Research article

Rapid characterization of ginsenosides in the roots and rhizomes of *Panax ginseng* by UPLC-DAD-QTOF-MS/MS and simultaneous determination of 19 ginsenosides by HPLC-ESI-MS

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A R T I C L E   I N F O

A B S T R A C T

Background: Ginsenosides are the characteristic and principal components which manifest a variety of the biological and pharmacological activities of the roots and rhizomes of *Panax ginseng* (GRR). This study was carried out to qualitatively and quantitatively determine the ginsenosides in the cultivated and forest GRR.
Methods: A rapid and sensitive ultra-high-performance liquid chromatography coupled with diode-array detector and quadrupole/time of flight tandem mass spectrometry (UPLC-DAD-QTOF-MS/MS) was applied to the qualitative analysis of ginsenosides and a 4000 QTRAP triple quadrupole tandem mass spectrometer (HPLC-ESI-MS) was applied to quantitative analysis of 19 ginsenosides.
Results: In the qualitative analysis, all ingredients were separated in 10 min. A total of 131 ginsenosides were detected in cultivated and forest GRR. The method for the quantitative determination was validated for linearity, precision, and limits of detection and quantification. 19 representative ginsenosides were quantitated. The total content of all 19 ginsenosides in the forest GRR were much higher than those in the cultivated GRR, and were increased with the growing ages.
Conclusion: This newly developed analysis method could be applied to the quality assessment of GRR as well as the distinction between cultivated and forest GRR.

1. Introduction

Asian ginseng, *Panax ginseng* Meyer, is a deciduous perennial herb. It belongs to the family Araliaceae, which is distributed in Northeast China, Korea and the Russian Far East. The roots and rhizomes of ginseng (GRR) are known as the lord or king of herbs. This drug has been an important component of Chinese medicine for over 3,000 yr and is now widely used around the world [1]. Ginseng is also becoming popular in the public food field. It has been approved by the Chinese government as a new food resource in 2012 (http://news.xinhuanet.com/fortune/2012-09/05/c_112970866.htm). Nowadays, wild harvest has depleted the natural populations of ginseng to such a degree that it has become threatened with extinction in certain regions. It is necessary to cultivate the most commonly used ones to guarantee supplies. During the long-term natural and artificial selection, three cultivated types have formed, namely garden ginseng, forest ginseng and transplanted wild ginseng. Garden ginseng is produced as a type grown purely under artificial conditions, and its growth usually spans only 4—7 yr. Forest ginseng is developed by sowing seeds of garden ginseng into natural environments and letting them grow without any artificial disturbance or management, and its growth usually spans over 10 yr. Transplanted wild ginseng is domesticated by transplanting seedlings of wild ginseng into artificial or

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semiartificial environments. Because forest ginseng may become an alternative source of wild ginseng, China and Korea are vigorously developing forest ginseng. Ginsenosides [2] are the characteristic and principal components which manifest a variety of the biological and pharmacological activities of GRR [3–5] and have been an important index in assessing the quality of GRR and its products [6]. Naturally occurring ginsenosides can be further classified into three major types, namely types of protopanaxatriol (PPT), protopanaxadiol (PPD) and oleanolic acid (OA), according to their sapogenins with a dammarane or oleanane skeleton (Fig. 1). Many analytical approaches have been developed to quantify ginsenosides, including TLC [7], HPLC coupled with a UV detector [8,9] or an evaporative light scattering detector (ELSD) [10–12], and high-performance LC-MS [13]. Because of the diversity, similarity and complexity of the chemical structures, the analysis of ginsenosides is a great challenge. Liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) is a powerful tool for the ginsenosides analysis. Song et al [14] have identified three pairs of ginsenoside (G) isomers (G-Rg2 and G-Rg3, G-Rg1 and G-F11 as well as G-Rd and G-Re) and Miao et al [15] have studied the fragmentation pathway of 9 ginsenosides, namely G-Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, Rg2, and F11 by LC-MS/MS. Because MS can provide the information of molecular formula and fragmentation ions, some researchers have identified ginsenosides in red ginseng by LC-ESI-MS/MS methods. For instance, Zhang et al [16] characterized 25 ginsenosides in 152 min while Xie et al [9] identified 28 ginsenosides in 80 min. In these reports, the methods established were suitable for the analysis of the main ion peaks in

total ion current (TIC) of total ginsenosides. So the ginsenosides detected were limited and the characterization of 28 ginsenosides gave the perfect results. Usually, ginsenosides in minor or trace amounts cannot be detected. Otherwise, the analytical time is very long, which is not convenient to rapidly qualify the ginsenosides in ginseng. In order to rapidly clarify the basic chemical substances of GRR, a rapid and sensitive method, which can thoroughly detect the main and minor or trace amounts of ginsenosides, should be established. In the present study, a new rapid and sensitive ultra-high-performance liquid chromatography coupled with diode-array detector and quadrupole/time of flight tandem mass spectrometry (UPLC-DAD-QTOF-MS/MS) method was established to identify the basic chemical substances. As a result, a total of 131 ginsenosides were characterized. Also, a sensitive and practical HPLC−MS/MS^n method was developed to simultaneously determine 19 ginsenosides in GRR for the first time. This newly developed qualitative and quantitative method could be applied to the holistic quality assessment of GRR.

2. Materials and methods

2.1. GRR samples

All GRR samples are listed in Table 1. The botanical origins of samples were identified by Professor Da-Qing Zhao, of the Changchun University of Chinese Medicine, China. A voucher specimen has been deposited in the State Key Laboratory of Natural and
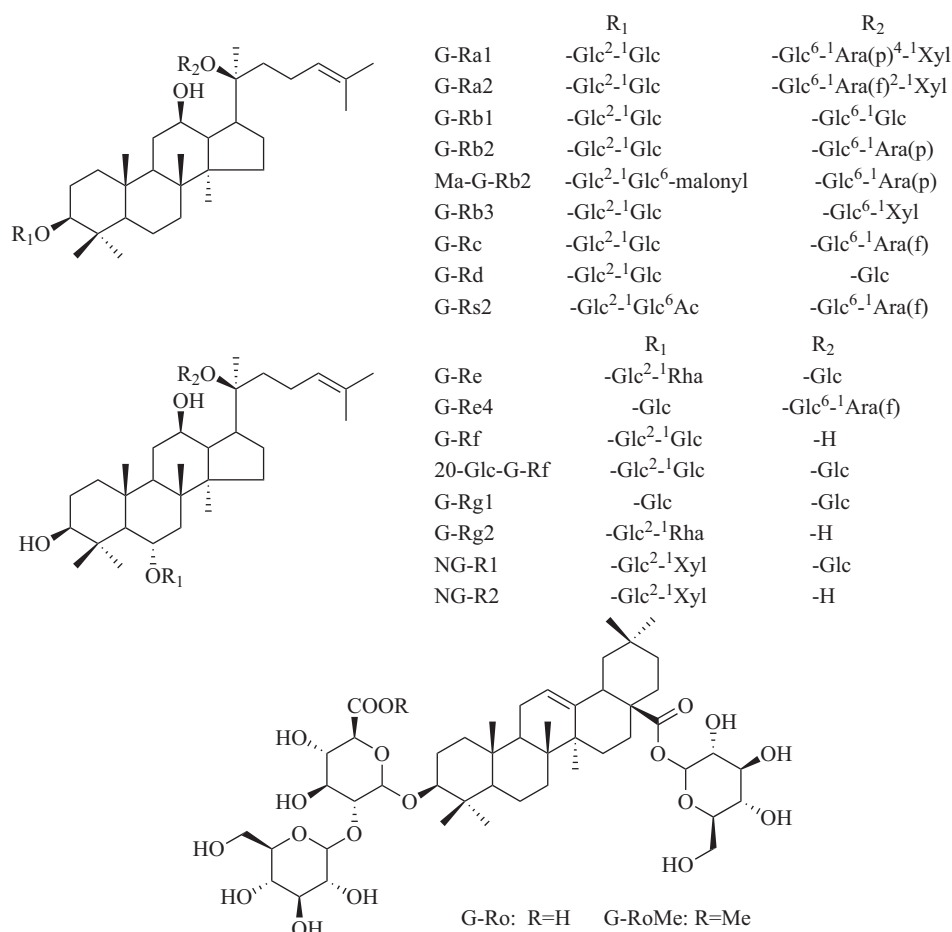

	R$_1$	R$_2$
G-Ra1	-Glc^2-^{1}Glc	-Glc^6-^{1}Ara(p)^4-^{1}Xyl
G-Ra2	-Glc^2-^{1}Glc	-Glc^6-^{1}Ara(f)^2-^{1}Xyl
G-Rb1	-Glc^2-^{1}Glc	-Glc^6-^{1}Glc
G-Rb2	-Glc^2-^{1}Glc	-Glc^6-^{1}Ara(p)
Ma-G-Rb2	-Glc^2-^{1}Glc^6-malonyl	-Glc^6-^{1}Ara(p)
G-Rb3	-Glc^2-^{1}Glc	-Glc^6-^{1}Xyl
G-Rc	-Glc^2-^{1}Glc	-Glc^6-^{1}Ara(f)
G-Rd	-Glc^2-^{1}Glc	-Glc
G-Rs2	-Glc^2-^{1}Glc^{6}Ac	-Glc^6-^{1}Ara(f)

	R$_1$	R$_2$
G-Re	-Glc^2-^{1}Rha	-Glc
G-Re4	-Glc	-Glc^6-^{1}Ara(f)
G-Rf	-Glc^2-^{1}Glc	-H
20-Glc-G-Rf	-Glc^2-^{1}Glc	-Glc
G-Rg1	-Glc	-Glc
G-Rg2	-Glc^2-^{1}Rha	-H
NG-R1	-Glc^2-^{1}Xyl	-Glc
NG-R2	-Glc^2-^{1}Xyl	-H

G-Ro: R=H G-RoMe: R=Me

Fig. 1. The chemical structures of 19 reference standards.

