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Research Article

Simultaneous determination and difference evaluation of 14 ginsenosides in *Panax ginseng* roots cultivated in different areas and ages by high-performance liquid chromatography coupled with triple quadrupole mass spectrometer in the multiple reaction–monitoring mode combined with multivariate statistical analysis

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ABSTRACT

Background: Ginsenosides are not only the principal bioactive components but also the important indexes to the quality assessment of *Panax ginseng* Meyer. Their contents in cultivated ginseng vary with the growth environment and age. The present study aimed at evaluating the significant difference between 36 cultivated ginseng of different cultivation areas and ages based on the simultaneously determined contents of 14 ginsenosides.

Methods: A high-performance liquid chromatography (HPLC) coupled with triple quadrupole mass spectrometer (MS) method was developed and used in the multiple reaction–monitoring (MRM) mode (HPLC-MRM/MS) for the quantitative analysis of ginsenosides. Multivariate statistical analysis, such as principal component analysis and partial least squares-discriminant analysis, was applied to discriminate ginseng samples of various cultivation areas and ages and to discover the differentially accumulated ginsenoside markers.

Results: The developed HPLC-MRM/MS method was validated to be precise, accurate, stable, sensitive, and repeatable for the simultaneous determination of 14 ginsenosides. It was found that the 3- and 5-yr-old ginseng samples were differentiated distinctly by all means of multivariate statistical analysis, whereas the 4-yr-old samples exhibited similarity to either 3- or 5-yr-old samples in the contents of ginsenosides. Among the 14 detected ginsenosides, Rg1, Rb1, Rb2, Rc, 20(S)-Rf, 20(S)-Rh1, and Rb3 were identified as potential markers for the differentiation of cultivation ages. In addition, the 5-yr-old samples were able to be classified in cultivation area based on the contents of ginsenosides, whereas the 3- and 4-yr-old samples showed little differences in cultivation area.

Conclusion: This study demonstrated that the HPLC-MRM/MS method combined with multivariate statistical analysis provides deep insight into the accumulation characteristics of ginsenosides and could be used to differentiate ginseng that are cultivated in different areas and ages.

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1. Introduction

Ginseng is the dried root of the perennial herb *Panax ginseng* Meyer (*P. ginseng*), which belongs to the *Panax* genus under the Araliaceae family [1,2]. Extensive modern research has revealed that ginseng has a variety of pharmacological activities, such as the

modulation of the cardiovascular, endocrine, and immune systems and the potential for cancer prevention [3,4]. These activities and efficacies make ginseng one of the most valuable medicinal resources in traditional Chinese medicine, being used for thousands of years. *P. ginseng* is native to East Asian countries and is mainly cultivated in Northeast China, Korea, and Russian Far East to supply

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the worldwide commercial market [5]. Its growth is greatly influenced by germplasm, soil, age, climate, and water source, all of which lead to the different chemical components in *P. ginseng* populations and, thereby, the diversity in quality [6–8]. Therefore, a practicable method for differencing *P. ginseng* of various cultivation areas and ages is important and valuable for its evaluation and quality control.

Ginsenosides are considered as the principal bioactive components responsible for the pharmacological activities of *P. ginseng* [9,10]. Their contents are the main indexes to the quality assessment of ginseng. More than 100 ginsenosides have been isolated and identified from ginseng and its processed products, and most of them are generally classified into ocotillol type, oleanolic acid (OA) type, protopanaxadiol (PPD) type, and protopanaxatriol (PPT) type, according to the aglycone moieties [11]. The heterogeneity in the structure of ginsenoside is remarkable and significant to the medicinal value of *P. ginseng* [12,13]. The contents of major ginsenosides in cultivated ginseng may vary distinctly with the cultivation areas and ages, even between the ginseng samples within a single population. Our previous report indicated that the individual content of five major ginsenosides differed significantly with the ginseng samples, which were collected from three different cultivation regions [14]. In addition, commercial ginseng products such as red ginseng and black ginseng are normally pharmacologically superior to their original forms due to the rich content of rare ginsenosides [12,13,15,16]. However, rare ginsenosides are scarcely naturally occurring in cultivated ginseng and can be obtained only by the transformation of major ones [17]. Therefore, the accurately quantitative determination of ginsenoside is essential for the assessment of ginseng products and also for the difference evaluation of ginseng samples cultivated in different areas and ages.

A few analytical methods have been developed to quantitatively determine the ginsenosides in cultivated ginseng, such as high-performance liquid chromatography (HPLC) combined with evaporative light-scattering detector, ultra violet, and mass spectrometer (MS) [7,18–20]. However, HPLC-based methods are unable to baseline separate all the components due to the similarity and heterogeneity of ginsenosides in structures. The major ginsenosides Re and Rg1 are the typical example that usually cannot be separated readily in reversed column [6]. This limitation decreases the number of detected components and the accuracy of peak integration, probably leading to an inaccurate quantification. Accordingly, the accurate analysis of the individual ginsenoside remains challenging. Multiple reaction–monitoring MS (MRM/MS) provides superior performance in the quantitative analysis of complex samples comparing with conventional MS analytical methods. Its combination with HPLC has been considered the golden standard in the metabolomics study [21]. MRM/MS is usually performed on a triple quadrupole (QqQ) MS or a hybrid QqQ linear ion trap MS. As it only scans the predefined characteristic precursor and product ions, MRM/MS avoids the interference from the coeluting components and has advantages of great repeatability, sensitivity, and wide dynamic range for accurately measuring complex samples [22–24]. Therefore, HPLC-MRM/MS is considered to fit well with the aim of accurate quantification of ginsenosides in ginseng. Recently, Wang et al [5] reported the qualitative and quantitative analysis of 19 ginsenosides using an MRM-based HPLC-ESI-MS method, which made a great progress in the rapid quantification of ginsenosides and in the differentiation of cultivated and forest ginseng.

