



# Identification of ginsenoside markers from dry purified extract of *Panax ginseng* by a dereplication approach and UPLC-QTOF/MS analysis



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## ARTICLE INFO

### Article history:

Received 13 January 2015

Received in revised form 16 February 2015

Accepted 17 February 2015

Available online 25 February 2015

### Keywords:

*Panax ginseng*

Ginsenoside

UPLC-QTOF

Dereplication

## ABSTRACT

A dry purified extract of *Panax ginseng* (PEG) was prepared using a manufacturing process that includes column chromatography, acid hydrolysis, and an enzyme reaction. During the manufacturing process, the more polar ginsenosides were altered into less polar forms via cleavage of their sugar chains and structural modifications of the aglycones, such as hydroxylation and dehydroxylation. The structural changes of ginsenosides during the intermediate steps from dried ginseng extract (DGE) to PEG were monitored by ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectroscopy (UPLC-QTOF/MS). 22 ginsenosides isolated from PEG were used as the reference standards for determining of unknown ginsenosides and further suggesting of the metabolic markers. The elution order of 22 ginsenosides based on the type of aglycones, and the location and number of sugar chains can be used for the structural elucidation of unknown ginsenosides. This information could be used in a dereplication process for quick and efficient identification of ginsenoside derivatives in ginseng preparations. A dereplication approach helped the identification of the metabolic markers in the UPLC-QTOF/MS chromatograms during the conversion process with multivariate analyses, including principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) plots. These metabolic markers were identified by comparing with the dereplication information of the reference standards of 22 ginsenosides, or they were assigned using the pattern of the MS/MS fragmented ions. Consequently, the developed metabolic profiling approach using UPLC-QTOF/MS and multivariate analysis represents a new method for providing quality control as well as useful criteria for a similarity evaluation of the manufacturing process of ginseng preparations.

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## 1. Introduction

Ginseng (*Panax ginseng* C.A. Meyer) has been used for thousands of years in Korean traditional medicine as an herbal medication for a

variety of disorders. Ginseng is believed to be an important medicinal resource and health supplement for enhancing bodily functions, maintaining human health, and balancing bodily conditions [1,2]. Recently, ginseng has become popular as a dietary health supplement and as an additive in foods and beverages. Ginsenosides, the major pharmacologically active ingredients in ginseng, are triterpenoidal saponins that are primarily attributed to a wide range of pharmacological and therapeutic properties, i.e., maintaining homeostasis of the body, improving brain function, preventing cancer, enhancing the immune system, and adjusting blood pressure. Ginsenosides also exhibit anti-aging, anti-obesity, and anti-diabetic effects [3,4]. Ginsenosides are classified into three groups according to the type of aglycones, i.e., dammarane, oleanane triterpenes. Furthermore, the dammarane type to which most

**Abbreviations:** DGE, dry ginseng extract; MeOH, methanol; MS, mass spectrometer; OPLS-DA, orthogonal partial least squared discriminant analysis; PCA, principle component analysis; PEG, the final dry purified extract of *P. ginseng*; PPD, protopanaxadiol; PPT, protopanaxatriol; QTOF, quadruple time-of-flight; UPLC, ultra performance liquid chromatography.

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ginsenosides belong can be generally classified as protopanaxadiol (PPD; ginsenosides Rb1 (Rb1), Rc, Rd, Rg3, Rh2, etc.) or protopanaxatriol (PPT; Rg1, Re, Rg2, Rh1, etc.) [5,6]. Although the sugar chains in the PPD-type group are attached to C-3 or C-20, the sugar chains in the PPT-type group are linked to a hydroxyl moiety at C-6 or C-20 [7]. In addition, the two types can be further differentiated based on the types of sugar chains and the aliphatic chain at C-17. The less polar ginsenosides (F2, Rg2, Rg3, Rh1, Rh2, compound K, etc.), which are rarely present or even absent in wild ginseng, can be produced by transformation of the more polar ginsenosides (Rb1, Rb2, Rc, Rd, Re, etc.), which account for more than 80% of the total ginsenosides in wild ginseng [7]. Biotransformation methods, i.e., hydrolysis, steaming, heating, and enzymatic and microbial transformations, have been attempted to produce less polar ginsenosides with less than two sugar chains, which show more potent pharmacological activities than the more polar varieties [8,9]. In particular, Rh2 (one glucose at C-3) and Rg3 (two glucoses at C-3) have been well studied as anti-cancer agents in various cell lines [10–14].

Recently, ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectroscopy (UPLC-QTOF/MS) has been applied as a powerful analytical tool for rapid analysis of the complex components or metabolites and chemical transformations in ginseng-related products [15,16]. The development of the UPLC-QTOF/MS system offers much higher resolution for the separation of single components in ginseng extracts and also facilitates quantification with high sensitivity and selectivity for identification of the components in ginseng extract [17,18]. The value of accurate mass acquired from QTOF/MS can be the most important information and have been applied to predict and find the known components in natural products extracts as a dereplication strategy.