Table 1
The sources of roots and rhizomes of *Panax ginseng*

No.	Samples	Age (yr)
1[1]	Ji'an city, Jilin province, China	5
2[1]	Ji'an city, Jilin province, China	5
3[1]	Quanyang Jiangdong village, Fusong county, Jilin province, China	5
4[1]	Yongqing tree farm, Lushuihe, Fusong county, Jilin province, China	5
5[1]	Shangri village, Dunhua city, Jilin province, China	5
6[1]	Fuxing village, Mengjiang town, Jingyu county, Jilin province, China	5
7[1]	Liangjiang town, Antu county, Jilin province, China	5
8[1]	Majiagang village, Baoquanshan town, Changbai county, Jilin province, China	5
9[1]	Changling farm, Qianjin town, Jiaohe city, Jilin province, China	5
10[1]	Gonghe town, Mudanjiang city, Heilongjiang province, China	4
11[1]	Geumsan-gun, Korea	4
12[1]	Geumsan-gun, Korea	4
13[2]	Lengchang hamlet, Yanjiang village, Fusong county, Jilin province, China	15
14[2]	Lengchang hamlet, Yanjiang village, Fusong county, Jilin province, China	20
15[2]	Lengchang hamlet, Yanjiang village, Fusong county, Jilin province, China	25
16[2]	Lengchang hamlet, Yanjiang village, Fusong county, Jilin province, China	30

[1] Cultivated GRR
[2] Forest GRR

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2.2. Standard samples, chemicals, and reagents

Thirty-six reference standards of ginsenosides (G)-Ra1 (71), Ra2 (62), Ra3 (64), Rb1 (65), Rb2 (76), Rc (72), Rd (95), Re (14), Re1 (2), Re2 (10), Re3 (8), Re4 (4), Rf (57), Rg1 (15), Rg2 (74), Ro (73), Rs2 (93), 20-*O*-glucopyranosylginsenoside Rf (20-glc-G-Rf, 5), ginsenoside Ro methyl ester (G-RoMe, 111), notoginsenoside (NG)-Fe (119), N (13), R1 (9), R2 (66), R4 (50), quinquenoside (PQ)-R1 (84), and koryoginsenoside (KG)-R1 (56) were isolated from GRR in our previous research [17,18]. G-Rb3 (77), Rg3 (127), Rh1 (79), Rh2 (131), Rs1 (98), (20*R*)-G-Rg3 (128), 20(*R*)-G-Rh1 (99), malonylginsenoside (Ma-G)-Rb2 (80), polyacetyleneginsenoside-Ro (PG-Ro, 130), and chikusetsusaponin (CS) IV (83) were supplied by the sample bank of natural products at the State Key Laboratory of Natural and Biomimetic Drugs of Peking University. Their chemical structures were elucidated by MS and 2D NMR spectra or by comparison of spectroscopic data (IR, MS, ^{1}H-NMR, and ^{13}C-NMR) with the literature data. The purities of all reference standards were above 99.0%, as determined by an LC−DAD method. The chemical structures of 19 quantitative ginsenosides are shown in Fig. 1. LC-MS grade acetonitrile (MeCN) was obtained from J.T. Baker (Phillipsburg, NJ, USA). LC-grade MeCN and methanol (MeOH) were obtained from Dikma Tech. Inc. (Beijing, China). LC-grade formic acid was purchased from Dikma Tech. Inc. Water (H_2O) was obtained from a Milli-Q Ultra-pure water system in our laboratory (Millipore, Billerica, MA, USA). Other reagents were of analytical grade.

2.3. Sample and reference standards solutions preparation

The GRR was pulverized into powder (40 mesh). The accurately weighed powder (1.0 g) was suspended in 20 mL of 70% aqueous MeOH and was ultrasonically extracted (40 kHz, 200W) for 30 min at 30°C. The extracted solutions were then filtered. This extraction was repeated two additional times. The combined filtrate was evaporated to dryness using a rotary evaporator at 40°C. The residue was dissolved in 5 mL of 70% aqueous MeOH. The diluted solutions were filtered through a 0.22 μm syringe filter prior to qualitative and quantitative analysis. The 19 quantitative ginsenoside reference compounds were dissolved in MeOH and they were stored at 4°C until analysis. A quantity of the stock solutions of these 19 reference compounds were mixed and diluted with MeOH to obtain a series of mixture solutions containing the standard reference compounds. The solutions were filtered through a 0.22 μm syringe filter prior to qualitative and quantitative analysis.

2.4. Qualitative analysis

The Agilent 1290 Infinity Liquid Chromatography system (Agilent, MA, USA), equipped with a binary pump, an online vacuum degasser, an autosampler and a thermostatic column compartment was used to perform the separation of the multicomponents. Desirable chromatographic separation of ginsenosides in GRR was obtained on a Agilent ZORBAX RRHD Eclipse Plus C_{18} column (100 × 3 mm id, 1.8 μm) connected with a Phenomenex Security Guard ULTRA Cartridge (UHPLC C18, 2.1 mm id) by use of the mobile phase A (0.1% formic acid aqueous solution) and mobile phase B (0.1% formic acid-MeCN) in a gradient elution program: 0→5 min, 5→40%B; 5→10 min, 40→95%B. The flow rate was 0.8 mL/min and the diversion ratio was 1:1. The wavelength was set at 203 nm and the temperature was set at 45°C. The inject volume was 1 μL.

The high accuracy mass spectrometric data were recorded on an Agilent QTOF 6540 mass spectrometer (Agilent Technologies, Waldbronn, Germany) equipped with an ESI source with Agilent Jet Steam (AJS) technology in negative ion mode. The optimized parameters were obtained as follows: gas temperature: 300°C, gas flow: 5 L/min, nebulizer: 35 psi, sheath gas temperature: 400°C, sheath gas flow: 12 L/min, capillary voltage: 3,500 V, nozzle voltage: 1,500 V, fragmentor: 280 V, collision energy: 60 eV. Internal references (Purine and HP-0921) were adopted to modify the measured masses in real time, and the reference masses in negative ion mode were at *m/z* 119.0363 and 1,033.9881. The mass spectrometer was in full scan ranges of *m/z* 100−1,700 for MS and MS/MS. The acquisition rate was 1 spectrum/s for MS and 2 spectra/s for MS/MS. Data acquisition was controlled by the Agilent MassHunter Workstation Software (Version B.06.00, Agilent Technologies, Waldbronn, Germany).

2.5. Quantitative analysis

The quantitative analysis was performed using an analytical DIONEX Ultimate 3000 HPLC system consisting of a Ultimate 3000 pump, a DIONEX Ultimate 3000 Autosampler and a DIONEX Ultimate 3000 Compartment. The Applied Biosystems 4000QTRAP triple quadrupole tandem mass spectrometer (Applied Biosystems/

MDS Sciex, Canada) was equipped with an ESI source for the mass analysis and detection. All data collected were analyzed and processed using Analyst 1.5.1 software (Applied Biosystems/MDS Sciex). The separation was performed on a Diamonsil ODS C_{18} column (250 × 4.6 mm i.d., 5 μm; Dikma). The mobile phase consisted of (A) MeCN and (B) MeCN:H_2O:0.1% formic acid aqueous solution (5:90:8; v/v/v) with gradient elution (0→20 min, 10→20% A; 20→30 min, 20→22%A; 30→40 min, 22→31%A; 40→75 min, 31→33%A; 75→80 min, 33→40%A; 80→90 min, 40→50%A; 90→100 min, 50→60%A; 100→110 min, 60→70%A). The flow rate was changed with gradient (0→32 min, 0.8 mL/min; 32.1→110 min, 0.5 mL/min). The wavelength was set at 203 nm and the temperature was set at room temperature. The inject volume was 10 μL. Turbo ionspray source was set in a negative ionization mode. Multiple reaction monitoring (MRM) was used for detection transitions. The selective ion-pair, DP, and eV of the 19 ginsenosides are shown in Table 2. The ion spray voltage was set at 5,500 V and the source temperature was set at 550°C. Pin voltage was set at −4,000 V, and gas 1 and gas 2 were set at 50 psi and 55 psi, respectively.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

In the qualitative analysis of ginsenosides, desirable chromatographic separation was obtained by optimizing the column types [Agilent Extend C_{18} column (50 × 2.1 mm id, 1.8 μm); Agilent ZORBAX RRHD Eclipse Plus C_{18} column (100 × 3 mm id, 1.8 μm)], the gradient elution procedure, the flow rate (0.4 mL/min, 0.6 mL/min, and 0.8mL/min, respectively) and the temperature (35°C, 40°C, and 45°C, respectively). Finally, an Agilent ZORBAX RRHD Eclipse Plus C_{18} column (100 × 3 mm id, 1.8 μm) was used, and the mobile phase consisting of A (0.1% formic acid aqueous solution) and B (0.1% formic acid aqueous solution-MeCN) was employed to perform gradient elution. All MS parameters were optimized to achieve high sensitivity of ginsenosides. The base peak chromatogram (BPC) profiles are shown in Fig. 2.

In quantitative analysis, to achieve good separation of as many peaks as possible, the column types (Diamonsil ODS C_{18}, Varian Microsorb-MV C_8, Symmetry ODS C_{18} column), the mobile phase compositions (MeOH-H_2O, MeCN-H_2O, and formic acid aqueous solution), the gradient elution procedure, and the flow rate of mobile phase (1.0 mL/min, 0.8 mL/min, 0.5 mL/min) were optimized, respectively. Finally, a Diamonsil ODS C_{18} column was used, and the mobile phase consisting of (A) MeCN and (B) MeCN:H_2O:0.1% formic acid aqueous solution (5:90:8; v/v/v) was employed to perform gradient elution. The flow rate was also changed with gradient (0→32 min, 0.8 mL/min; 32.1→110 min, 0.5 mL/min).