The present study aimed at evaluating the differences between the cultivated ginseng of various cultivation areas and ages based on the quantitative results of 14 ginsenosides, which were simultaneously determined by an HPLC-MRM/MS method. Thirty-six ginseng samples that aged from 3 to 5 years were collected from

four main cultivation areas of Northeast China. Multivariable statistical analysis techniques, including unsupervised principal component analysis (PCA) and supervised partial least squares-discriminant analysis (PLS-DA) associating with hierarchical cluster analysis (HCA), and analysis of variance (ANOVA), were employed to evaluate the differences in the contents contributed by the cultivation area and age and to discover the differentially accumulated ginsenoside markers. Although there are several reports describing the analysis of ginsenosides [5–8,14–20,23,24], the work combining the absolute quantification and the statistical analysis of 14 ginsenosides in 3- to 5-yr-old cultivated ginseng was rarely reported, especially for the ginseng that was cultivated in the four major cultivation areas of Northeast China. Moreover, compared with the difference between cultivated, forest, and American ginseng, the difference within cultivated ginseng may be more difficult to distinguish. The aim of the present study is to provide a direct insight into the significant effects of cultivation area and age on the accumulation of ginsenosides and also to provide an analytical platform for the differentiation and determination of cultivated ginseng.

2. Materials and methods

2.1. Materials and chemicals

Thirty-six cultivated *P. ginseng* samples were grown from seeds and were collected during July and August of 2015. All the samples were harvested at the age of 3, 4, and 5 years from four different cultivation areas (Antu country, Changbai country, Dunhua city, and Ji'an city) in Jilin province, China (Table 1). Their botanical origins were authenticated by Professor Shumin Wang of Changchun University of Chinese Medicine, China. Fourteen commercially available authentic standards, including ginsenosides Re, Rg1, 20(S)-Rf, 20(S)-Rg2, 20(S)-Rh1, Rb1, Rc, Rb2, Rb3, Rd, 20(S)-Rg3, and Ro as well as notoginsenosides (NG)-R1 and 20(S)-R2, with more than 98% purity were purchased from Shanghai Yuanye Biological Technology Co., Ltd. (Shanghai, China). HPLC-grade methanol and acetonitrile were acquired from Tedia Company, Inc. (Fairfield, USA), while HPLC-grade formic acid was acquired from Thermo Fisher Scientific (Waltham, USA). Analytical grade methanol, diethyl ether, and *n*-butanol were obtained from Beijing Chemical Industry Group Co., Ltd. (Beijing, China). All the reagents were used as received without further purification. Ultrapure water (Millipore, Bedford, USA) was used throughout the experiments.

2.2. Preparation of sample and standard solutions

The extraction method of ginsenoside was established and optimized according to the previously reported method [14]. All the

Table 1
Details of the *P. ginseng* samples

No.	Age	Origin
A01–A03	3	Antu (AT) country
A04–A06	4	Antu (AT) country
A07–A09	5	Antu (AT) country
C01–C03	3	Changbai (CB) country
C04–C06	4	Changbai (CB) country
C07–C09	5	Changbai (CB) country
D01–D03	3	Dunhua (DH) city
D04–D06	4	Dunhua (DH) city
D07–D09	5	Dunhua (DH) city
J01–J03	3	Ji'an (JA) city
J04–J06	4	Ji'an (JA) city
J07–J09	5	Ji'an (JA) city

ginseng samples were washed, dried to constant weight, and then pulverized to 40 mesh. One gram of the ginseng powder was refluxed with 50 mL of diethyl ether for 2 h in a Soxhlet extractor to remove the lipid-soluble components. The powder was sequentially extracted using 50 mL of methanol. The extraction was concentrated to dryness by vacuum evaporation. The residue was dissolved in 10 mL of water and treated with 30 mL of water-saturated *n*-butanol three times. The *n*-butanol fractions were evaporated and transferred into a 10 mL volumetric flask and then HPLC-grade methanol was added to the desired volume.

An ultrasonic extraction method was employed to clarify the origination of ginsenoside 20(S)-Rg3 in the extraction. One gram of the ginseng powder was dissolved in 15 mL of 70% methanol aqueous solution and extracted under sonication for 20 min at room temperature followed by filtration. The extraction was repeated three times. Then, the filtrate was combined and evaporated slowly in vacuum at 40°C. The residue was dissolved in HPLC-grade methanol to 10 mL.

The 14 authentic ginsenoside standards were weighted accurately and dissolved in methanol individually to a concentration of 50 µg/mL. These single standard solutions were used to optimize the MRM parameters. Mixed standard stock solution was prepared by dissolving all the 14 weighted authentic standards in 2 mL of methanol. Afterward, a series of mixed standards were obtained by diluting the stock solution to different concentration levels and then used to build the calibration curves. All the prepared solutions were filtered through a 0.22 µm membrane before being subjected to MS analysis.

2.3. HPLC-MS conditions

HPLC analysis was conducted on a Dionex Ultimate 3000 system (Thermo Fisher Scientific Inc, San Jose, CA, USA) equipped with a quaternary bump, an online vacuum degasser, an autosampler, and a column compartment. Analytes were separated on a Thermo Scientific Synchronis C₁₈ column (100 mm × 2.1 mm inner diameter, 1.7 mm) at 35°C. The mobile phase consisted of solvent A (0.1% formic acid aqueous solution, v/v) and solvent B (acetonitrile) and were set in a gradient elution program: 0–5 min (25–30% B); 5–8 min (30–32% B); 8–9 min (32–36% B); 9–16 min (36–37% B); 16–18 min (37–70% B); and 18–20 min (70–95% B) with a flow rate of 0.25 mL/min. The injection volume was 1 µL.

HPLC system was online coupled with a TSQ Endura triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc.) via an electrospray ionization (ESI) ion source. Nitrogen gas was supplied as sheath, auxiliary, and sweep gas in the ESI ion source at a flow rate of 38, 11, and 1 arbitrary unit, respectively. The ion transfer temperature, vaporizer temperature, and spray voltage were set to 329°C, 296°C, and –2500 V, respectively. The MRM parameters of each ginsenoside were optimized by introducing the prepared single standard solutions into the ion source via an infusion pump at a flow rate of 5 µL/min. Two pairs of precursor and product ions and the corresponding collision energy were selected (Table 2). Ultrapure argon with a pressure of 3 mTorr was used as the collision gas. The full scan mode was operated from *m/z* 100 to 2000 with a scan speed of 1000 Da/s. All the MS data were automatically recorded in centroid mode by a customized sequence subroutine implemented under Xcalibur software (Thermo Fisher Scientific, version 2.2 SP1.48).

