1) Ginsenoside Rs_1 is unique for PG, while p-F_{11} is characteristic for PQ; (2) The presence of Ro, Rf, Ra_3, Rs_1, m-Rc, m-Rb_2, and the absence of p-F_{11} are diagnostic for PG; the presence of p-F_{11}, Ro, and the absence of Rf, Rs_1, Ra_3 could allow the identification of PQ. PN contains Ra_3, but not p-F_{11}, Rs_1, Ro, m-Rc, and m-Rb_2; (3) Ginsenosides 20-*O*-glc-Rf, Re, Rg_1, Rc, Rb_2, and Rd are rich in PG; Re and Rd are abundant in PQ; noto-R_1, Rg_1, and Rd are abundant in PN.

3.4. Identification of the three Panax species in TCM compound preparations

In total 17 diagnostic chemical marker compounds, including 7 characteristic markers (Rf, Rs_1, p-F_{11}, Ro, m-Rc, m-Rb_2, and Ra_3, species specific) and 10 significantly differential markers (chikusetsusaponin IV, 20-*O*-glc-Rf, Re, Rb_2, Rb_1, Rc, Rg_1, Rd, noto-R_1, and noto-R_2, showing significant abundance variance between species) were discovered. These markers were used to identify PG, PQ, and PN in 40 different TCM compound preparations (60 batches).

All 60 batches of samples were analyzed by LC–MS, and the peak areas for the markers were obtained by extracting the $[M–H]^−$ ions. For the 7 characteristic markers, their presence or absence was used to identify the *Panax* species. For the 10 significantly differential markers, the relative peak area ratios for each compound in the extracted ion chromatograms against that of Rb_1 were calculated. The results are shown in Supplementary Table 4.

By monitoring multiple diagnostic chemical markers, the *Panax* species used for the manufacturing of TCM compound