The final dry purified extract of *P. ginseng* (PEG) product was produced from dry ginseng extract (DGE) via patented technologies [19,20]. It was designed for elevating the content of many less polar ginsenosides, especially Rh2 and Rg3, in the three steps of the manufacturing processes, i.e., column chromatography, acid and enzyme hydrolysis, and pressurized heating.

Recently, a dereplication process using LC/MS was demonstrated as a powerful technique for rapid isolation and identification of known compounds from natural product extracts including ginsenosides [21,22]. The chromatograms and spectroscopic data acquired from the LC/MS technique could be used to monitor changes in the chemical profile during the manufacturing processes in natural product preparations [23–26]. With this perspective, we attempted to discovery of the metabolic markers during the manufacturing steps from DGE to PEG. The metabolic markers could be identified via a dereplication process using the spectroscopic features of 22 ginsenosides isolated from PEG as the reference standards. The suggested metabolic markers and a dereplication approach might be used in quality control as well as for observation of the metabolic changes in ginsenosides during the manufacturing process of ginseng preparation.

## 2. Material and methods

### 2.1. Chemicals

The preparations of ginseng extracts and reference standards used in this study were gifted from Green Cross Health Science, Inc. (Sungnam, Korea) [19,20]. The manufacturer provided four dry ginseng extracts (DGE), seven of the first intermediate products (I-1), eight of the second intermediate products (I-2), and eleven of the final products (PEG) produced from different batches and prepared with patented technology. In brief, the harvested crude ginseng was ground and repeatedly extracted with aqueous ethanol followed

by evaporation in vacuo to yield DGE. Next, DGE was suspended in distilled water and subjected to HP-20 resin column chromatography to yield I-1. The I-2 was obtained from I-1 by reaction with an enzyme containing ginsenoside- $\beta$ -glucosidase. After mild acid hydrolysis (acetic acid) of the mixture of I-1 and I-2, the reactant was purified with HP-20 resin followed by washing with aqueous ethanol. Aqueous ethanol extract was concentrated and designated as PEG, also named *P. ginseng* dry purified extract.

### 2.2. Preparation of samples and reference standards

The powdered samples were resolved in 80% MeOH to obtain a concentration of 5 mg/ml and filtered through a 0.2- $\mu$ m membrane filter prior to the analysis. The reference standards of Rb1, Rb2, Rc, Rd, Re, and compound K were provided by Green Cross Health Science, Inc., and 22 ginsenosides (Table 2) were isolated using a series of column chromatography techniques according to the procedure in the previous study [26]. The reference standards were dissolved in 80% MeOH and mixed at a concentration of 100  $\mu$ g/ml for injection into the UPLC-QTOF/MS system. The volume injected to the column was 1  $\mu$ l, and each sample was injected three times. The run sequence was randomly generated, and the blank (80% MeOH) was injected once every five runs.

### 2.3. UPLC-QTOF/MS analysis

The UPLC-QTOF/MS analyses were performed on a Waters Acquity UPLC system (Waters Co., Milford, MA, USA), which consists of a binary solvent delivery system and an auto sampler. The UPLC column was a Waters Acquity UPLC BEH C18 (150 mm  $\times$  2.1 mm, 1.7  $\mu$ m). The mobile phases were 0.1% formic acid in H<sub>2</sub>O (A) and acetonitrile (B), with the following gradient: 15% B (0–2 min), 15–50% B (2–10 min), 50–70% B (10–17 min), 70–90% B (17–20 min), 90% B (20–21 min), and 15% B (21–23 min). The flow rate was set at 400  $\mu$ l/min. The temperatures in the auto sampler and in the column oven were set at 10 °C and 45 °C, respectively. The MS experiments were performed on a Waters Xevo G2 QTOF mass spectrometer (Waters MS Technologies, Manchester, UK) connected to the UPLC system through an electrospray ionization (ESI) interface. The ESI conditions were set as follows: negative ion mode, capillary voltage of 2.5 kV, cone voltage of 45 V, source temperature of 100 °C, desolvation temperature of 350 °C, cone gas flow of 50 l/h, and desolvation gas flow of 800 l/h. The ion acquisition rate was 0.2 s with resolution in excess of 20,000 FWHM. The energy for collision-induced dissociation (CID) was set to 4 V for the precursor ion, and the MS/MS fragment information was obtained using a collision energy ramp from 40 eV to 45 eV in MSe mode. The instrument was calibrated using sodium formate solution as the calibration standard suggested by the manufacturer allowing for mass accuracies of <5 ppm. To ensure the mass accuracy and reproducibility of the optimized MS condition, leucine encephalin (*m/z* 554.2615 in negative mode) was used as the reference lock mass at a concentration of 200 pg/ $\mu$ l and a flow rate of 5  $\mu$ l/min and was sprayed into the MS instrument every 10 s.