2.4. Validation of methodology

The calibration curves were obtained by plotting the integrated peak areas against the concentration of each ginsenoside in the mixed standard solutions at six different levels. The limit of

Table 2

The ion-Pair list of precursor and product ions and the collision energy of the 14 ginsenosides

Ginsenoside	Precursor ion (<i>m/z</i>)	Product ion I (<i>m/z</i>)/Collision energy (eV)	Product ion II (<i>m/z</i>)/Collision energy (eV)
Re	945.5	637.3/38.01	475.3/49.74
Rg1	799.5	637.3/23.96	475.3/34.42
20(S)-Rf	799.5	637.3/30.38	475.3/39.12
Rb1	1107.5	945.3/41.90	783.3/46.05
Rc	1077.5	945.3/42.97	783.3/45.04
Rb2	1077.5	945.3/42.26	621.3/52.07
Rb3	1077.5	783.3/44.13	621.3/51.21
Rd	945.5	783.3/35.99	621.3/40.54
20(S)-Rg3	783.5	621.4/30.98	459.3/39.33
20(S)-Rg2	783.4	637.3/28.40	619.3/31.39
20(S)-Rh1	637.4	475.3/23.75	391.2/41.90
Ro	955.4	793.3/42.00	569.3/55.00
NG-R1	931.5	799.3/29.47	637.3/37.15
NG-20(S)-R2	769.4	637.3/27.04	475.3/36.14

NG, notoginsenosides.

detection (LOD) and limit of quantification (LOQ) were determined at the signal to noise ratio of 3 and 10, respectively. Precision was evaluated through intraday and interday variations, which were performed by analyzing an extracted sample six times within a single day and in triplicate on 3 consecutive days, respectively. Variations were expressed by relative standard deviation (RSD). For the repeatability test, one sample was equally divided into six portions, and each portion was extracted individually to analyze the contents of the 14 ginsenosides. For the stability test, the 14 ginsenosides in a freshly extracted sample solution were analyzed at 0 h, 4 h, 8 h, 12 h, 16 h, 20 h, and 24 h, respectively. A recovery test was carried out to assess the accuracy of the extraction and analytical method. The mixed authentic standard solutions at three amount levels (approximately equal to 80%, 100%, and 120% of each ginsenoside amount in the extracted sample) were added into the pulverized sample and then extracted and analyzed using the HPLC-MRM/MS method. Three replicates on each amount level were examined. The recovery was calculated as follows:

$$\text{Recovery (\%)} = 100 \times (\text{found amount} - \text{original amount}) / \text{spiked amount}$$

2.5. Data processing and statistical analysis

The peak areas of the 14 ginsenosides in all the 36 ginseng samples were automatically integrated using Qual Browser of Xcalibur software. The contents of ginsenosides were obtained by substituting the peak areas into the calibration curves. The quantitative analyses of each sample were conducted with three replicates. The contents were expressed by the means and standard deviations. These calculated mean contents were then subjected to multivariable statistical analysis. PCA was carried out using the SPSS statistics software (version 19, SPSS Inc., Chicago, IL, USA). ANOVA was performed with the general line model procedure of the SPSS software to evaluate the significant differences in the contents resulted from the cultivation area and age. The level of statistical significance was set as $p < 0.05$ and $p < 0.01$. Before being subjected to HCA, the data matrix of the contents of ginsenosides was normalized using Z-score transformation. HCA with a heatmap was conducted with R (version 3.2.5., R Foundation for Statistical Computing [25]) using a complete linkage method to measure the Euclidean distance. PLS-DA was performed on SIMCA-P software (version 11.5, Umetrics, Umeå, Sweden). The 3- and 5-yr-old samples were set as group 1 and 2, respectively. Permutation tests were conducted to validate the PLS-DA model.

3. Results and discussion

3.1. Identification of 14 ginsenosides in ginseng samples

A typical total ion chromatogram of the extracted ginseng sample is shown in Fig. 1A. The investigated ginsenosides are labeled with peak 1–14. They were identified by the tandem mass spectrometry analysis and comparison of their retention behaviors with those of the authentic ginsenoside standards. In the ESI ion source, the negative ion mode provided more direct structural information than its positive counterpart, and hence it was employed to identify the ginsenosides herein [17]. The MS spectra of ginsenosides are normally dominated by the modifier adduct $[M + HCOO]^-$ ion and/or the deprotonated molecular $[M-H]^-$ ion. This ion pair is used to determine the molecular weight and empirical molecular formula. In collision-induced dissociation experiment, the saccharide substituent of ginsenoside is usually lost via the glycosidic bond cleavage, leading to the deprotonated aglycone $[Aglycone-H]^-$ ion. The neutral loss information provides preliminary information of the fragmented saccharide substituent, such as 162 Da (glucosyl), 146 Da (rhamnosyl), and 132 Da (arabinosyl or xylosyl) [23]. Taking peak 4 as an example, its molecular

weight was calculated to be 800.5 Da with an empirical molecular formula of $C_{42}H_{72}O_{14}$. The MS^2 spectrum on its $[M + HCOO]^-$ ion exhibited three fragments at m/z 799.5, m/z 637.4, and m/z 475.4 (Fig. 1B), indicating the presence of two glucosyl. The ion at m/z 475.4 is the characteristic $[Aglycone-H]^-$ ion of PPT-type ginsenoside as the ions at m/z 459.4 and m/z 455.4 are that of PPD- and OA-type ginsenoside [5]. In comparison with the authentic standard, peak 4 was unambiguously assigned as ginsenoside 20(S)-Rf. Accordingly, peak 1–14 were identified in sequence as NG-R1, Re, Rg1, 20(S)-Rf, NG-20(S)-R2, Rb1, 20(S)-Rg2, 20(S)-Rh1, Rc, Ro, Rb2, Rb3, Rd, and 20(S)-Rg3. Their structures are listed in Fig. 2, and the chemical information and major fragment ions are summarized in Table S1.