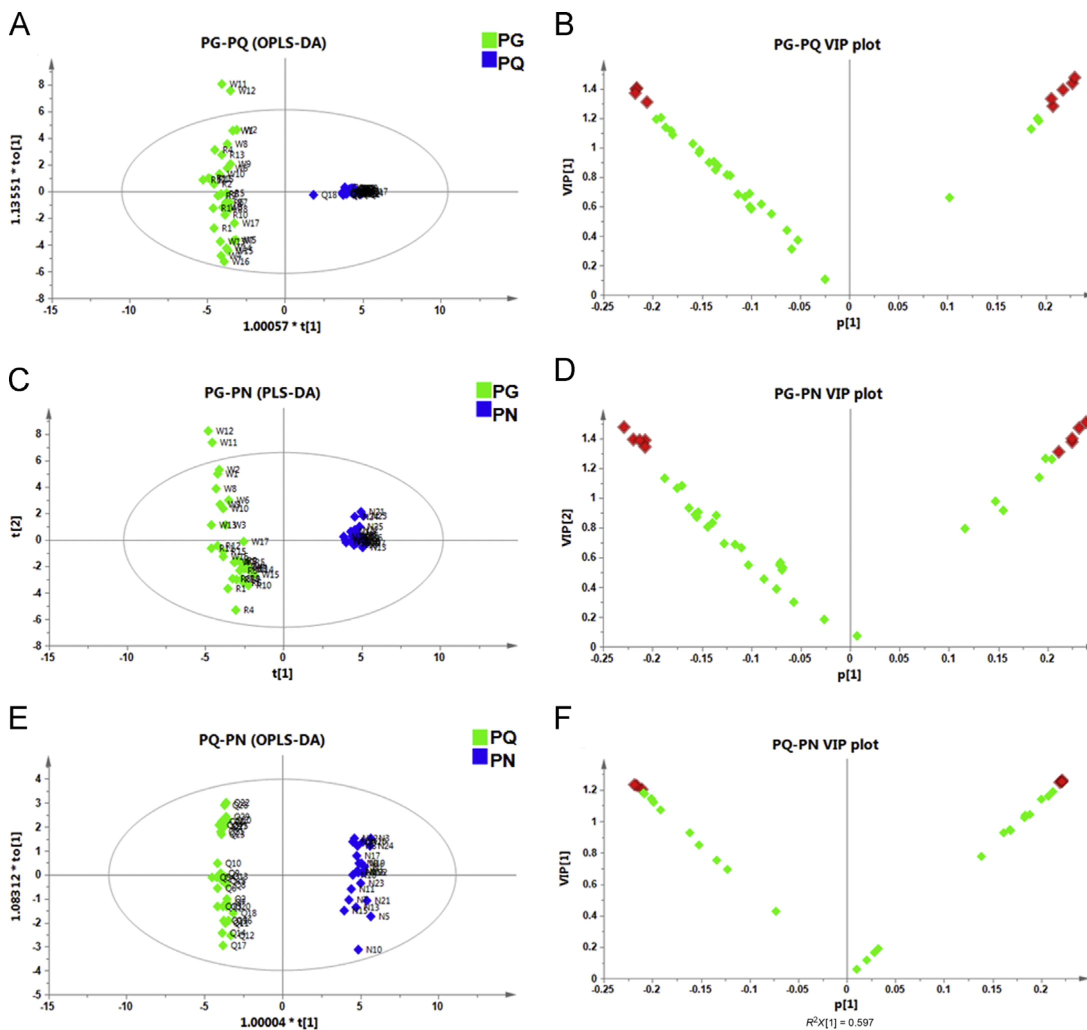

Figure 5 Statistic analyses and discovery of potential markers to differentiate PG/PQ, PG/PN, and PQ/PN. PG, *P. ginseng*; PQ, *P. quinquefolium*, PN, *P. notoginseng*. (A) OPLS-DA score plot of PG and PQ; (B) VIP plot of PG and PQ showing 9 significantly differential components while VIP cutoff was set at 1.3; (C) PLS-DA score plot of PG and PN; (D) VIP plot of PG and PN showing 10 significantly differential components with VIP values higher than 1.3; (E) OPLS-DA score plot of PQ and PN; (F) VIP plot of PQ and PN showing 7 significantly differential components while VIP cutoff was set at 1.2.

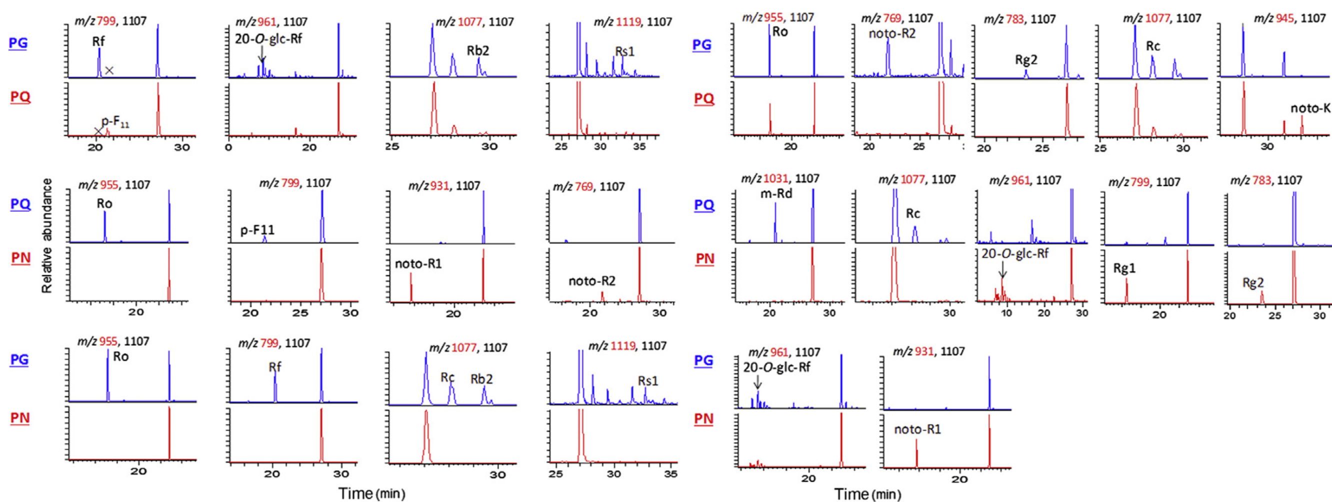

Figure 6 Extracted ion chromatograms of the 17 diagnostic chemical markers to differentiate PG, PN and PQ crude drugs.