### 2.4. Data processing and multivariate statistical analysis

For the chemical profiles of the samples (DGE, I-1, I-2 and PEG), accurate and reproducible MS data acquired from UPLC-QTOF/MS were processed using MassLynx™ software (Ver. 4.1, Waters Co., Milford, MA, USA). For identification of the peaks, all possible molecular formulae (elements C, H, O, tolerance of 5 mDa, at least 2 carbons) were extracted with the Element Composition analysis software provided by the manufacturer.

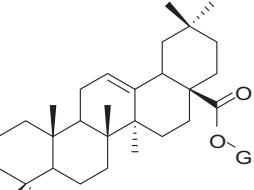
For multivariate statistical analysis, the chromatographic data and MS spectral data for each peak were extracted in MakerLynx™

**Table 1**  
Structures of **1–22** ginsenosides isolated from PEG.

Name	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
25-OH-20S-Rh1 ( <b>1</b> )	OH	O-Glc <sup>a</sup>	β-OH
25-OH-20R-Rh1 ( <b>2</b> )	OH	O-Glc	α-OH
25-OH-20S-Rh2 ( <b>10</b> )	O-Glc	H	β-OH
25-OH-20R-Rh2 ( <b>11</b> )	O-Glc	H	α-OH
Name	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
20S-Rg2 ( <b>4</b> )	OH	O-Glc-Rha <sup>b</sup>	β-OH
20S-Rh1 ( <b>5</b> )	OH	O-Glc	β-OH
20R-Rg2 ( <b>6</b> )	OH	O-Glc-Rha	α-OH
20R-Rh1 ( <b>7</b> )	OH	O-Glc	α-OH
20S-AcetylRg2 ( <b>8</b> )	OH	O-AcetylGlc <sup>c</sup> -Rha	β-OH
20R-AcetylRg2 ( <b>9</b> )	OH	O-AcetylGlc-Rha	α-OH
20S-Rg3 ( <b>14</b> )	O-Glc-Glc	H	β-OH
20R-Rg3 ( <b>15</b> )	O-Glc-Glc	H	α-OH
20S-Rh2 ( <b>19</b> )	O-Glc	H	β-OH
20R-Rh2 ( <b>20</b> )	O-Glc	H	α-OH
20S-AcetylRh2 ( <b>21</b> )	O-AcetylGlc	H	β-OH
20R-AcetylRh2 ( <b>22</b> )	O-AcetylGlc	H	α-OH
25-OH-Rh4 ( <b>3</b> )			
Name	R <sub>1</sub>	R <sub>2</sub>	
Rk3 ( <b>12</b> )	OH	O-Glc	
Rk1 ( <b>17</b> )	O-Glc-Glc	H	

Table 1 (Continued)

Name	R <sub>1</sub>	R <sub>2</sub>
Rh4 ( <b>13</b> )	OH	O-Glc
Rg5 ( <b>18</b> )	O-Glc-Glc	H


  
**Oleanolic acid 28-O- $\beta$ -D-glucopyranose (**16**)**

<sup>a</sup> Glc:  $\beta$ -D-glucose.<sup>b</sup> Glc-Rha: -2'-O- $\alpha$ -L-rhamnose.<sup>c</sup> AcetylGlc:  $\beta$ -D-6'-O-acetyl-glucose.

software (Ver. 4.1, Waters Co., Milford, MA, USA). The method parameters for the acquisition of the data matrix were a retention time range of 1–23 min, a mass range of 400–1200 Da, and an XIC window of 0.02 Da. Ions from different samples were considered to be the same ion if they demonstrated the same *t*<sub>R</sub> (tolerance of 0.2 min) and *m/z* value (tolerance of 0.04 Da) as the collection parameters. The peak was determined using a peak width of 5% of the peak height of 5 s, a noise elimination of 10, and an intensity threshold of 30. The ion intensities for each detected peak were normalized against the sum of the peak intensities within that sample using MarkerLynx™. After the collection by MarkerLynx™, these three-dimensional data composed of peak number (*t*<sub>R</sub>-*m/z* pair), sample name, and ion intensity were analyzed using principle component analysis (PCA) and orthogonal partial least squared discriminant analysis (OPLS-DA) with the SIMCA-P software (Ver. 13, Umetrics, Umeå, Sweden). To increase the importance of low-abundance ions without significantly amplifying the noise, the data matrix was transformed via Pareto scaling. The quality of the OPLS-DA models was evaluated using *R*<sup>2</sup> (the goodness-of-fit parameter) and *Q*<sup>2</sup> (the predictive ability parameter). The metabolic markers significantly contributed to the discrimination between each step, as determined by the S plot based on the OPLS-DA plots. Among the markers provided by the multivariate analyses, metabolite peaks were determined by comparison with the reference standards or the isolated compounds and were tentatively assigned by MS/MS analysis compared with the literature values or by searching the accurate masses using the on-line chemical databases (SciFinder®; <http://www.scifinder.org>, Reaxys®; <http://www.reaxys.com>).