Although more than 100 ginsenosides have been isolated from ginseng and its product, many of them are the transformation products and are absent in cultivated ginseng root, such as Rk1, Rg5, Rf2, etc [11]. For the naturally occurring ginsenosides, not all of their authentic standards are commercially available, which is a major hindrance of absolute quantification. In this study, Re, Rg1, Rb1, Rc, and Rb2 are undoubtedly selected for quantitative analysis as they are acknowledged as the major ginsenosides. Ro is the typical OA-type ginsenosides. It is present in a relatively high

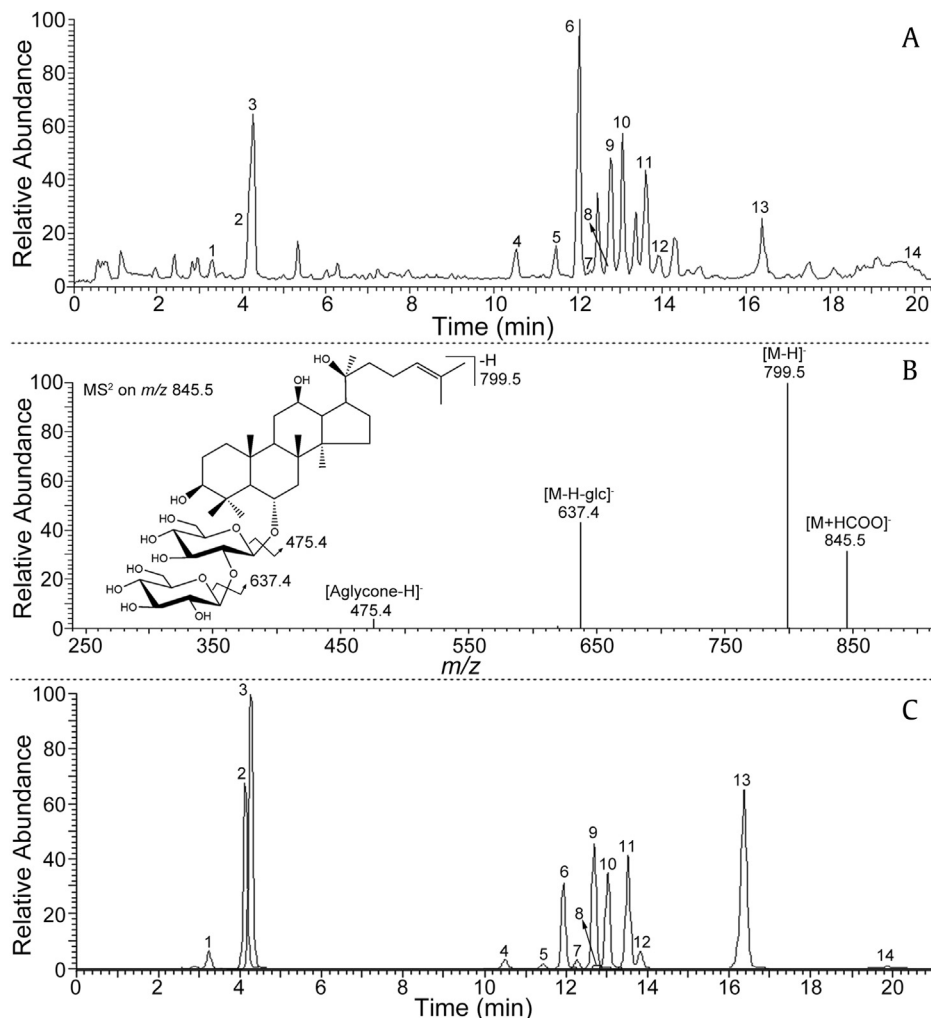


Fig. 1. HPLC-MS analysis of the extraction of the ginseng sample (J09). (A) Representative total ion chromatogram (TIC) detected by HPLC-MS in full scan mode. (B) MS^2 spectrum of the $[M + HCOO]^-$ ion at m/z 845.5 and the fragmentation pathway of peak 4 [20(S)-Rf]. (C) Reconstructed MRM chromatogram detected by HPLC-MRM/MS analysis.

HPLC-MS, high-performance liquid chromatography mass spectrometer. HPLC-MRM/MS, high-performance liquid chromatography coupled with triple quadrupole mass spectrometer in the multiple reaction-monitoring mode.

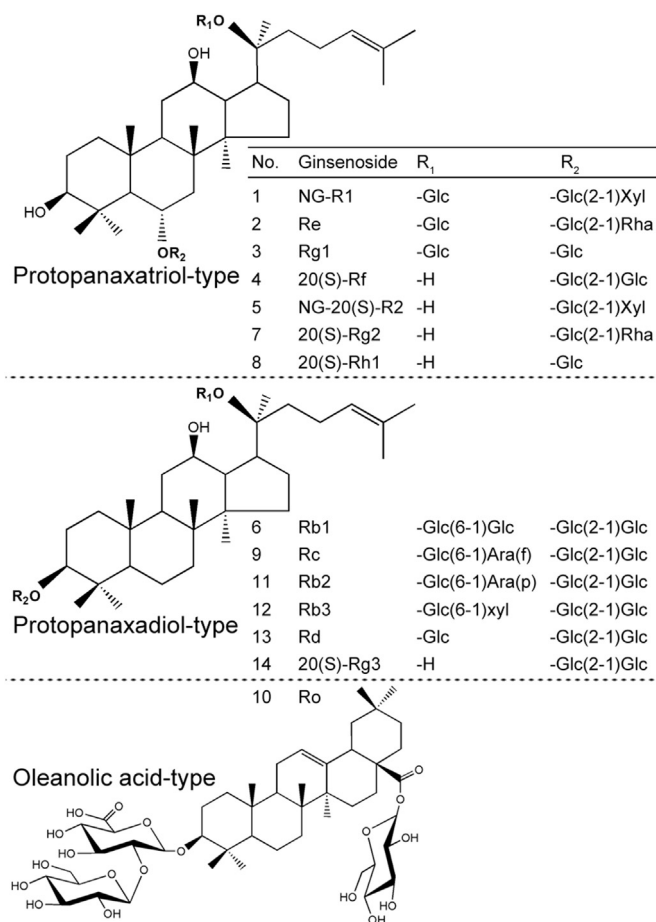


Fig. 2. Chemical structures of the 14 investigated ginsenosides which belong to PPD-, PPT-, and OA-type ginsenoside. OA, oleanolic acid; PPD, protopanaxadiol; PPT, protopanaxatriol.

content in cultivated ginseng and contributes a lot to the total amount of ginsenoside. For NG-R1, NG-20(S)-R2, 20(S)-Rf, 20(S)-Rg2, 20(S)-Rg3, 20(S)-Rh1, Rb3, and Rd, their contents are relatively lower than the major ones, but they may still contribute to the difference between different ginseng samples. Moreover, the authentic standards of all these 14 ginsenosides are commercially available, which is essential for the repeatability of the experiment and the HPLC-MRM/MS combined with multivariate statistical analysis strategy.