preparations could be identified. Among the 40 preparations we analyzed, the identification results were consistent with the specified species for 37 preparations. The only two exceptions were Wu-ji-bai-feng Pills (A6) and Sheng-mai-yin (B1). Although their chemical patterns were mostly similar to *P. ginseng* (which was the specified species), they showed very weak chromatographic peaks, and several marker compounds were not detected. Sample A8 (Yi-nian-jin) could be identified to contain *P. ginseng* according to its multi-marker chemical pattern. Two characteristic markers for PG (m-Rc and m-Rb_2) were not detected, probably due to poor thermostability of malonyl ginsenosides[23]. Similarly, C13 (Wei-kai-ling Capsules) and C14 (Jin-kang Capsules) could be identified to contain PN, by analyzing their multi-marker patterns, though the signals for ginsenoside Rf were very weak. In traditional Chinese medicine, the roots of *P. ginseng* could be used as white ginseng (dried after collection) or red ginseng (processed by steaming). By using the multiple chemical markers discovered in this study, both white and red ginseng could be correctly identified as *P. ginseng*. However, these two types could not be differentiated from each other.

4. Conclusions

To effectively differentiate the three *Panax* species, PG, PQ and PN, an LC–MS-based fingerprinting method coupled with multivariate data analysis was established. The peak areas for 40 ginsenosides were used for pattern recognition chemometric analysis by PLS-DA and OPLS-DA. A total of 17 diagnostic chemical marker compounds, including 7 characteristic markers (Rf, p-F_{11}, Ro, Rs_1, Ra_3, m-Rc, and m-Rb_2, species specific) and 10 significantly differential markers (20-*O*-glc-Rf, chikusetsusaponin IV, Re, Rg_1, Rd, Rc, Rb_2, noto-R_1, noto-R_2, and Rb_1, showing significant abundance variance between species) were discovered to differentiate PG, PQ, and PN. Ginsenoside Rs_1 could be a new marker to differentiate PG and PQ. By monitoring the above multiple diagnostic markers, the *Panax* species in 60 batches of TCM compound preparations could be effectively identified.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.apsb.2016.05.005.