### 3. Results and discussion

#### 3.1. Optimization of the UPLC-QTOF/MS chromatographic conditions

The UPLC-QTOF/MS conditions were optimized to obtain optimal peak capacity, stronger retention, and better resolution of major components in all samples. Within the gradient time (23 min) at a flow rate of 400  $\mu$ l/min, the gradient solvent condition consisting of 0.1% formic acid and acetonitrile was used to obtain the improved peak capacity and resolution of the more polar ginsenosides compared with the lower polarity ginsenosides at the optimal backpressure (less than 9000 psi). Compared with the positive ion mode, higher sensitivity and more accurate mass spectra of minor ginsenosides with low background noise could be more easily acquired in the negative ion mode [18]. In further studies, UPLC-QTOF/MS data for identification of structures, molecular formulae of components in samples, and multivariate analyses were acquired and processed in negative ion mode. To obtain better

resolution of the MS spectra, the ESI conditions were optimized as described in the Material and Methods section with 22 ginsenosides isolated from the final product, PEG, as the reference standards. The mass accuracy was set up with resolution power in excess of 20,000 FWHM within 5 ppm using external calibration setting. We used the MSe experiment mode for simultaneous acquisition of both non-fragmented parent ions and their fragmented daughter ions in a single run. The MS/MS fragmentation data for structural elucidation of ginsenosides were acquired in MSe mode with different collision energies (a low collision energy of 4 eV and a high collision energy ramp ranging from 40 to 45 eV).

#### 3.2. Identification and dereplication of the characteristic peaks from PEG

We isolated 22 ginsenosides from the final PEG product using a series of column chromatography steps (Table 1) [27]. The structures were elucidated with 1D and 2D NMR spectra data and compared with spectroscopic data in the literature (Table 2). These components had four structural features. First, all 22 ginsenosides obtained from PEG showed no sugar chain at C-20 and 1–2 sugar chains at C-3 (PPD-type) or C-6 (PPT-type). This structure might result from selective cleavage of sugar chains at C-20 by acid hydrolysis during the manufacturing process. It is known that the cleavage of the glucosyl bond at C-20 is hydrolyzed more easily than the bonds at C-3 and C-6 in mild acidic conditions [28]. Second, eight pairs of 20R/S isomers were isolated, i.e., 25-OH-20R/S-Rh1 (25-hydroxyl-20R/S-ginsenoside Rh1), 20R/S-Rg2, 20R/S-Rh1, 20R/S-AcetylRg2 (6'-O-acetyl-20R/S-ginsenoside Rg2), 25-OH-20R/S-Rg2, 20R/S-Rg3, 20R/S-Rh2, and 20R/S-AcetylRh2. This series of 20R-ginsenosides, which are not frequently found in wild ginseng, were most likely derived by attack of the hydroxyl group at C-20 after selective deglycosylation [29]. Third,  $\Delta$ 20 and  $\Delta$ 20(22) ginsenosides, i.e., Rg5, 25-OH-Rh4, Rh4, Rk1, and Rk3, and ginsenosides that are hydroxylated at C-25, i.e., 25-OH-20R/S-Rh1, 25-OH-20R/S-Rh2, and 25-OH-Rh4, were generated. These components may be derived from a combination of dehydration and hydration in the process of heating and by acidic or enzyme hydrolysis [30]. Finally, 20R/S-AcetylRg2 and 20R/S-AcetylRh2 with the acetyl group at C-6 of the glucose moiety were isolated as the reference standards. These ginsenosides were assumed to have been generated during the manufacturing process by decarboxylation of the malonyl moiety in the more polar ginsenosides or by acetylation via mild acidic hydrolysis or enzyme reactions [30,31]. 22 ginsenosides isolated as the reference compounds were used for optimizing of solvent gradient and MS acquisition conditions and further identifying of unknown ginsenoside by the comparison of their *m/z* values and fragmentation