Table 2
The selective ion-pair, DP, and eV of the 19 ginsenosides

Ginsenoside	DP	eV	Ion-pairs	
G-Ra1	−181.06	−72.13	1209.5	1077.5
G-Ra2	−167.77	−69.04	1209.5	1077.3
G-Rb1	−213.42	−67.09	1107.6	783.4
G-Rb2	−136.64	−59.94	1078.1	945.8
G-Rb3	−172.62	−60.18	1077.5	783.5
G-Rc	−172.00	−62.29	1077.9	783.7
G-Rd	−184.69	−60.03	945.9	621.7
G-Re	−159.47	−55.79	945.7	783.3
G-Re4	−136.13	−54.01	931.9	637.5
G-Rf	−168.93	−59.02	799.7	475.4
G-Rg1	−101.96	−35.28	799.5	637.5
G-Rg2	−163.08	−53.04	783.8	475.4
G-Ro	−153.46	−64.83	955.8	793.3
G-Rs2	−144.20	−57.76	1119.5	1077.2
G-RoMe	−102.69	−25.84	969.7	807.6
Ma-G-Rb2	−111.64	−30.92	1164.0	1120.1
20-Glc-G-Rf	−162.95	−69.98	961.7	475.4
NG-R1	−167.96	−55.19	931.8	637.7
NG-R2	−156.86	−53.04	769.8	475.3
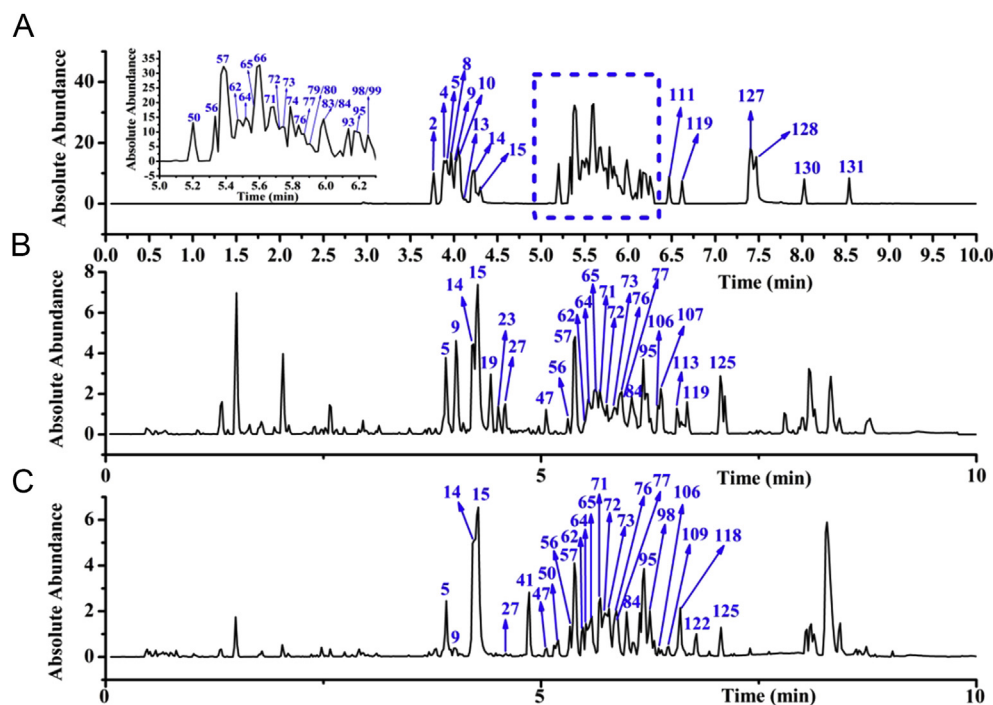
Fig. 2. The base peak chromatogram (BPC) profiles of reference standards and the roots and rhizomes of *Panax ginseng* (GRR) samples. (A) Reference standards; (B) forest GRR; (C) cultivated GRR.

Table 3
Ginsenosides identified from the roots and rhizomes of *Panax ginseng*

No.	t_R (min)	Compound name	Molecular formula	Measured value (*m/z*)	References
1	3.50	Unknown[1),2)]	$C_{53}H_{90}O_{23}$	1093.5780	—
2	3.76	G-Re1[1),2)]	$C_{48}H_{82}O_{19}$	961.5349	2
3	3.85	Floral G-M/Floral G-N[1),2)]	$C_{53}H_{90}O_{22}$	1077.5885	21–22
4	3.88	G-Re4[1),2)]	$C_{47}H_{80}O_{18}$	931.5238	2
5	3.91	20-glc-G-Rf[1),2)]	$C_{48}H_{82}O_{19}$	961.5352	2
6	3.95	Floral G-M/Floral G-N[1),2)]	$C_{53}H_{90}O_{22}$	1077.5840	21–22
7	3.95	NG-R1 isomer[1),2)]	$C_{47}H_{80}O_{18}$	931.5261	2
8	3.96	G-Re3[1),2)]	$C_{48}H_{82}O_{19}$	961.5343	2
9	4.03	NG-R1[1),2)]	$C_{47}H_{80}O_{18}$	931.5234	2
10	4.05	G-Re2[1),2)]	$C_{48}H_{82}O_{19}$	961.5353	2
11	4.11	NG-R1 isomer[1),2)]	$C_{47}H_{80}O_{18}$	931.5265	2
12	4.11	KG-R2/isomer[1),2)]	$C_{54}H_{92}O_{24}$	1123.5913	2
13	4.13	NG-N[1),2)]	$C_{48}H_{82}O_{19}$	961.5359	2
14	4.23	G-Re[1),2)]	$C_{48}H_{82}O_{18}$	945.5445	2
15	4.28	G-Rg1[1),2)]	$C_{42}H_{72}O_{14}$	799.4876	2
16	4.29	G-Re isomer[1),2)]	$C_{48}H_{82}O_{18}$	945.5436	2
17	4.35	KG-R2/isomer[1),2)]	$C_{54}H_{92}O_{24}$	1123.5926	2
18	4.39	Acetyl G-Re/isomer[1)]	$C_{50}H_{84}O_{19}$	987.5534	2
19	4.42	Acetyl G-Rg1/isomer[1)]	$C_{44}H_{74}O_{15}$	841.4936	2
20	4.43	KG-R2/isomer[1),2)]	$C_{54}H_{92}O_{24}$	1123.5920	2
21	4.50	G-Ia[1),2)]	$C_{42}H_{72}O_{14}$	799.4853	23
22	4.51	Floral G-P/isomer[1),2)]	$C_{53}H_{90}O_{23}$	1093.5815	22
23	4.52	Acetyl Re/isomer[1)]	$C_{50}H_{84}O_{19}$	987.5498	2
24	4.53	KG-R2/isomer[1),2)]	$C_{54}H_{92}O_{24}$	1123.5923	2
25	4.60	Unknown[1),2)]	$C_{47}H_{80}O_{17}$	915.5327	—
26	4.60	Floral G-P isomer[1),2)]	$C_{53}H_{90}O_{23}$	1093.5815	22
27	4.60	Acetyl G-Rg1/isomer[1),2)]	$C_{44}H_{74}O_{15}$	841.4981	2
28	4.61	KG-R2/isomer[1),2)]	$C_{54}H_{92}O_{24}$	1123.5920	2
29	4.63	KG-R2/isomer[1),2)]	$C_{54}H_{92}O_{24}$	1123.5914	2
30	4.65	Floral G-P isomer[1),2)]	$C_{53}H_{90}O_{23}$	1093.5787	22
31	4.67	Unknown[1),2)]	$C_{58}H_{98}O_{27}$	1225.6243	—
32	4.70	KG-R2/isomer[1),2)]	$C_{54}H_{92}O_{24}$	1123.5915	2
33	4.71	G-Re1/G-Re2/G-Re3/NG-N isomer[1),2)]	$C_{48}H_{82}O_{19}$	961.5356	2
34	4.73	Floral G-P/isomer[1),2)]	$C_{53}H_{90}O_{23}$	1093.5774	22
35	4.78	Unknown[1),2)]	$C_{47}H_{80}O_{17}$	915.5337	—
36	4.80	G-F3/G-F5[1),2)]	$C_{41}H_{70}O_{13}$	769.4721	24
37	4.80	Floral G-P isomer[1),2)]	$C_{53}H_{90}O_{23}$	1093.5788	22
38	4.83	G-Re1/G-Re2/G-Re3/NG-N isomer[1),2)]	$C_{48}H_{82}O_{19}$	961.5348	2
39	4.85	Acetyl G-Re/isomer[1),2)]	$C_{50}H_{84}O_{19}$	987.5545	2
40	4.86	G-F3/G-F5[1)]	$C_{41}H_{70}O_{13}$	769.4731	24
41	4.87	Yesanchinoside D isomer[1),2)]	$C_{44}H_{74}O_{15}$	841.4944	2
42	4.87	Malonyl KG-R2[1)]	$C_{57}H_{94}O_{27}$	1209.5939	2
43	4.93	G-Re1/G-Re2/G-Re3/NG-N isomer[1),2)]	$C_{48}H_{82}O_{19}$	961.5408	2
44	4.97	G-Re1/G-Re2/G-Re3/NG-N isomer[1),2)]	$C_{48}H_{82}O_{19}$	961.5335	2
45	5.00	KG-R2/isomer[1),2)]	$C_{54}H_{92}O_{24}$	1123.5928	2
46	5.02	G-Re1/G-Re2/G-Re3/NG-N isomer[1),2)]	$C_{48}H_{82}O_{19}$	961.5346	2
47	5.07	Acetyl G-Re/isomer[1),2)]	$C_{50}H_{84}O_{19}$	987.5518	2
48	5.15	Yesanchinoside D[1),2)]	$C_{44}H_{74}O_{15}$	841.4968	2
49	5.17	G-Re1/G-Re2/G-Re3/NG-N isomer[1),2)]	$C_{48}H_{82}O_{19}$	961.5408	2
50	5.20	NG-R4[1),2)]	$C_{59}H_{100}O_{27}$	1239.6370	2
51	5.23	G-Rb1 isomer[1),2)]	$C_{54}H_{92}O_{23}$	1107.5922	2
52	5.23	Acetyl panajaponol A[2)]	$C_{44}H_{74}O_{15}$	887.4973[3)]	25
53	5.25	Malonyl NG-R4[1),2)]	$C_{62}H_{102}O_{30}$	1325.6359	2
54	5.30	Unknown[1),2)]	$C_{60}H_{102}O_{28}$	1269.6467	—
55	5.32	NG-R4 isomer[1),2)]	$C_{59}H_{100}O_{27}$	1239.6354	2
56	5.33	KG-R1[1),2)]	$C_{46}H_{76}O_{15}$	867.5138	2
57	5.38	G-Rf[1),2)]	$C_{42}H_{72}O_{14}$	799.4842	2
58	5.42	G-Ra2 isomer[1),2)]	$C_{58}H_{98}O_{26}$	1209.6244	2
59	5.43	G-Ra3 isomer[1),2)]	$C_{59}H_{100}O_{27}$	1239.6349	2
60	5.45	Unknown[1),2)]	$C_{60}H_{102}O_{28}$	1269.6438	—
61	5.45	G-Rb1 isomer[1),2)]	$C_{54}H_{92}O_{23}$	1107.5906	2
62	5.48	G-Ra2[1),2)]	$C_{58}H_{98}O_{26}$	1209.6243	2
63	5.51	Acetyl panajaponol A[1)]	$C_{44}H_{74}O_{15}$	841.4935	25
64	5.52	G-Ra3[1),2)]	$C_{59}H_{100}O_{27}$	1239.6356	2
65	5.58	G-Rb1[1),2)]	$C_{54}H_{92}O_{23}$	1107.5987	2
66	5.60	NG-R2[1),2)]	$C_{41}H_{70}O_{13}$	769.4715	2
67	5.62	Malonyl G-Rb1[1),2)]	$C_{57}H_{94}O_{26}$	1193.5927	2
68	5.62	Acetyl G-Rb1/isomer[1),2)]	$C_{56}H_{94}O_{24}$	1149.6023	2
69	5.63	G-Ro isomer[1),2)]	$C_{48}H_{76}O_{19}$	955.4934	2
70	5.68	NG-R2 isomer[1),2)]	$C_{41}H_{70}O_{13}$	769.4748	2
71	5.68	G-Ra1[1),2)]	$C_{58}H_{98}O_{26}$	1209.6247	2
72	5.73	G-Rc[1),2)]	$C_{53}H_{90}O_{22}$	1077.5872	2
73	5.77	G-Ro[1),2)]	$C_{48}H_{76}O_{19}$	955.4936	2
74	5.78	G-Rg2[1),2)]	$C_{42}H_{72}O_{13}$	783.4883	2