References

1. Qi LW, Wang CZ, Yuan CS. Isolation and analysis of ginseng: advances and challenges. *Natl Prod Rep* 2011;**28**:467–95.
2. Chen CF, Chiou WF, Zhang JT. Comparison of the pharmacological effects of *Panax ginseng* and *Panax quinquefolium*. *Acta Pharmacol Sin* 2008;**29**:1103–8.
3. Chen XC, Liao BS, Song JY, Pang XH, Han JP, Chen SL. A fast SNP identification and analysis of intraspecific variation in the medicinal *Panax* species based on DNA barcoding. *Gene* 2013;**530**:39–43.
4. Chen P, Luthria D, Harrington PB, Harnly JM. Discrimination among *Panax* species using spectral fingerprinting. *J AOAC Int* 2011;**94**:1411–21.
5. Jiang C, Qu HB. A comparative study of using in-line near-infrared spectra, ultraviolet spectra and fused spectra to monitor *Panax notoginseng* adsorption process. *J Pharm Biomed Anal* 2015;**102**:78–84.
6. Edwards HGM, Munshi T, Page K. Analytical discrimination between sources of ginseng using Raman spectroscopy. *Anal Bioanal Chem* 2007;**389**:2203–15.
7. Nguyen HT, Lee DK, Choi YG, Min JE, Yoon SJ, Yu YH, et al. A ^{1}H NMR–based metabolomics approach to evaluate the geographical authenticity of herbal medicine and its application in building a model effectively assessing the mixing proportion of intentional admixtures: a case study of *Panax ginseng*: metabolomics for the authenticity of herbal medicine. *J Pharm Biomed Anal* 2016;**124**:120–8.
8. Yuk J, McIntyre KL, Fischer C, Hicks J, Colson KL, Lui E, et al. Distinguishing *Ontario ginseng* landraces and ginseng species using NMR-based metabolomics. *Anal Bioanal Chem* 2013;**405**:4499–509.
9. Sun JH, Chen P. Differentiation of *Panax quinquefolius* grown in the USA and China using LC/MS-based chromatographic fingerprinting and chemometric approaches. *Anal Bioanal Chem* 2011;**399**:1877–89.
10. Wang XM, Sakuma T, Asafu-Adjaye E, Shiu GK. Determination of ginsenosides in plant extracts from *Panax ginseng* and *Panax quinquefolius* L. by LC/MS/MS. *Anal Chem* 1999;**71**:1579–84.
11. Chan TWD, But PPH, Cheng SW, Kwok IMY, Lau FW, Xu HX. Differentiation and authentication of *Panax ginseng*, *Panax quinquefolius*, and ginseng products by using HPLC/MS. *Anal Chem* 2000;**72**:1281–7.
12. Li T, Zhuang SX, Wang YW, Wang YL, Wang WH, Zhang HH, et al. Flavonoid profiling of a traditional Chinese medicine formula of Huangqin Tang using high performance liquid chromatography. *Acta Pharm Sin B* 2016;**6**:148–57.
13. Lai YH, So PK, Lo SCL, Ng EWY, Poon TCW, Yao ZP. Rapid differentiation of *Panax ginseng* and *Panax quinquefolius* by matrix-assisted laser desorption/ionization mass spectrometry. *Anal Chim Acta* 2012;**753**:73–81.
14. Chen P, Harnly JM, Harrington PB. Flow injection mass spectroscopic fingerprinting and multivariate analysis for differentiation of three *Panax* species. *J AOAC Int* 2011;**94**:90–9.
15. Yang WZ, Ye M, Qiao X, Liu CF, Miao WJ, Bo T, et al. A strategy for efficient discovery of new natural compounds by integrating orthogonal column chromatography and liquid chromatography/mass spectrometry analysis: its application in *Panax ginseng*, *Panax quinquefolium* and *Panax notoginseng* to characterize 437 potential new ginsenosides. *Anal Chim Acta* 2012;**739**:56–66.
16. Li YJ, Wei HL, Qi LW, Chen J, Ren MT, Li P. Characterization and identification of saponins in *Achyranthes bidentata* by rapid-resolution liquid chromatography with electrospray ionization quadrupole time-of-flight tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2010;**24**:2975–85.
17. Besso H, Kasai R, Wei JX, Wang JF, Saruwatari YI, Fuwa T, et al. Further studies on dammarane-saponins of American ginseng, roots of *Panax quinquefolium* L. *Chem Pharm Bull* 1982;**30**:4534–8.
18. Kasai R, Besso H, Tanaka O, Saruwatari YI, Fuwa T. Saponins of red ginseng. *Chem Pharm Bull* 1983;**31**:2120–5.
19. Li WK, Gu CG, Zhang HJ, DVC Awang, Fitzloff JF, HHS Fong, et al. Use of high-peghormance liquid chromatography–tandem mass spectrometry to distinguish *Panax ginseng* C. A. Meyer (Asian ginseng) and *Panax quinquefolius* L. (North American ginseng). *Anal Chem* 2000;**72**:5417–22.
20. Li L, Luo GA, Liang QL, Hu P, Wang YM. Rapid qualitative and quantitative analyses of Asian ginseng in adulterated American ginseng preparations by UPLC/Q-TOF-MS. *J Pharm Biomed Anal* 2010;**52**:66–72.
21. Sun H, Ni B, Zhang AH, Wang M, Dong H, Wang XJ. Metabolomics study on Fuzi and its processed products using ultra-performance liquid-chromatography/electrospray-ionization synapt high-definition

mass spectrometry coupled with pattern recognition analysis. *Analyst* 2012;**137**:170–85.

22. Liang J, Wu WY, Sun GX, Wang DD, Hou JJ, Yang WZ, et al. A dynamic multiple reaction monitoring method for the multiple components quantification of complex traditional Chinese medicine preparations: Niuhuang Shangqing pill as an example. *J Chromatogr A* 2013;**1294**:58–69.
23. Wang YT, You JY, Yu Y, Qu CL, Zhang HR, Ding L, et al. Analysis of ginsenosides in *Panax ginseng* in high pressure microwave-assisted extraction. *Food Chem* 2008;**110**:161–7.