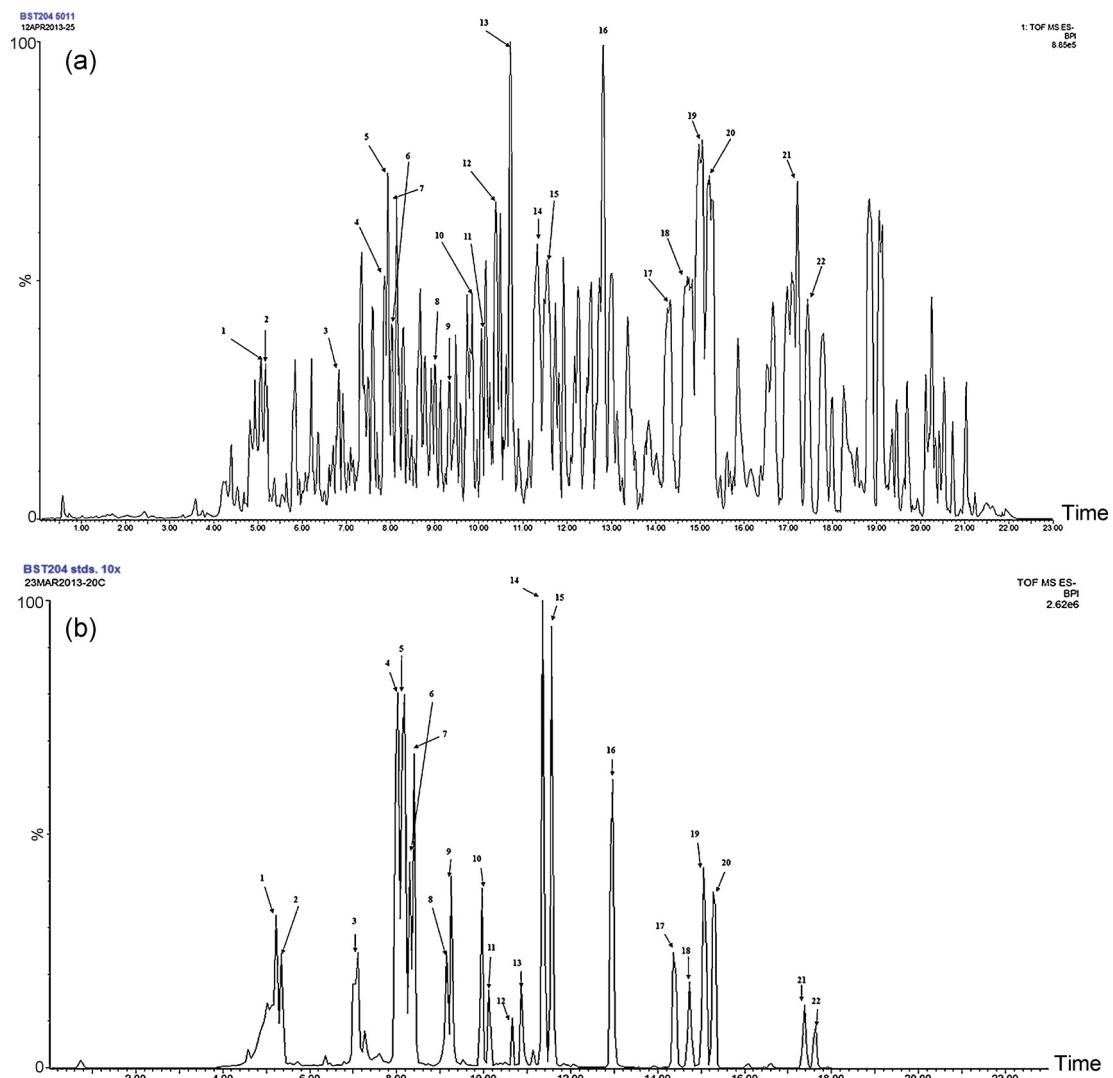
**Table 2**  
Isolated ginsenosides from PEG.