Ginsenoside 20(S)-Rg3 of trace amount is naturally present in *P. ginseng* and is generally thought to be the major thermal decomposition product [5,16,26]. To clarify the origination of the detected 20(S)-Rg3, a raw ginseng sample was extracted simultaneously by the ultrasonic extraction method and the reflux extraction method as ultrasonic vibration could avoid the decomposition of thermal-sensitive ginsenosides [3]. The content of 20(S)-Rg3 is $(7.15 \pm 0.15) \times 10^{-4}$ $\mu\text{g}/\text{mg}$ in the ultrasonic extraction, which is comparable with the content of $(7.57 \pm 0.21) \times 10^{-4}$ $\mu\text{g}/\text{mg}$ in the refluxed extraction. Therefore, the detected 20(S)-Rg3 is believed to come from the raw ginseng samples but not thermal degradation of other PPD-type ginsenosides.

3.2. Validation of quantitative method

It is observed in Fig. 1A that most of the 14 ginsenosides could be baseline separated from each other in 20 min, which is beneficial to improve the accuracy of HPLC-based method. Although

ginsenosides Re and Rg1 as well as Rc and Rh1 cannot be completely separated due to their similar polarity, they could also be quantified individually using the MRM mode of QqQ MS (Fig. 1C). Before being subjected to quantitative analysis, the developed HPLC-MRM/MS method was validated by evaluating its linearity, LOD, LOQ, precision, repeatability, stability, and recovery. As shown in Table S2, the correlation coefficient (R^2) of each ginsenoside was greater than 0.9990, except for 0.9988 of ginsenoside Re, indicating a good linear correlation between the concentration and integrated peak area. The linearity could be remained over three orders of magnitude. The LOD and LOQ were in the range of 0.0188–0.1307 $\mu\text{g}/\text{mL}$ and 0.0628–0.4356 $\mu\text{g}/\text{mL}$, respectively, which were sensitive enough to detect and quantify ginsenoside 20(S)-Rg3 of trace amount in cultivated ginseng. The intraday and interday precisions (RSD) were less than 3.91% and 3.28%, respectively, for all the 14 ginsenosides (Table S3). The repeatability of the employed analytical method was acceptable with an RSD less than 3.29%. The prepared sample solutions were relatively stable for at least 24 h with an overall variation less than 3.54%. For the accuracy assessment, the average recoveries were 95.37–104.16%, 94.95–103.26%, and 95.54–102.20% for the three amount levels of 80%, 100%, and 120% with the RSD less than 3.14%, 2.95%, and 3.31%, respectively. In all, these validated results proved the developed HPLC-MRM/MS method to be suitable for the simultaneous determination of ginsenosides in complex extraction of cultivated ginseng.

3.3. Quantitative determination of 14 ginsenosides in 36 ginseng samples

To test the applicability of the HPLC-MRM/MS method, it was then used to quantitatively determine the 14 ginsenosides in the 36 ginseng samples that were cultivated in different areas and ages. All the mean contents and standard deviation data are listed in Table S4, and the contents of PPD, PPT, and total ginsenosides are expressed as boxplots in Fig. 3. It is observed that the contents of ginsenosides varied among cultivation areas and increased with cultivation ages. At each age, Dunhua city (DH) and Ji'an city (JA) samples exhibited the highest contents of PPT- and PPD-type ginsenosides, respectively, whereas the contents of both type ginsenoside were the lowest in Antu country samples (Figs. 3A and 3B). In addition, the total contents in JA samples were lower than those in DH samples at the age of 4 yr, but they quickly accumulated to the highest in the 5th year (Fig. 3C). It can be further calculated that OA-, PPD-, and PPT-type ginsenosides accounted for an average of 13.4%, 49.2%, and 37.4% of the total contents, respectively, indicating a larger proportion taken up by PPD than PPT in cultivated ginseng. Moreover, the six major ginsenosides Re, Rg1, Rb1, Rc, Rb2, and Ro occupied 71.1–89.8% of the total contents of ginsenosides. Their contents may be used as the potential indexes to assess the quality of ginseng.

3.4. Principal component analysis

Two-way ANOVA was firstly conducted to investigate the effect of cultivation area and age on the content of individual ginsenoside. As shown in Table 3, the contents of most of the 14 ginsenosides were significantly affected by the cultivation area and age. Therefore, it is possible to differentiate these ginseng samples and to evaluate the difference between them based on their contents of ginsenosides.

PCA was used initially to obtain the understanding of the relationship between the 36 ginseng samples and the contents of the 14 ginsenosides. The PCA scores plots are shown in Fig. 4. The multidimensional data matrix was transformed to a two-