Table 3 (continued)

No.	t_R (min)	Compound name	Molecular formula	Measured value (m/z)	References
75	5.80	Acetyl G-Rb1/isomer[1],[2]	$C_{56}H_{94}O_{24}$	1149.6050	2
76	5.84	G-Rb2[1],[2]	$C_{53}H_{90}O_{22}$	1077.5814	2
77	5.87	G-Rb3[1],[2]	$C_{53}H_{90}O_{22}$	1077.5796	2
78	5.90	G-Ra1 isomer[2]	$C_{58}H_{98}O_{26}$	1209.6252	2
79	5.90	G-Rh1[1],[2]	$C_{36}H_{62}O_9$	637.4346	2
80	5.90	Malonyl G-Rb2[1],[2]	$C_{56}H_{92}O_{25}$	1163.5839	2
81	5.92	G-Ro isomer[1],[2]	$C_{48}H_{76}O_{19}$	955.4922	2
82	5.93	Acetyl G-Rb2[1],[2]	$C_{55}H_{92}O_{23}$	1119.5944	2
83	5.97	CS IV[1],[2]	$C_{47}H_{74}O_{18}$	925.4769	25
84	5.98	PQ-R1[1],[2]	$C_{56}H_{94}O_{24}$	1149.6054	2
85	5.99	G-Rd isomer[1]	$C_{48}H_{82}O_{18}$	945.5437	2
86	6.02	Pseudo-G-RT1[1],[2]	$C_{47}H_{74}O_{18}$	925.4810	25
87	6.03	Yesanchinoside D isomer[1],[2]	$C_{44}H_{74}O_{15}$	841.4926	2
88	6.03	G-Rb2/G-Rb3/G-Rc isomer[1],[2]	$C_{53}H_{90}O_{22}$	1077.5865	2
89	6.04	Acetyl G-Rc[1],[2]	$C_{55}H_{92}O_{23}$	1119.6001	2
90	6.05	G-Ra5[1],[2]	$C_{60}H_{100}O_{27}$	1251.6393	2
91	6.08	PQ-R1 isomer[1],[2]	$C_{56}H_{94}O_{24}$	1149.6036	2
92	6.12	G-Ro isomer[1]	$C_{48}H_{76}O_{19}$	955.4947	2
93	6.13	G-Rs2[1],[2]	$C_{55}H_{92}O_{23}$	1119.5986	2
94	6.14	Unknown[1]	$C_{52}H_{88}O_{21}$	1047.5728	—
95	6.18	G-Rd[1],[2]	$C_{48}H_{82}O_{18}$	945.5453	2
96	6.22	CS IVa[1],[2]	$C_{42}H_{66}O_{14}$	793.4354	25
97	6.22	PQ-R1 isomer[2]	$C_{56}H_{94}O_{24}$	1149.6039	2
98	6.25	G-Rs1[1],[2]	$C_{55}H_{92}O_{23}$	1119.5976	2
99	6.27	20(R)-G-Rh1[1],[2]	$C_{36}H_{62}O_9$	637.4307	2
100	6.28	G-Ra5 isomer[1],[2]	$C_{60}H_{100}O_{27}$	1251.6376	2
101	6.29	G-Rd isomer[1],[2]	$C_{48}H_{82}O_{18}$	945.5433	2
102	6.29	G-Ro isomer[1]	$C_{48}H_{76}O_{19}$	955.4924	2
103	6.30	G-Ra6[1],[2]	$C_{58}H_{96}O_{24}$	1175.6234	2
104	6.35	Acetyl G-Rb2[1],[2]	$C_{55}H_{92}O_{23}$	1119.5984	2
105	6.35	Malonyl G-Rd[1],[2]	$C_{51}H_{84}O_{21}$	1031.5449	2
106	6.38	G-Rd isomer[1],[2]	$C_{48}H_{82}O_{18}$	945.5449	2
107	6.43	Acetyl G-Rd[1],[2]	$C_{50}H_{84}O_{19}$	987.5564	2
108	6.43	G-Re6[1],[2]	$C_{46}H_{76}O_{15}$	867.5137	2
109	6.45	G-Ra7/G-Ra8/G-Ra9[1],[2]	$C_{57}H_{94}O_{23}$	1145.6134	2
110	6.47	Acetyl G-Rb3[1],[2]	$C_{55}H_{92}O_{23}$	1119.5977	2
111	6.47	G-Ro methyl ester[1],[2]	$C_{49}H_{78}O_{19}$	969.5033	2
112	6.48	G-Ro isomer[1]	$C_{48}H_{76}O_{19}$	955.4890	2
113	6.48	Vinaginsenoside R16[1],[2]	$C_{47}H_{80}O_{17}$	915.5351	20
114	6.50	Gypenoside XVII[1],[2]	$C_{48}H_{82}O_{18}$	945.5450	2
115	6.52	G-Ra6 isomer[1],[2]	$C_{58}H_{96}O_{24}$	1175.6235	2
116	6.53	Pseudo-G-RC1[1],[2]	$C_{50}H_{84}O_{19}$	987.5547	2
117	6.57	G-Ra7/G-Ra8/G-Ra9[1],[2]	$C_{57}H_{94}O_{23}$	1145.6130	2
118	6.60	Pseudo-G-RC1 isomer[1],[2]	$C_{50}H_{84}O_{19}$	987.5520	2
119	6.62	NG-Fe[1],[2]	$C_{47}H_{80}O_{17}$	915.5330	26
120	6.65	G-Ra6 isomer[2]	$C_{58}H_{96}O_{24}$	1175.6243	2
121	6.70	Pseudo-G-RC1 isomer[1],[2]	$C_{50}H_{84}O_{19}$	987.5545	2
122	6.78	Acetyl gypenoside XVII[1],[2]	$C_{50}H_{84}O_{19}$	987.5509	2
123	6.80	G-Ra7/G-Ra8/G-Ra9[1],[2]	$C_{57}H_{94}O_{23}$	1145.6144	2
124	6.95	Unknown[1],[2]	$C_{48}H_{82}O_{17}$	929.5453	—
125	7.07	CS IVa isomer[1],[2]	$C_{42}H_{66}O_{14}$	793.4353	25
126	7.39	Acetyl G-Rg3[1]	$C_{44}H_{74}O_{14}$	825.5018	2
127	7.40	G-Rg3[1],[2]	$C_{42}H_{72}O_{13}$	783.4872	2
128	7.47	(20R)-G-Rg3[1],[2]	$C_{42}H_{72}O_{13}$	783.4918	2
129	7.92	PG-Ro isomer[1],[2]	$C_{65}H_{100}O_{21}$	1215.6632	2
130	8.02	PG-Ro[1],[2]	$C_{65}H_{100}O_{21}$	1215.6661	2
131	8.54	G-Rh2[1],[2]	$C_{36}H_{62}O_8$	667.4427[3]	2

[1] Ginsenosides detected in forest roots and rhizomes of *Panax ginseng*

[2] Ginsenosides detected in cultivated roots and rhizomes of *Panax ginseng*

[3] $[M+HCOO]^-$

3.2. Identity assignment and confirmation of the ginsenosides in GRR

To date, more than 50 ginsenosides [2] have been isolated and unambiguously characterized from GRR. 36 reference ginsenosides were used not only to optimize the mass chromatographic conditions but also to obtain the fragmentation pathways of ginsenosides. Because ginsenosides had not only higher sensitivity but also clearer mass spectra in the negative ion mode, data monitored in negative ion mode were used for the component detection and characterization. This made it easier to detect ginsenosides of lower content and confirm molecular ions or quasi-molecular ions in the identification of each peak. According to the t_R (retention time), ESI-MS (molecular weight) and MS/MS (fragment ion) information, the chromatographic behaviors and MS spectra of 36 reference standards were obtained (Tables 3 and S1), which were the basis for identifying the other ingredients in GRR. The negative MS/MS spectra were obtained from the deprotonated molecular $[M-H]^-$ ions, and the mass spectra of the product ion of $[M-H]^-$ exhibited a fragmentation pattern corresponding to the successive loss of the
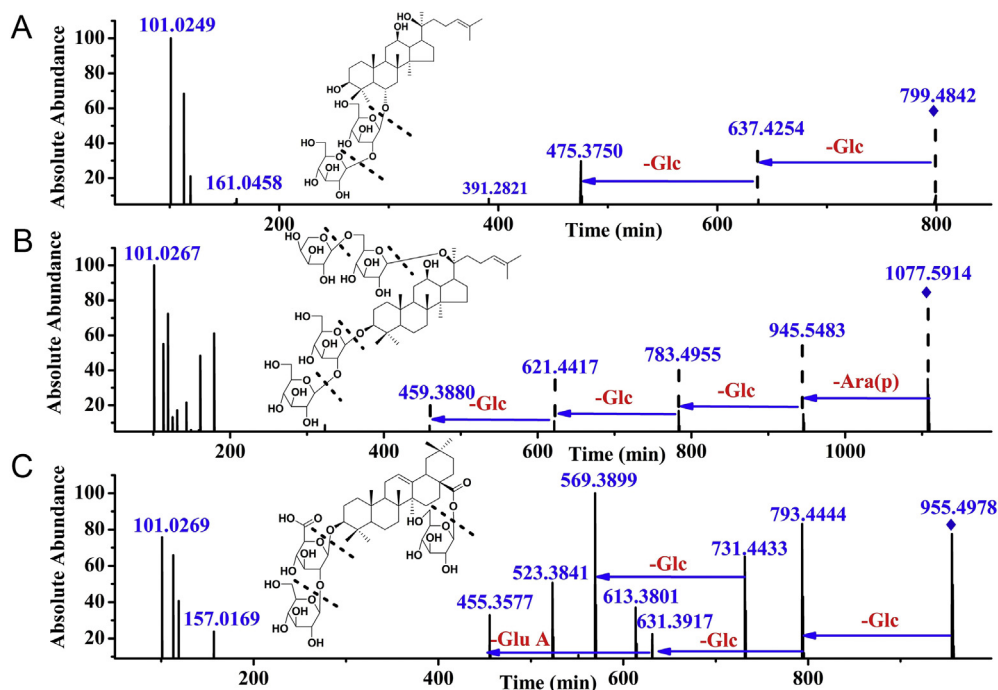
Fig. 3. The MS/MS spectra of ginsenosides in the roots and rhizomes of *Panax ginseng*. (A) The MS/MS spectrum of G-Rf, the aglycone of which was yielded by successive losses of Glc and Glc from the precursor ion at *m/z* 799.4842. (B) The MS/MS spectrum of G-Rb2, the aglycone of which was yielded by successive losses of Ara(p), Glc, Glc and Glc from the precursor ion at *m/z* 1,077.5914. (C) The MS/MS spectrum of G-Ro, the aglycone of which was yielded by successive losses of Glc, Glc and Glu A from the precursor ion at *m/z* 955.4978.