No.	<i>t</i> <sub>R</sub>	Measured mass	Theoretical mass	ΔmDa	<i>m/z</i> ion	Molecular formula	MS/MS fragmentation (ΔmDa)	ID	References
1	5.23	655.4417	655.4421	-0.4	C <sub>36</sub> H <sub>63</sub> O <sub>10</sub> [M-H] <sup>-</sup>	C <sub>36</sub> H <sub>64</sub> O <sub>10</sub>	493.3885 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.8) 493.3893 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.0)	25-OH-20S-Rh1 25-OH-20R-Rh1 25-OH-Rh4	[40]
2	5.34	655.4427	655.4421	0.6	C <sub>36</sub> H <sub>63</sub> O <sub>10</sub> [M-H] <sup>-</sup>	C <sub>36</sub> H <sub>64</sub> O <sub>10</sub>	493.3893 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.0)	25-OH-20R-Rh1	[40]
3	7.11	637.4311	637.4316	-0.5	C <sub>36</sub> H <sub>61</sub> O <sub>9</sub> [M-H] <sup>-</sup>	C <sub>36</sub> H <sub>62</sub> O <sub>9</sub>	683.4374 [M+COOH] <sup>-</sup> , 475.3788 [M-6(Glc-H <sub>2</sub> O)-H] <sup>-</sup> (0.1)	25-OH-Rh4	[41]
4	8.03	783.4899	783.4895	0.4	C <sub>42</sub> H <sub>71</sub> O <sub>13</sub> [M-H] <sup>-</sup>	C <sub>42</sub> H <sub>72</sub> O <sub>13</sub>	637.4311 [M-(Rha-H <sub>2</sub> O)-H] <sup>-</sup> (-0.5), 475.3784 [M-(Rha-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.3)	20S-Rg2	[42]
5	8.18	683.4371	683.4370	0.1	C <sub>37</sub> H <sub>63</sub> O <sub>11</sub> [M+COOH] <sup>-</sup>	C <sub>36</sub> H <sub>62</sub> O <sub>9</sub>	475.3783 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.4)	20S-Rh1	[42]
6	8.29	783.4893	783.4895	-0.2	C <sub>42</sub> H <sub>71</sub> O <sub>13</sub> [M-H] <sup>-</sup>	C <sub>42</sub> H <sub>72</sub> O <sub>13</sub>	637.4313 [M-(Rha-H <sub>2</sub> O)-H] <sup>-</sup> (-0.3), 475.3778 [M-(Rha-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.1)	20R-Rg2	[41]
7	8.40	683.4367	683.4370	-0.3	C <sub>37</sub> H <sub>63</sub> O <sub>11</sub> [M+COOH] <sup>-</sup>	C <sub>36</sub> H <sub>62</sub> O <sub>9</sub>	475.3782 M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.5)	20R-Rh1	[40]
8	9.15	825.5003	825.5000	0.3	C <sub>44</sub> H <sub>73</sub> O <sub>14</sub> [M-H] <sup>-</sup>	C <sub>44</sub> H <sub>74</sub> O <sub>14</sub>	783.4891 [M-COCH <sub>3</sub> -H] <sup>-</sup> 637.4306 [M-COCH <sub>3</sub> -(Rha-H <sub>2</sub> O)-H] <sup>-</sup> (-1.0), 475.377891 [M-(Rha-H <sub>2</sub> O)-(AcetylGlu-H <sub>2</sub> O)] <sup>-</sup> (-0.9)	20S-AcetylRg2	[43]
9	9.26	825.5013	825.5000	1.3	C <sub>44</sub> H <sub>73</sub> O <sub>14</sub> [M-H] <sup>-</sup>	C <sub>44</sub> H <sub>74</sub> O <sub>14</sub>	783.4905 [M-COCH <sub>3</sub> -H] <sup>-</sup> (1.0), 637.4320 [M-COCH <sub>3</sub> -(Rha-H <sub>2</sub> O)-H] <sup>-</sup> (0.4), 475.3782 [M-(Rha-H <sub>2</sub> O)-(AcetylGlu-H <sub>2</sub> O)] <sup>-</sup> (-0.5)	20R-AcetylRg2	[43]
10	9.96	685.4529	685.4527	0.2	C <sub>37</sub> H <sub>65</sub> O <sub>11</sub> [M+COOH] <sup>-</sup>	C <sub>36</sub> H <sub>64</sub> O <sub>9</sub>	639.4474 [M-H] <sup>-</sup> (0.6), 477.3940 [M-(Glu-H <sub>2</sub> O)] <sup>-</sup> (-0.2)	25-OH-20S-Rh2	[26]
11	10.12	685.4541	685.4527	1.4	C <sub>37</sub> H <sub>65</sub> O <sub>11</sub> [M+COOH] <sup>-</sup>	C <sub>36</sub> H <sub>64</sub> O <sub>9</sub>	639.4487 [M-H] <sup>-</sup> (0.5), 477.3948 [M-(Glu-H <sub>2</sub> O)] <sup>-</sup> (0.4)	25-OH-20R-Rh2	[26]
12	10.66	621.4370	621.4366	0.4	C <sub>36</sub> H <sub>61</sub> O <sub>8</sub> [M-H] <sup>-</sup>	C <sub>36</sub> H <sub>60</sub> O <sub>8</sub>		Rk3	[44]
13	10.87	621.4371	621.4366	0.5	C <sub>36</sub> H <sub>61</sub> O <sub>8</sub> [M-H] <sup>-</sup>	C <sub>36</sub> H <sub>60</sub> O <sub>8</sub>		Rh4	[40]
14	11.36	783.4896	783.4895	0.1	C <sub>42</sub> H <sub>71</sub> O <sub>13</sub> [M-H] <sup>-</sup>	C <sub>42</sub> H <sub>72</sub> O <sub>13</sub>	621.4368 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.2), 459.3832 [M-2(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.4)	20S-Rg3	[45]
15	11.57	783.4904	783.4895	0.9	C <sub>42</sub> H <sub>71</sub> O <sub>13</sub> [M-H] <sup>-</sup>	C <sub>42</sub> H <sub>72</sub> O <sub>13</sub>	621.4371 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.5), 459.3837 [M-2(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.1)	20R-Rg3	[31]
16	12.97	663.4110	663.4108	0.2	C <sub>37</sub> H <sub>59</sub> O <sub>10</sub> [M+COOH] <sup>-</sup>	C <sub>36</sub> H <sub>58</sub> O <sub>8</sub>	617.4054 [M-H] <sup>-</sup> (0.5), 455.3517 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.8)	oleanolic acid-28-Glu	[46]
17	14.36	765.4818	765.4789	2.9	C <sub>42</sub> H <sub>69</sub> O <sub>12</sub> [M-H] <sup>-</sup>	C <sub>42</sub> H <sub>70</sub> O <sub>12</sub>	603.4271 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (1.0)	Rk1	[45]
18	14.74	765.4807	765.4789	1.8	C <sub>42</sub> H <sub>69</sub> O <sub>12</sub> [M-H] <sup>-</sup>	C <sub>42</sub> H <sub>70</sub> O <sub>12</sub>	603.4258 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.3)	Rg5	[45]
19	15.06	667.4424	667.4421	0.3	C <sub>37</sub> H <sub>63</sub> O <sub>10</sub> [M+COOH] <sup>-</sup>	C <sub>36</sub> H <sub>62</sub> O <sub>8</sub>	621.4365 [M-H] <sup>-</sup> (-0.1), 459.3831 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.5)	20S-Rh2	[47]
20	15.28	667.4426	667.4421	0.5	C <sub>37</sub> H <sub>63</sub> O <sub>10</sub> [M+COOH] <sup>-</sup>	C <sub>36</sub> H <sub>62</sub> O <sub>8</sub>	621.4370 [M-H] <sup>-</sup> (0.4), 459.3840 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.2)	20R-Rh2	[48]
21	17.38	709.4526	709.4527	-0.1	C <sub>39</sub> H <sub>65</sub> O <sub>11</sub> [M+COOH] <sup>-</sup>	C <sub>38</sub> H <sub>64</sub> O <sub>9</sub>	663.4462 [M-H] <sup>-</sup> (-1.0), 621.4351 [M-COCH <sub>3</sub> -H] <sup>-</sup> (-1.6), 459.3828 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-1.0)	20S-AcetylRh2	[49]
22	17.65	709.4531	709.4527	0.4	C <sub>39</sub> H <sub>65</sub> O <sub>11</sub> [M+COOH] <sup>-</sup>	C <sub>38</sub> H <sub>64</sub> O <sub>9</sub>	663.4460 [M-H] <sup>-</sup> (-1.2), 621.4365 [M-COCH <sub>3</sub> -H] <sup>-</sup> (-0.1), 459.3832 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.4)	20R-AcetylRh2	No reference