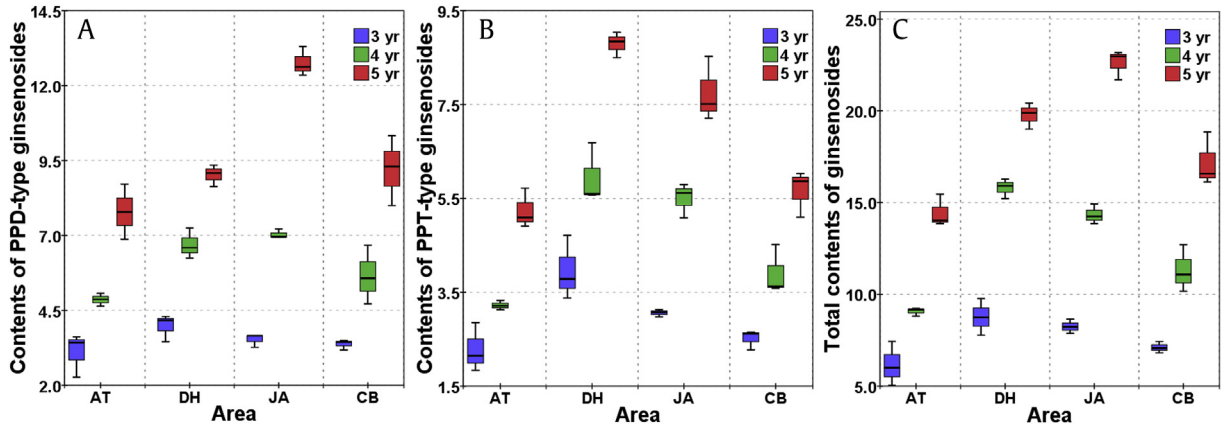


Fig. 3. Boxplots of the contents of ginsenosides in the 36 ginseng samples harvested from different cultivation areas and ages. (A) Boxplots of the contents of PPD-type ginsenosides in the 36 ginseng samples harvested from different cultivation areas and ages. (B) Boxplots of the contents of PPT-type ginsenosides in the 36 ginseng samples harvested from different cultivation areas and ages. (C) Boxplots of the total contents of the 14 ginsenosides in the 36 ginseng samples harvested from different cultivation areas and ages. PPD, protopanaxadiol; PPT, protopanaxatriol.

Table 3
ANOVA of the effects of cultivation area and age on the contents of the 14 ginsenosides

Factor	NG-R1	Re	Rg1	20(S)-Rf	NG-20(S)-R2	Rb1	20(S)-Rg2	20(S)-Rh1	Rc	Ro	Rb2	Rb3	Rd	20(S)-Rg3
Area	*1)	**3)	**	**	—	**	**	—	**	**	*	**	**	**
Age	—2)	**	**	**	—	**	**	**	**	**	**	**	**	—

NG, notoginsenoside.

1) Significant difference at the level of $p < 0.05$.

2) No significant difference.

3) Significant difference at the level of $p < 0.01$.

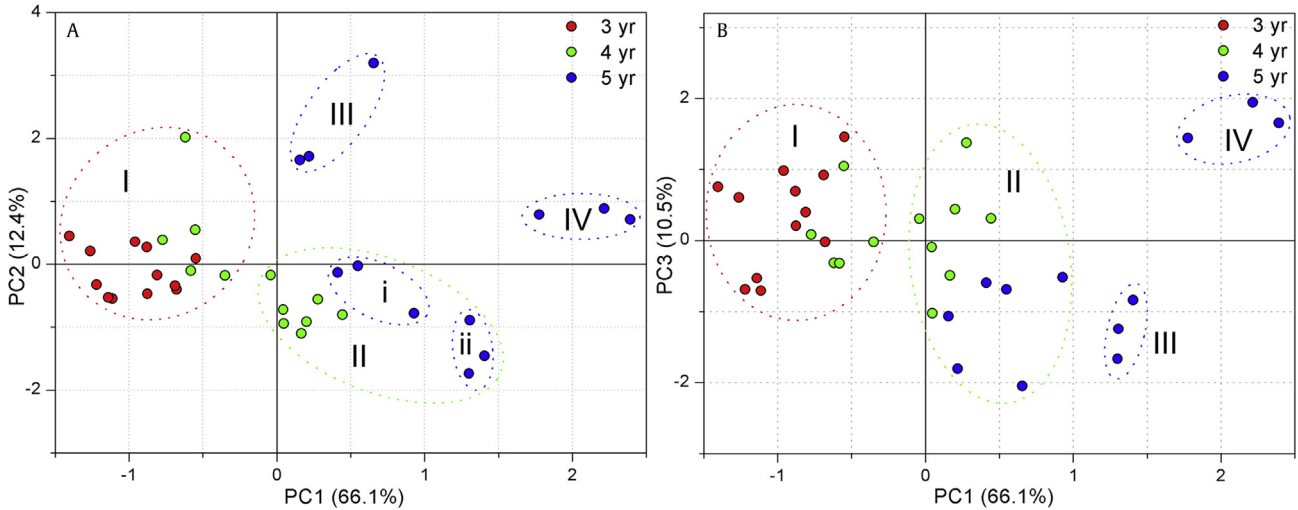


Fig. 4. PCA scores plots of the 36 ginseng samples harvested from four different areas and aged from 3 to 5 years. (A) The scores plot is constructed by plotting PC1 versus PC2. (B) The scores plot is constructed by plotting PC1 versus PC3. PC1, first principal component; PC2, second principal component; PC3, third principal component; PCA, principal component analysis.

dimensional dataset by plotting the principal components which enabled the samples to be differentiable [27]. The first and second principal component (PC1 and PC2) described 66.1% and 12.4%, respectively, of the total variability in the data matrix of the contents. And hence, Fig. 4A represented 78.5% of the total variability. All the 36 samples were clearly classified into four groups. The 3-yr-old samples were separated distinctly from the 5-yr-old samples by PC1, indicating the significant differences in the contents of ginsenosides between them. The 4-yr-old samples could not be distinguished from the 3- and 5-yr-old samples. Five of them were

gathered with the 3-yr-old samples in Group I by the negative values of PC1, whereas seven of them were gathered with parts of the 5-yr-old samples in Group II. The 5-yr-old samples in Group II could be further divided into subgroup i and ii, containing CB and DH samples. In addition, Antu country and JA samples of 5 yr old were clustered into Group III and IV, respectively, by the positive values of PC1 and PC2. Therefore, it is noted that the 5-yr-old samples exhibited an obvious difference in the content of ginsenosides affected by cultivation area, whereas the 3- and 4-yr-old samples did not. Moreover, the third principal component

represented the maximum amount of variability that was not contained by PC1 and PC2. The 36 samples were further analyzed by the PCA scores plot of PC1 versus the third principal component (Fig. 4(B)). They also could be differentiated and separated completely into four groups by PC1. Parts of the 4-yr-old samples were still similar to the 3- or 5-yr-old samples. The total contents of 14 ginsenosides in the samples of Group I, II, III, and IV were in the ranges of 5.02–11.08 mg/g, 12.71–18.84 mg/g, 18.99–20.42 mg/g, and 21.69–23.17 mg/g, respectively, which increased with PC1 values. From these results of PCA, it is seen that the differences in the contents of ginsenosides between the samples from the four different cultivation areas are gradually visible with increased cultivation age, which is due to the different accumulation characteristic of ginsenosides and growth environment in each area. The content of ginsenoside in the 4-yr-old ginseng samples could be even less than that in the 3-yr-old samples or higher than that in the 5-yr-old samples. Therefore, it is difficult to distinguish the ginseng cultivated in 2 consecutive years and different areas.