glycosidic units until the formation of [aglycon−H]$^-$ ions. According to the structural properties, PPT type ginsenosides possessed an aglycon ion at *m/z* 475 which was visible for 2, 4, 5, 8, 9, 10, 13, 14, 15, 56, 57, 66, 74, 79, and 99. As illustrated in Fig. 3A, in the MS/MS spectrum, for example ginsenoside Rf (57) gave [(20S)-proto-panaxatriol−H]$^-$ at *m/z* 475.3750 ($C_{30}H_{51}O_3$) via successive losses of Glc (162 Da) and Glc (162 Da). While PPD-type ginsenosides, including 50, 62, 64, 65, 71, 72, 76, 77, 80, 84, 93, 95, 98, 119, 127, 128 and 131, produced an aglycon ion at *m/z* 459. As illustrated in Fig. 3B, in the MS/MS spectrum, ginsenoside Rb2 (76) gave [(20S)-

protopanaxadiol−H]$^-$ at *m/z* 459.3880 ($C_{30}H_{51}O_4$) via the successive elimination of one arabinose and three glucoses. And OA type ginsenosides, including 73, 83, 111, and 130, produced an aglycon ion at *m/z* 455 ($C_{30}H_{47}O_3$), corresponding to [oleanolic acid−H]$^-$. For instance, ginsenoside Ro (73) shown in Fig. 3C gave diagnostic ions [oleanolic acid−H]$^-$ at *m/z* 455.3577 ($C_{30}H_{47}O_3$), which was formed via the losses of Glc, Glc, and Glu A. Therefore, the aglycones could be easily identified by finding these diagnostic fragment ions initially. The obtained neutral loss could be used to elucidate sugar unit moiety. The amount and the type of saccharide units were

Table 4
The regression equation, linear range, limits of detection and limits of quantification of 19 ginsenosides

Ginsenoside	Regression equations	Correlation coefficients (r^2)	Linear ranges (ng/mL)	LOD (ng)	LOQ (ng)
G-Ra1	$y = 262.23x + 533.92$	0.9999	12−384	0.791	2.637
G-Ra2	$y = 330.48x − 569.63$	0.9994	6−192	1.043	3.478
G-Rb1	$y = 163.3x − 453.87$	0.9992	15.625−500	4.360	14.535
G-Rb2	$y = 158.19x − 2281.6$	0.9993	35−1120	9.052	30.172
G-Rb3	$y = 160.54x − 133.47$	0.9997	6−192	1.636	5.455
G-Rc	$y = 181.19x − 1229.5$	0.9995	25−800	5.714	19.048
G-Rd	$y = 180.6x − 1613.9$	0.9991	12−384	2.133	7.111
G-Re	$y = 359.63x − 45.256$	0.9992	18.75−600	2.980	9.934
G-Re4	$y = 181.15x − 84.803$	0.9993	1.5−48	0.400	1.333
G-Rf	$y = 2243.4x − 3447.9$	0.9999	9.375−300	0.374	1.246
G-Rg1	$y = 510.21x − 1085.4$	0.9995	15.625−500	2.042	6.801
G-Rg2	$y = 1864.5x − 2866.4$	0.9992	2.5−80	0.615	2.051
G-Ro	$y = 1090.4x + 10948$	0.9993	24−768	0.850	2.832
G-Rs2	$y = 402.9x − 1839.7$	0.9994	12−384	2.504	8.348
G-RoMe	$y = 2666.1x − 837.11$	0.9993	0.625−20	0.159	0.530
20-Glc-G-Rf	$y = 388.13x + 156.29$	0.9998	7.5−240	1.565	5.217
Ma-G-Rb2	$y = 333.73x + 184.91$	0.9994	4.5−144	1.125	3.750
NG-R1	$y = 613.88x + 372.02$	0.9995	1.875−60	0.441	1.471
NG-R2	$y = 5901.6x − 5532$	0.9991	1.5−48	0.169	0.565

LOD, limit of detection; LOQ, limit of quantification

Table 5
The recovery of the 19 ginsenosides

| Ginsenoside	Original (ng)	Spiked (ng)	Found (ng)	Recovery (%)	RSD (%)
G-Ra1	138.96	120.00	260.64 ± 6.05	101.40	2.32
G-Ra2	10.07	10.00	20.25 ± 0.38	101.80	1.87
G-Rb1	139.70	120.00	255.08 ± 4.05	96.15	1.59
G-Rb2	86.47	100.00	184.71 ± 2.72	98.24	1.47
G-Rb3	36.98	30.00	65.72 ± 2.60	95.82	3.95
G-Rc	97.70	100.00	196.82 ± 4.80	99.12	2.44
G-Rd	81.83	90.00	173.58 ± 2.62	101.95	1.51
G-Re	179.93	180.00	362.78 ± 8.30	101.58	2.29
G-Re4	2.74	2.00	4.71 ± 0.18	98.33	3.73
G-Rf	29.26	30.00	58.98 ± 1.10	99.08	1.86
G-Rg1	90.40	90.00	179.34 ± 1.47	98.84	0.82
G-Rg2	21.46	20.00	40.88 ± 1.41	97.09	3.45
G-Ro	73.01	80.00	153.27 ± 2.99	100.31	1.95
G-Rs2	16.50	15.00	31.47 ± 0.49	99.83	1.57
G-RoMe	0.90	1.00	1.87 ± 0.03	97.07	1.81
20-Glc-G-Rf	10.73	9.00	19.26 ± 0.59	94.87	3.08
Ma-G-Rb2	12.15	10.00	21.79 ± 0.97	96.38	4.47
NG-R1	12.29	14.00	26.21 ± 1.04	99.39	3.99
NG-R2	8.28	8.00	16.48 ± 0.64	102.45	3.91

RSD, relative standard deviation

determined in which a mass difference of 162 Da indicating the presence of a glucosyl (Glc) group, 132 Da indicating the presence of a pentosyl group [arabinose (Ara) (pyranose or furanose) or xylose (Xyl)], 146 Da indicating the presence of an rhamnosyl (Rha) group, and 176 Da indicating the presence of a glucuronyl (Glu A) group.

Thirty-six ginsenosides (2, 4, 5, 8, 9, 10, 13−15, 50, 56, 57, 62, 64−66, 71−74, 76, 77, 79, 80, 83, 84, 93, 95, 98, 99, 111, 119, 127, 128, 130,

and 131) were unambiguously identified by comparison with the reference standards. The others were tentatively assigned by matching the empirical molecular formulas with those of the published known ginsenosides and the fragmentation features as well as the retention sequence of isomeric ginsenosides [19].

Peaks 3 and 6 were eluted at 3.85 min and 3.95 min, respectively. Their $[M-H]^-$ ions were observed at m/z 1,077.5885, indicating that their molecular formula was $C_{53}H_{90}O_{22}$. The MS/MS spectrum showed their aglycone ion at m/z 475.3825, suggesting that they were PPT-type ginsenosides. Their fragmentation ions at m/z 945.4295, 783.3972, 637.3243 and 475.3825 suggesting that Ara, Glc, Rha and Glc were successively eliminated from the $[M-H]^-$ ion. Thus, Peaks 3 and 6 were deduced as Floral G-M or Floral G-N, respectively. Peak 113 (t_R = 6.48 min) gave an $[M-H]^-$ ion at m/z 915.5351 indicating that its molecular formula was $C_{47}H_{80}O_{17}$. The MS/MS spectrum showed the aglycone ion at m/z 459.3825, suggesting that Peak 113 was a PPD-type ginsenoside. The fragmentation pattern $915.5351 \rightarrow 783.4955 \rightarrow 621.4434 \rightarrow 459.3825$ suggested the successive losses of Xyl (132 Da), Glc (162 Da), and Glc (162 Da) from the $[M-H]^-$ ion. Thus, Peak 113 was deduced to be vinaginsenoside R16, which was isolated previously from the underground part of *Panax vietnamensis* Ha et Grushv. [20]. Peak 96 (t_R = 6.22 min) gave the $[M-H]^-$ ion at m/z 793.4354, indicating the molecular formula was $C_{42}H_{66}O_{14}$. The MS/MS spectrum showed the aglycone ion was at m/z 455.3552, suggesting that Peak 96 was an OA-type ginsenoside. The fragmentation ions at m/z 631.3881 and 455.3552 indicated that Glc (162 Da) and Glu A (176 Da) were successively eliminated from the $[M-H]^-$ ion. Based on the information above, Peak 96 was deduced as CS Iva. In the same way, Peaks 36 and 40 were deduced as G-F3 or G-F5, respectively. Peaks
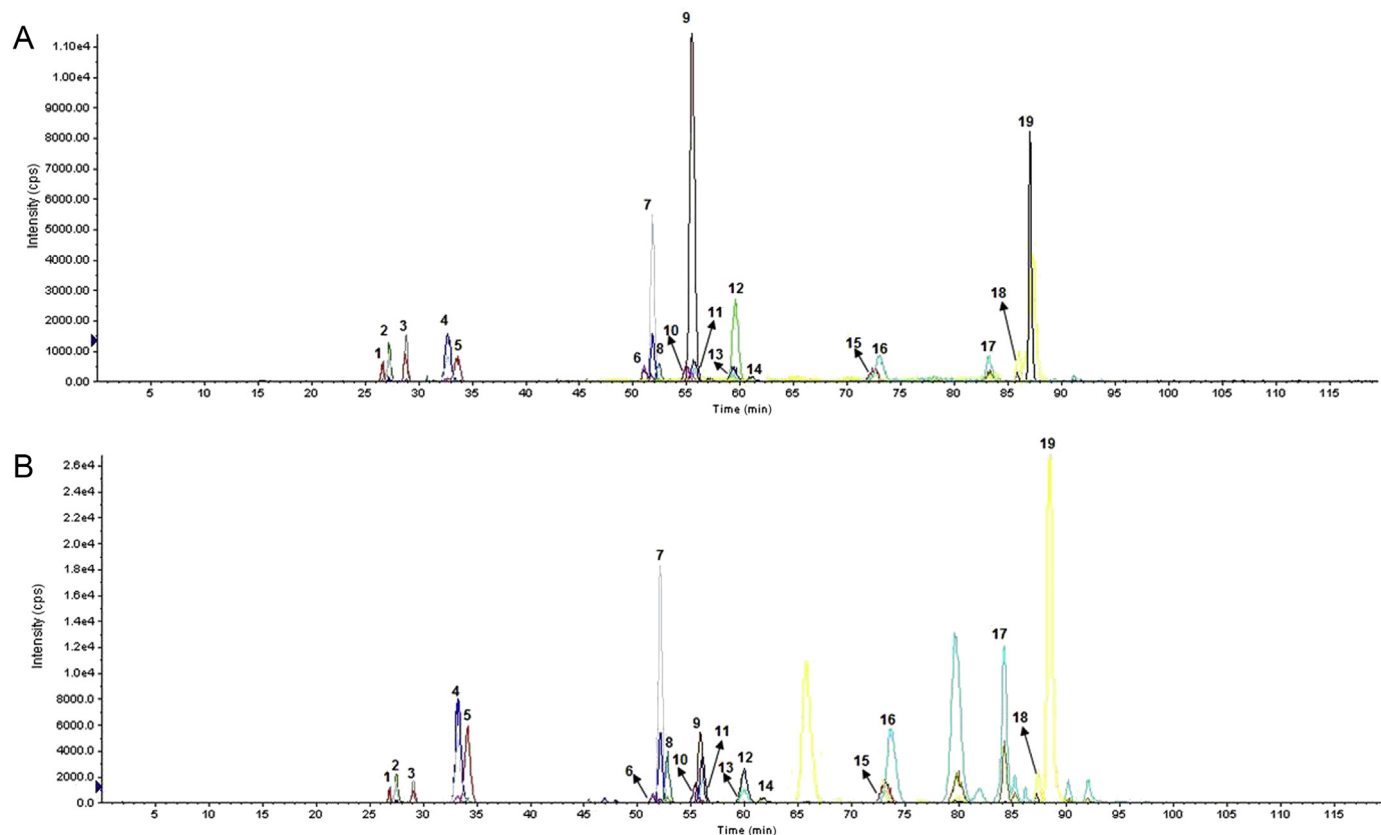
Fig. 4. The chemical profiling of (A) reference standards and (B) the roots and rhizomes of *Panax ginseng* samples. 1, G-Re4; 2, 20-glc-G-Rf; 3, NG-R1; 4, G-Rg1; 5, G-Re; 6, G-Ra2; 7, G-Rf; 8, G-Rb1; 9, NG-R2; 10, G-Ra1; 11, G-Rc; 12, G-Rg2; 13, G-Rb2; 14, G-Rb3; 15, G-Rd; 16, G-Rs2; 17, Ma-G-Rb2; 18, G-RoMe; 19, G-Ro.