Glu, β-D-glucose; Rha, α-L-rhamnose; GlcA, β-D-glucuronic acid.

patterns. They were unambiguously matched with the peaks in the chromatogram of PEG via comparison with the retention time and accurate mass (Fig. 1). Interestingly, 20S-ginsenosides were always eluted before 20R-ginsenosides among pairs of identical planner structures (25-OH-20S-Rh1 > 25-OH-20R-Rh1,

20S-Rg2 > 20R-Rg2, 20S-Rh1 > 20R-Rh1, 20S-AcetylRg2 > 20R-AcetylRg2, 25-OH-20S-Rg2 > 25-OH-20R-Rg2, 20S-Rg3 > 20R-Rg3, 20S-Rh2 > 20R-Rh2 and 20S-AcetylRh2 > 20R-AcetylRh2). Furthermore, Δ20 ginsenosides were eluted before their Δ20(22) derivatives (Rk3 > Rh4, Rk1 > Rg5). The structural and spectrometry



**Fig. 1.** UPLC–QTOF/MS chromatograms of PEG (a) and a mixture of 22 ginsenosides (b). The column was Waters Acuity UPLC BEH C18 (150 mm × 2.1 mm, 1.7 µm). The mobile phases were 0.1% formic acid in 5% acetonitrile (A) and 0.1% formic acid in 100% acetonitrile (B) with the following gradient: 15% B (0–2 min), 15–50% B (2–10 min), 50–70% B (10–17 min), 60–90% B (17–20 min), 90% B (20–21 min), and 15% B (21.1–23 min) at 400 µl/min. The detailed conditions for the UPLC–QTOF/MS analysis are described in the Experimental section. Compounds 1–22 are listed in Table 1.