3.5. Hierarchical cluster analysis

HCA was further conducted to reveal the relatively homogeneous clusters according to the similarity in the contents of ginsenosides in all the samples [28,29]. As shown in Fig. 5, the 36 ginseng samples were divided into four major clusters. The 3-yr-old samples were all included in Cluster I and separated clearly from the 5-yr-old samples in Cluster II and IV. The 4-yr-old samples, A04, A05, A06, C04, and C05, exhibited great similarity to the 3-yr-old samples and were included in Cluster I. Meanwhile, the other 4-yr-old samples were similar to each other and were classified in Cluster III except for J06. Cluster III demonstrated much shorter distance to Cluster II and IV than Cluster I, indicating that these 4-yr-old samples were much more similar to the 5-yr-old samples. In addition, the data matrix of the contents was visualized in the heatmap. The variation in the contents of ginsenosides with cultivation age was noticeable as the increase of ginsenoside content (Re, Rg1, Rb1, Rc, Rb2, and Ro) from Cluster I to Cluster II and IV was visible, whereas the contents of 20(S)-Rg3, 20(S)-Rh1, 20(S)-Rg2, NG-R1, and NG-20(S)-R2 were relatively steady with the increased cultivation age. Therefore, it could be predicted that the increase in the contents of ginsenosides along with cultivation age is mainly due to the accumulation of major ginsenosides, whereas the rare ginsenosides contribute relatively little to the increased total contents.

3.6. Partial least squares-discriminate analysis

PLS-DA, which is a standard and reliable supervised pattern recognition technique [30], was applied for further investigating the contributions of each ginsenoside to the difference between ginseng samples of different ages. It is found that the 4-yr-old samples could not be distinguished from the 3- and 5-yr-old samples even under the mandatory grouping conditions of PLS-DA, as exhibited in Fig. S1. Therefore, their contents of ginsenosides were not included in the dataset for PLS-DA. The PLS-DA model was then generated with the parameters of $R^2 = 0.95$ and $Q^2 = 0.93$ based on cross-validation. R^2 and Q^2 indicate the ability of the model to describe the data and to predict the new observations, respectively. The closer they get to 1.0, the higher goodness of fit and better predictability the model has [31]. Moreover, permutation tests were performed with the established PLS-DA modes to assess their validity. The results showed that R^2 and Q^2 values were higher in the original PLS-DA mode than in the permuted modes, and the regression line of the permuted Q^2 -points intersects the vertical axis below 0 (Fig. S2). These results demonstrated both

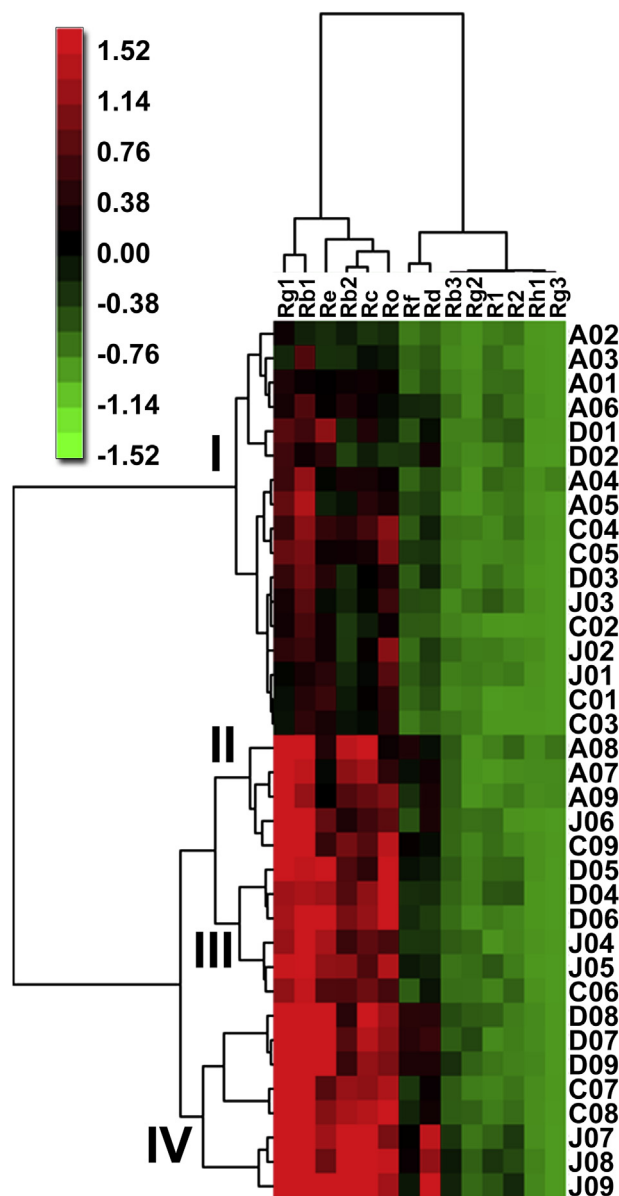


Fig. 5. Dendrogram and heatmap for the 36 cultivated ginseng samples according to the contents of the 14 ginsenosides generated by HCA with a complete linkage method. HCA, hierarchical cluster analysis.

high goodness of fit and predictability for the established PLS-DA mode.