Table 6
The amounts of 19 main ginsenosides in the roots and rhizomes of *Panax ginseng* from different sources

Ginsenoside	Content (mg/g)						
	1	2	3	4	5	6	7
G-Ra1	1.760 ± 0.020	0.624 ± 0.027	0.148 ± 0.002	0.102 ± 0.001	1.737 ± 0.032	0.480 ± 0.015	0.809 ± 0.037
G-Ra2	0.618 ± 0.006	0.195 ± 0.009	0.056 ± 0.001	0.051 ± 0.001	0.122 ± 0.002	0.087 ± 0.004	0.244 ± 0.009
G-Rb1	2.918 ± 0.049	2.607 ± 0.100	2.947 ± 0.087	4.897 ± 0.081	1.746 ± 0.051	1.970 ± 0.084	3.824 ± 0.181
G-Rb2	1.398 ± 0.031	1.277 ± 0.052	2.713 ± 0.010	3.891 ± 0.135	1.085 ± 0.047	2.448 ± 0.027	3.274 ± 0.140
G-Rb3	0.630 ± 0.002	0.445 ± 0.009	0.436 ± 0.007	0.406 ± 0.018	0.462 ± 0.009	0.363 ± 0.004	0.702 ± 0.019
G-Rc	2.051 ± 0.065	1.568 ± 0.071	2.610 ± 0.041	3.120 ± 0.119	1.221 ± 0.054	2.146 ± 0.039	3.623 ± 0.172
G-Rd	1.220 ± 0.046	0.312 ± 0.006	1.541 ± 0.034	1.304 ± 0.057	1.023 ± 0.023	1.555 ± 0.063	1.839 ± 0.082
G-Re	2.034 ± 0.084	1.423 ± 0.067	1.545 ± 0.062	2.989 ± 0.125	2.249 ± 0.090	1.817 ± 0.029	2.120 ± 0.020
G-Re4	0.048 ± 0.002	0.077 ± 0.003	0.073 ± 0.001	0.046 ± 0.002	0.034 ± 0.001	0.065 ± 0.000	0.109 ± 0.005
G-Rf	0.864 ± 0.002	0.619 ± 0.024	0.847 ± 0.023	0.817 ± 0.036	0.448 ± 0.017	0.667 ± 0.020	0.996 ± 0.042
G-Rg1	1.753 ± 0.008	2.224 ± 0.063	2.755 ± 0.115	2.915 ± 0.072	1.130 ± 0.043	2.136 ± 0.032	2.927 ± 0.091
G-Rg2	0.390 ± 0.010	0.135 ± 0.002	0.118 ± 0.001	0.288 ± 0.009	0.268 ± 0.011	0.175 ± 0.005	0.269 ± 0.003
G-Ro	2.529 ± 0.077	1.883 ± 0.033	4.055 ± 0.158	2.573 ± 0.084	0.930 ± 0.046	2.339 ± 0.102	2.709 ± 0.125
G-Rs2	0.187 ± 0.000	0.192 ± 0.002	0.210 ± 0.03	0.156 ± 0.000	0.206 ± 0.003	0.222 ± 0.002	0.217 ± 0.002
G-RoMe	0.017 ± 0.001	0.014 ± 0.001	0.048 ± 0.001	0.018 ± 0.001	0.011 ± 0.000	0.018 ± 0.001	0.029 ± 0.001
20-glu-G-Rf	0.137 ± 0.003	0.249 ± 0.013	0.421 ± 0.012	0.404 ± 0.018	0.134 ± 0.004	0.368 ± 0.010	0.331 ± 0.002
Ma-G-Rb2	0.157 ± 0.002	0.371 ± 0.017	0.368 ± 0.006	0.831 ± 0.021	0.152 ± 0.001	0.352 ± 0.006	0.484 ± 0.024
NG-R1	0.033 ± 0.002	0.039 ± 0.001	0.305 ± 0.014	0.564 ± 0.023	0.154 ± 0.007	0.168 ± 0.008	0.028 ± 0.000
NG-R2	0.031 ± 0.001	0.030 ± 0.001	0.214 ± 0.001	0.295 ± 0.008	0.102 ± 0.001	0.203 ± 0.002	0.044 ± 0.001
Total amounts	18.774 ± 0.411	14.285 ± 0.499	21.410 ± 0.601	25.667 ± 0.809	13.216 ± 0.441	17.580 ± 0.453	24.578 ± 0.956

21, 48, 86, 90, 103, 108, 114 and 116 were deduced as G-Ia, Yesanchinoside D, Pseudo-G-RT1, G-Ra5, G-Ra6, G-Re6, Gypenoside XVII, and Pseudo-G-RC1, respectively. Peaks 109, 117 and 123 were assigned as G-Ra7, G-Ra8 or G-Ra9, respectively.

Peak 67 gave the precursor ion at m/z 1,193.5927, indicating its molecular formula was $C_{57}H_{94}O_{26}$. In the MS/MS spectra, the mass difference between m/z 1,193.5927 and m/z 1,107.5932 suggested that malonyl was eliminated from the [M−H]$^-$ ion. Other fragmentation ions at m/z 945.5413, 783.4921, 621.4384 and 459.3833 were formed via successive losses of Glc, Glc, Glc and Glc from the [M−H−malonyl]$^-$ ion. After losing the malonyl group, the fragmentation pathway was similar to that of G-Rb1. Thus, peak 67 was deduced as malonyl G-Rb1. Similarly, peaks 42, 53, and 105 were deduced as malonyl KG-R2, malonyl NG-R4 and malonyl G-Rd, respectively. Peak 107 gave the dehydrogenation ion at m/z 987.5564, suggesting its molecular formula was $C_{50}H_{84}O_{19}$. After losing the Ac group, the fragmentation ions at m/z 783.4980, 621.3635, and 459.3897 were formed via successive losses of Glc, Glc and Glc. The fragmentation pathway was similar to that of G-Rd. Thus, peak 107 was deduced as acetyl G-Rd. Similarly, peaks 18, 23, 39 and 47 were tentatively assigned as acetyl G-Re and its isomers, whereas peaks 19 and 27 were tentatively deduced as acetyl G-Rg1 and its isomers. Peaks 52 and 63 were tentatively assigned as acetyl panajaponol A. Peaks 68 and 75 were deduced as acetyl G-Rb1 or its isomer, whereas peaks 82 and 104 were deduced as acetyl G-Rb2, respectively. Peak 89, 110, 122 and 126 were assigned as acetyl G-Rc, acetyl G-Rb3, acetyl gypenoside XVII, and acetyl G-Rg3, respectively.

During the identification of ginsenosides, there were a lot of isomers which had the same aglycone and sugar moiety. Therefore, these isomers could not be unambiguously identified. Peaks 51 (t_R = 5.23 min) and 61 (t_R = 5.45 min) gave the same [M−H]$^-$ ion at m/z 1,107.5922 ($C_{54}H_{92}O_{23}$). In their MS/MS spectra, the diagnostic ion at m/z 459 indicated the structures of peaks 51 and 61 were PPD-type ginsenosides. Their fragmentation pathway was also the same as that of G-Rb1, exhibiting fragmentation pathway of 1,107→945→783→621→459. Their fragmentation pathways suggested Glc (162 Da), Glc (162 Da), Glc (162 Da) and Glc (162 Da) were successively eliminated from the [M−H]$^-$ ion.

Thus, peaks 51 and 61 were deduced as G-Rb1 isomers. Peaks 7 and 11 were tentatively assigned as NG-R1 isomers due to their fragmentation pathways being the same as that of NG-R1. Similarly, peaks 12, 17, 20, 24, 28, 29, 32, and 45 were tentatively deduced as KG-R2 and its isomers. Peaks 22, 26, 30, 34, and 37 were tentatively assigned as floral G-P and its isomers, whereas peaks 33, 38, 43, 44, 46, and 49 were tentatively deduced as isomers of G-Re1/G-Re2/G-Re3/NG-N. Peaks 41 and 87 were tentatively assigned as yesanchinoside D isomers, whereas peaks 91 and 97 were tentatively deduced as PQ-R1 isomers. Three isomers of G-Rd (peaks 85, 101, and 106) and five isomers of G-Ro (peaks 69, 81, 92, 102, and 112) were also detected. Peaks 115 and 120 were tentatively assigned as G-Ra6 isomers, whereas peaks 118 and 121 were tentatively deduced as pseudo-G-RC1 isomers. In addition, peaks 16 (G-Re isomer), 55 (NG-R4 isomer), 58 (G-Ra2 isomer), 59 (G-Ra3 isomer), 70 (NG-R2 isomer), 78 (G-Ra1 isomer), 88 (isomer of G-Rb2/G-Rb3/G-Rc), 100 (G-Ra5 isomer), 125 (CS IVa isomer), and 129 (PG-Ro isomer) were also tentatively assigned.

Fortunately, some potential new compounds were also detected. For example, the dehydrogenation ion of peak 1 was observed at m/z 1,093.5780, indicating the molecular formula was $C_{53}H_{90}O_{23}$. The aglycone ion was observed at m/z 475.3864 suggesting peak 1 was a PPT-type ginsenoside. The fragmentation ions at m/z 931.4853, 799.4458, 637.3989, and 475.3864 were formed via successive losses of Glc, Ara or Xyl, Glc and Glc from the [M−H]$^-$ ion. Based on the data above, peak 1 was deduced as PPT-type ginsenoside and the aglycone ion linked with 3Glc and Ara or Xyl. Similarly, a further 7 potential new compounds, including peaks 25, 31, 35, 54, 60, 94 and 124 were tentatively assigned.

According to the result of qualitative analysis, a total of 131 ginsenosides were identified. Among them, 115 ginsenosides presented in both cultivated and forest GRR. Only 4 peaks, which did not exist in forest GRR, including peaks 52 (acetyl-panajaponol A), 78 (G-Ra1 isomer), 97 (PQ-R1 isomer), and 120 (G-Ra6 isomer) were detected in cultivated GRR. A further 12 ginsenosides including peaks 18 (acetyl G-Re/isomer), 19 (acetyl G-Rg1/isomer), 23 (acetyl G-Re/isomer), 40 (G-F3/G-F5), 42 (malonyl KG-R2), 63 (acetyl panajaponol A), 85 (G-Rd isomer), 92 (G-Ro isomer), 94

				Content (mg/g)				
8	9	10	11	12	13	14	15	16
1.879 ± 0.038	0.712 ± 0.024	0.532 ± 0.007	0.161 ± 0.005	0.469 ± 0.017	0.444 ± 0.003	0.809 ± 0.037	1.879 ± 0.038	0.712 ± 0.024
1.237 ± 0.001	0.293 ± 0.002	0.212 ± 0.003	0.156 ± 0.003	0.166 ± 0.004	0.033 ± 0.000	0.449 ± 0.002	0.414 ± 0.004	0.585 ± 0.004
3.966 ± 0.018	3.722 ± 0.054	1.649 ± 0.057	3.427 ± 0.112	1.596 ± 0.041	5.361 ± 0.021	5.397 ± 0.004	5.211 ± 0.010	5.457 ± 0.016
1.031 ± 0.026	1.919 ± 0.001	1.757 ± 0.007	2.396 ± 0.049	1.488 ± 0.061	3.924 ± 0.030	4.081 ± 0.028	4.382 ± 0.003	5.552 ± 0.006
0.720 ± 0.003	0.398 ± 0.002	0.523 ± 0.001	0.601 ± 0.010	0.454 ± 0.004	1.329 ± 0.002	0.778 ± 0.004	1.845 ± 0.024	2.128 ± 0.006
1.267 ± 0.049	2.005 ± 0.007	1.790 ± 0.015	2.492 ± 0.051	1.336 ± 0.052	2.477 ± 0.008	1.428 ± 0.028	2.241 ± 0.010	4.541 ± 0.006
0.899 ± 0.037	1.020 ± 0.004	0.780 ± 0.025	0.896 ± 0.011	0.356 ± 0.003	0.635 ± 0.004	0.960 ± 0.024	0.890 ± 0.038	1.188 ± 0.011
1.661 ± 0.017	1.589 ± 0.043	1.432 ± 0.025	1.819 ± 0.035	1.465 ± 0.030	3.646 ± 0.004	3.130 ± 0.004	3.448 ± 0.003	3.829 ± 0.004
0.048 ± 0.002	0.037 ± 0.001	0.022 ± 0.001	0.081 ± 0.002	0.030 ± 0.002	0.057 ± 0.000	0.046 ± 0.002	0.080 ± 0.002	0.099 ± 0.002
1.291 ± 0.013	0.802 ± 0.001	0.292 ± 0.005	0.939 ± 0.021	0.555 ± 0.028	0.764 ± 0.001	1.087 ± 0.004	0.785 ± 0.002	1.063 ± 0.001
3.639 ± 0.027	2.605 ± 0.040	0.711 ± 0.004	2.006 ± 0.058	1.823 ± 0.014	2.791 ± 0.014	3.872 ± 0.001	3.377 ± 0.005	4.916 ± 0.010
0.067 ± 0.002	0.230 ± 0.003	0.250 ± 0.023	0.285 ± 0.001	0.339 ± 0.003	0.304 ± 0.000	0.364 ± 0.003	0.412 ± 0.001	0.425 ± 0.001
3.024 ± 0.087	1.724 ± 0.009	1.812 ± 0.067	1.499 ± 0.032	1.368 ± 0.053	1.972 ± 0.002	3.680 ± 0.004	2.964 ± 0.003	4.264 ± 0.004
0.197 ± 0.003	0.194 ± 0.003	0.493 ± 0.001	0.155 ± 0.001	0.103 ± 0.002	0.306 ± 0.001	0.385 ± 0.000	0.391 ± 0.001	0.450 ± 0.001
0.028 ± 0.000	0.025 ± 0.001	0.024 ± 0.001	0.011 ± 0.000	0.020 ± 0.000	0.021 ± 0.000	0.027 ± 0.000	0.028 ± 0.000	0.045 ± 0.000
0.690 ± 0.023	0.307 ± 0.004	0.100 ± 0.022	0.325 ± 0.014	0.256 ± 0.002	0.673 ± 0.000	0.756 ± 0.002	0.666 ± 0.002	0.944 ± 0.012
0.153 ± 0.003	0.232 ± 0.007	0.123 ± 0.005	0.982 ± 0.032	0.594 ± 0.005	0.218 ± 0.013	0.358 ± 0.017	0.371 ± 0.038	0.538 ± 0.081
0.325 ± 0.002	0.063 ± 0.001	0.106 ± 0.003	0.032 ± 0.001	0.147 ± 0.001	0.967 ± 0.003	0.599 ± 0.001	1.103 ± 0.001	1.517 ± 0.002
0.059 ± 0.000	0.026 ± 0.001	0.076 ± 0.002	0.061 ± 0.002	0.102 ± 0.001	0.579 ± 0.000	0.489 ± 0.000	0.552 ± 0.000	0.973 ± 0.000
22.181 ± 0.350	17.904 ± 0.206	12.684 ± 0.274	18.323 ± 0.439	12.667 ± 0.324	26.503 ± 0.108	28.466 ± 0.128	29.197 ± 0.147	38.547 ± 0.168

(unknown), 102 (G-Ro isomer), 112 (G-Ro isomer), and 126 (acetyl G-Rg2) were detected in forest GRR. These results indicated that ginsenosides in GRR exhibited chemical diversity with the ages growing and due to different ecological factors.

3.3. Validation of quantitative analytical method

During quantitative analysis, 19 marker ginsenosides were unambiguously identified by comparison with the reference standards. The HPLC-ESI-MS^n quantitative analysis method was validated by defining the linearity, limits of quantification (LOQ) and detection (LOD), repeatability, precision, stability, and recovery. All calibration curves were plotted on the basis of linear regression analysis of the integrated peak areas (y) versus concentrations (x, μg) of the 19 marker ginsenosides in the standard solution at six different concentrations. The regression equations, coefficient of determination, and linear ranges for the analysis of the 19 marker ginsenosides are shown in Table 4. The stock solution containing 19 reference compounds was diluted to a series of appropriate concentrations with MeOH, and an aliquot of the diluted solutions was injected into HPLC-ESI-MS for analysis. The LOD and LOQ under the present chromatographic conditions were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively.

Intra- and inter-day variations were chosen to determine the precision of the developed assay. The known concentrations of 19 standard ginsenoside solutions were tested. For the intraday variability test, the mixed standard solutions were analyzed within 1 d, while for interday variability test, the solutions were examined in duplicate over a consecutive 3 d period. Variations were expressed by relative standard deviation (RSD). The validation studies showed overall intra- and inter-day variations (RSD) of less than 4.44% and 4.58%, respectively.

For the stability test, the contents of 19 ginsenosides in sample solutions were analyzed at 0 h, 2 h, 4 h, 8 h, 12 h, 24 h, and 48 h. RSD values of the contents of 19 ginsenosides were less than 3.75%. To confirm the repeatability of the test, five replicates of the same samples were extracted and analyzed as mentioned above. The RSD values of the contents of 19 ginsenosides were less than 3.55%.

The recovery was used to evaluate the accuracy of the method. A known amount of ginsenoside standards was added into a certain amount (1.0 g) of sample. The mixture was extracted and analyzed using the method mentioned above. Three replicates were performed for the test. The developed method had good accuracy with the overall recovery of 94.87−102.45% and the RSD ranging from 0.82−3.99% (Table 5). These results indicated that the HPLC-ESI-MS method is precise, accurate, and sensitive for the quantitative determination of 19 ginsenosides in GRR.

3.4. Constituents analysis of samples

Chemical profiling and quantification of the 19 ginsenosides from 16 samples of GRR using the HPLC-ESI-MS method were carried out (Fig. 4). Each sample was analyzed three times to determine the mean contents and the results are shown in Table 6. These results indicated that the contents of 19 ginsenosides varied greatly among the samples collected from different locations.