As shown in Fig. 6A, the 3-yr-old samples were distinctly separated from the 5-yr-old samples in the PLS-DA scores plot, indicating the contents of the 14 ginsenosides varied significantly during the 2 years of growth. The 3-yr-old samples were clustered much more closely than the 5-yr-old samples, suggesting that the accumulation of ginsenosides in the former was less affected by cultivation area. The contribution of each ginsenoside to differentiate the ginseng samples was calculated and ranked by the variable importance for the projection (VIP) values. VIP shows the importance of the variable to explain the dataset and to correlate with the groups, i.e., the critical influence of each ginsenoside on clustering [32]. In association with the loading plot (Fig. 6B), the ginsenoside with a VIP value exceeding 1.0 was selected as differential marker. Finally, seven ginsenosides Rg1, Rb1, Rb2, Rc, 20(S)-Rf, 20(S)-Rh1, and Rb3 were obtained (Fig. S3). The student *t* test showed that

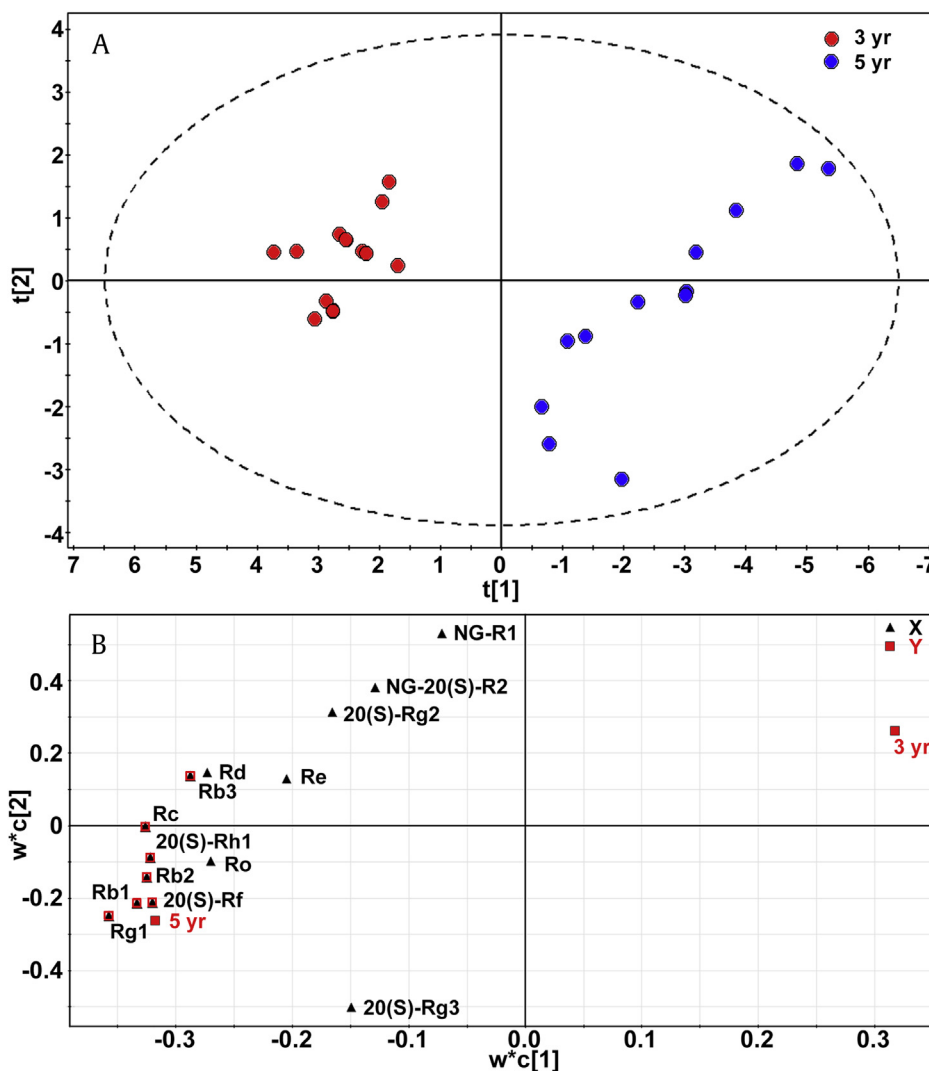


Fig. 6. PLS-DA of 3- and 5-yr-old ginseng samples. (A) PLS-DA scores plot of 3- and 5-yr-old ginseng samples. (B) The corresponding loading plot derived from the content of ginsenoside detected by HPLC-MRM/MS. In the loading plot, the closer to the origin, the smaller contribution a ginsenoside makes to the discrimination.

HPLC-MRM/MS, high-performance liquid chromatography coupled with triple quadrupole mass spectrometer in the multiple reaction–monitoring mode PLS-DA, partial least squares-discriminant analysis.

their contents were significantly different ($p < 0.05$) between the 3- and 5-yr-old samples. It is noticeable that, except for 20(S)-Rh1, the other six ginsenosides are original in cultivated ginseng and exist naturally. The original ginsenosides can be transformed into minor ones during the growth of ginseng [6]. Therefore, we may predict that the difference in ginsenosides between cultivated ginseng in the initial 5 years of growth was partly derived from the differential accumulation of the original ginsenosides.

4. Conclusions

In the present study, we proposed a strategy to simultaneously determine 14 ginsenosides using an HPLC-MRM/MS method. As a proof of concept, 36 cultivated ginseng samples were analyzed. In combination with multivariate statistical analysis, we were able to differentiate ginseng samples of different cultivation areas and ages to investigate the effects of cultivation area and age on the accumulation of ginsenosides and to identify the differentially accumulated markers. In particular, the 5-yr-old JA samples had the highest total content of ginsenosides among all the cultivated ginseng. PCA and HCA demonstrated that the 3- and 5-yr-old

samples were significantly different in the contents of ginsenosides, whereas the 4-yr-old samples showed a significant similarity to either of them. Cultivation area had a significant effect on the 5-yr-old samples but not others. Moreover, seven ginsenosides Rg1, Rb1, Rb2, Rc, 20(S)-Rf, 20(S)-Rh1, and Rb3 were identified as the differentially accumulated markers that contributed to the differentiation of cultivation age. Finally, these results render the developed HPLC-MRM/MS combined with multivariable statistical analysis an applicable platform to simultaneously determine multiple ginsenosides and to evaluate the quality of cultivated ginseng.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jgr.2017.12.001>.

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