3.5. Principal component analysis

The HPLC-ESI-MS contents of 19 ginsenosides were used for the assessment of substantial compositional difference between the 4−5 yr old cultivated Panax ginseng (collected from Jilin and Heilongjiang provinces as well as Korea) and the 15 yr, 20 yr, 25 yr, and 30 yr old forest Panax ginseng (collected from Jilin province). The contents of 19 ginsenosides were subjected to principal component analysis (PCA) to differentiate the production area of the ginseng roots. The results were shown in Fig. 5. The first principal component (PC1) contains the most variance in the data and the second principal component (PC2) represents the maximum amount of variance not explained by PC1. The two ranking PCs, PC1 and PC2, described 46.6% and 14.0% of the total variability in the original observations, respectively and they can account for 60.6% of the total variance. The scores plots for PC1 versus PC2 (Fig. 5A) showed the ability to distinguish these samples. The scores plot (Fig. 5A) showed that 16 samples of GRR were clarified into five groups (Groups I−V) and Groups II, IV and V were separated distinctly according to PC1. Group II (containing samples 1, 2, 6, 9, and 11) was
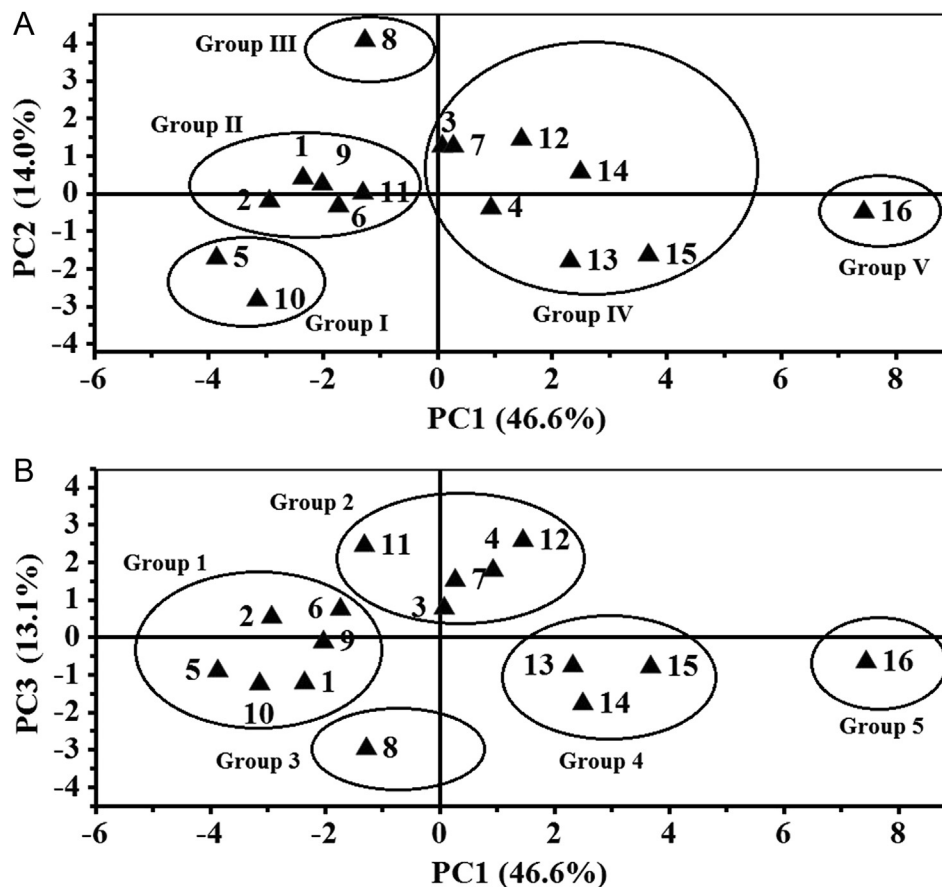
**Fig. 5.** The principal component analysis (PCA) showed that 16 samples can be divided into four groups.

clustered by negative values of PC1, while Group V (containing sample 16) and Group IV (containing 3, 4, 7, as well as 12–15) were separated by positive values of PC1. Group I (including samples 5 and 10), Group II and Group III (including sample 16) were distinctly separated according to PC2. Group I was clustered by negative values of PC2, while Group III was clustered by positive values of PC2. Except sample 12, the total contents of ginsenosides in samples 3, 4, 7, and 13–15 were much higher than other cultivated samples, and all were more than 21.410 mg/g. However, the total content of ginsenosides in sample 16 was much higher than those in Group IV (38.547 mg/g), and was solely divided into one group. In contrast, the total contents of 19 ginsenosides in samples 5 and 10 were much lower than others, and were no more than 13.216 mg/g. The third principal component (PC3) contains the remaining variance not explained by PC1 and PC2 by analogy and PC3 can describe 13.1% of the total variability in the original observations and consequently all the PCs accounts for 73.7% of the total variance. The scores plots for PC1 versus PC3 (Fig. 5B) also showed the ability to differentiate these 16 samples. The cultivated *Panax ginseng* (Group 2) and the forest *Panax ginseng* (Group 3) were distinctly separated according to PC3, which were not separated in the scores plot for PC1 versus PC2. The contents of ginsenosides in forest GRR which formed Group 3 were different from the cultivated GRR probably because of the different growth years, the localities, and the cultivation techniques. From the scores plots of PC1 versus PC2 and PC1 versus PC3, we found that samples 11 and 12 collected from Korea cannot be completely separated from

the cultivated *Panax ginseng*. The loading plots for PC1 versus PC2 as well as PC1 versus PC3 were shown in Fig. 6A and 6B. A more detailed interpretation of the loadings can be done from plots showing the loadings separately (shown in Fig. 7). In Fig. 7A–C, we can see the influence of each variable (S1~S19) on the 1st component, 2nd component, and 3rd component. Any ginsenoside can influence the discrimination of the samples from different localities.

In summary, a new rapid and sensitive UPLC-DAD-QTOF-MS/MS method was established to qualify the ginsenosides in GRR. With the optimized conditions, a total of 131 ginsenosides were detected in 10 min. Thirty-six ginsenosides were confirmed by comparing the mass spectra and retention times with those of the reference ginsenosides, whereas the others were tentatively assigned by matching the empirical molecular formulas with those of the published known ginsenosides and the fragmentation features. In order to quantify the 19 ginsenosides in GRR, an LC-MS method was developed and was applied to determine the contents of ginsenosides in 16 GRR samples. All 19 ginsenosides could be quantitated at the nanogram on-column level. The established qualitative and quantitative methods can be applied to assess the quality of GRR. In addition, the analysis method developed could also be applied to distinguish the cultivated GRR from the forest GRR. Further, the results provide some important guidelines for the design of LC-MS guided isolation of ginsenosides from GRR and the subsequent elucidation of the exact or complete chemical structure by NMR spectroscopic methods because many chemical structures of
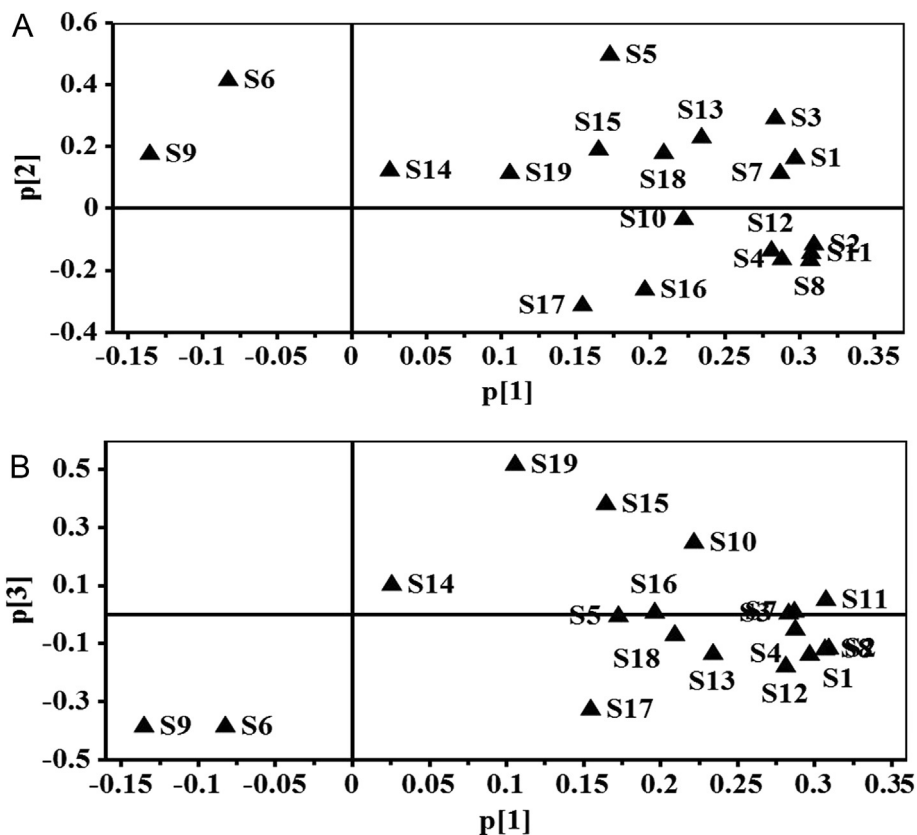
Fig. 6. The loading plots of PC1 versus PC2 (A) as well as PC1 versus PC3 (B) for 19 ginsenosides in their LC-MS profiles of 16 roots and rhizomes of *Panax ginseng* samples. S1, G-Re4; S2, 20-glc-G-Rf; S3, NG-R1; S4, G-Rg1; S5, G-Re; S6, G-Ra2; S7, G-Rf; S8, G-Rb1; S9, NG-R2; S10, G-Ra1; S11, G-Rc; S12, G-Rg2; S13, G-Rb2; S14, G-Rb3; S15, G-Rd; S16, G-Rs2; S17, Ma-G-Rb2; S18, G-RoMe; S19, G-Ro.
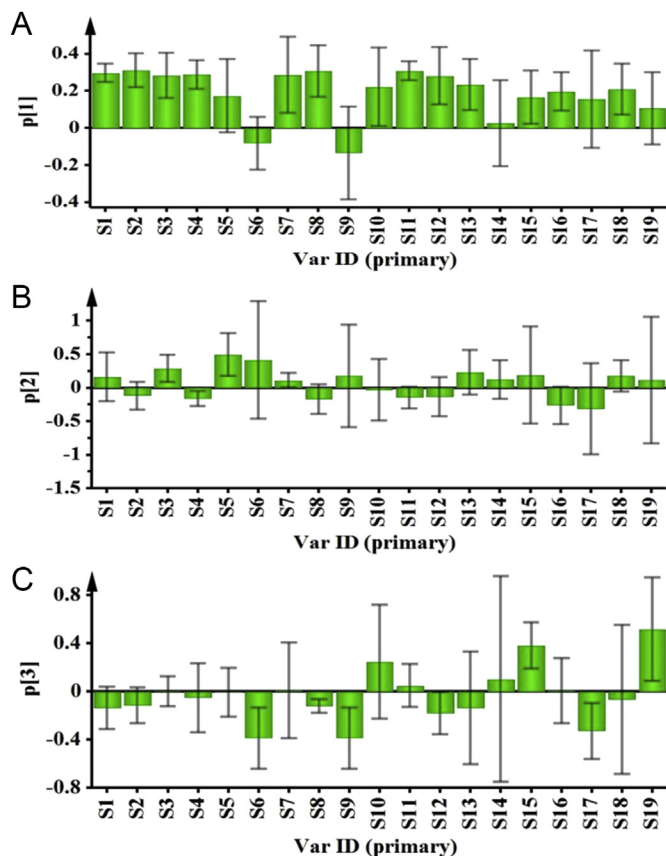
Fig. 7. The influences of each variable on (A) the first component, (B) the second component and (C) the third component.

ginsenosides, as shown in Table 3, are still ambiguous. The detailed studies are currently in progress.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http:// dx.doi.org/10.1016/j.jgr.2015.12.001.

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