data from the isolated ginsenosides were expected to be efficient for identifying or distinguishing among previously reported components. This information will be useful for saving time during the isolation, purification, and spectrometric identification of known ginsenosides in a dereplication process. Additionally, this approach could support the determination of two previously unreported ginsenoside derivatives (Table 3; Fig. 2). The fragmentation patterns of two peaks at 12.24 min and 12.50 min suggested that their structures contain a glucose, an acetylated glucose, and a 12, 25-dihydroxydammara-20-ene or 12,25-dihydroxydammara-20(22)-ene backbone. Because  $\Delta$ 20 ginsenosides are more polar than their  $\Delta$ 20(22) counterparts and the acetyl moiety always occurred on a hydroxyl moiety attached to C-6' and not C-6" in the glucose groups, they were tentatively identified as ( $3\beta,12\beta$ )-12,25-dihydroxydammara-20-en-3-O- $\beta$ -6'-O-acetyl-D-glucosyl-(1→2)-O- $\beta$ -D-glucose (**1**) and ( $3\beta,12\beta,20E$ )-12,25-dihydroxydammara-20(22)-en-3-O- $\beta$ -6'-O-acetyl-D-glucosyl-(1→2)-O- $\beta$ -D-glucose (**2**), respectively. In addition, 20R-AcetylRh2 (( $3\beta,12\beta,20R$ )-12, 20-dihydroxydammara-24-en-3-O- $\beta$ -6'-O-acetyl-D-glucose) (**3**) isolated from PEG was identified by this approach and confirmed by the MS/MS fragmentation (Fig. 2c and d).

### 3.3. Characteristic changes in the chemical profile during the manufacturing process

Under the established UPLC–QTOF/MS conditions, the changes in the chemical profile throughout the manufacturing process were remarkable. During the process, the base peak intensity (BPI) chromatogram profiles were notably altered. The peak intensities of the hydrophilic (more polar) components gradually diminished or disappeared, and those of the non-hydrophilic (less polar) ones significantly increased. These changes were expected due to the metabolism of the polar ginsenosides to less polar ginsenosides via biotransformation, i.e., dehydration and deglycosylation under acidic or enzymatic conditions [32]. This trend was also observed in the multivariate analysis (Fig. 3). In the PCA plot based on the MS data including  $m/z$  values, retention times and peak intensities the base peak intensity (BPI) chromatogram, each sample was clearly clustered in the same group and separated from other groups except for DGE and I-1. Although samples of DGE and I-1 were discriminated from others by principal component (PC) 1 and PC2, it was difficult to distinguish the samples from each other using the PCA plot. As the simple column chromatography was performed for removing the primary metabolites, i.e. sugars,

**Table 3**  
Novel ginsenoside derivatives from PEG (1–2).

No.	<i>t</i> <sub>R</sub>	Measured mass	Theoretical mass	ΔmDa	<i>m/z</i> ion	Molecular formula	MS/MS fragmentation (ΔmDa)	ID
1	12.24	825.5021	825.5000	2.1	C <sub>44</sub> H <sub>73</sub> O <sub>14</sub> [M-H] <sup>-</sup>	C <sub>44</sub> H <sub>74</sub> O <sub>14</sub>	783.4913 [M-COCH <sub>3</sub> -H] <sup>-</sup> (1.8), 621.4369 [M-COCH <sub>3</sub> -(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.3), 459.3841 [M-(AcetylGlu <sup>b</sup> -H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.3)	(3β,12β)-12,25-dihydroxydammara-20-en-3-O-β-6'-O-acetyl-d-glucosyl-(1→2)-O-β-D-glucose
2	12.50	825.5011	825.5000	1.1	C <sub>44</sub> H <sub>73</sub> O <sub>14</sub> [M-H] <sup>-</sup>	C <sub>44</sub> H <sub>74</sub> O <sub>14</sub>	783.4903 [M-COCH <sub>3</sub> -H] <sup>-</sup> (0.8), 621.4363 [M-COCH <sub>3</sub> -(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.3), 459.3827 [M-(AcetylGlu-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-1.1)	(3β,12β,20E)-12,25-dihydroxydammara-20(22)-en-3-O-β-6'-O-acetyl-d-glucosyl-(1→2)-O-β-D-glucose
3	17.44	709.4534	709.4527	0.7	C <sub>39</sub> H <sub>65</sub> O <sub>11</sub> [M+COOH] <sup>-</sup>	C <sub>38</sub> H <sub>64</sub> O <sub>9</sub>	663.4470 [M-H] <sup>-</sup> (0.2), 621.4377 [M-COCH <sub>3</sub> -H] <sup>-</sup> (1.0), 459.3779 [M-(AcetylGlu-H <sub>2</sub> O)-H] <sup>-</sup> (-3.9)	20R-AcetylRh2

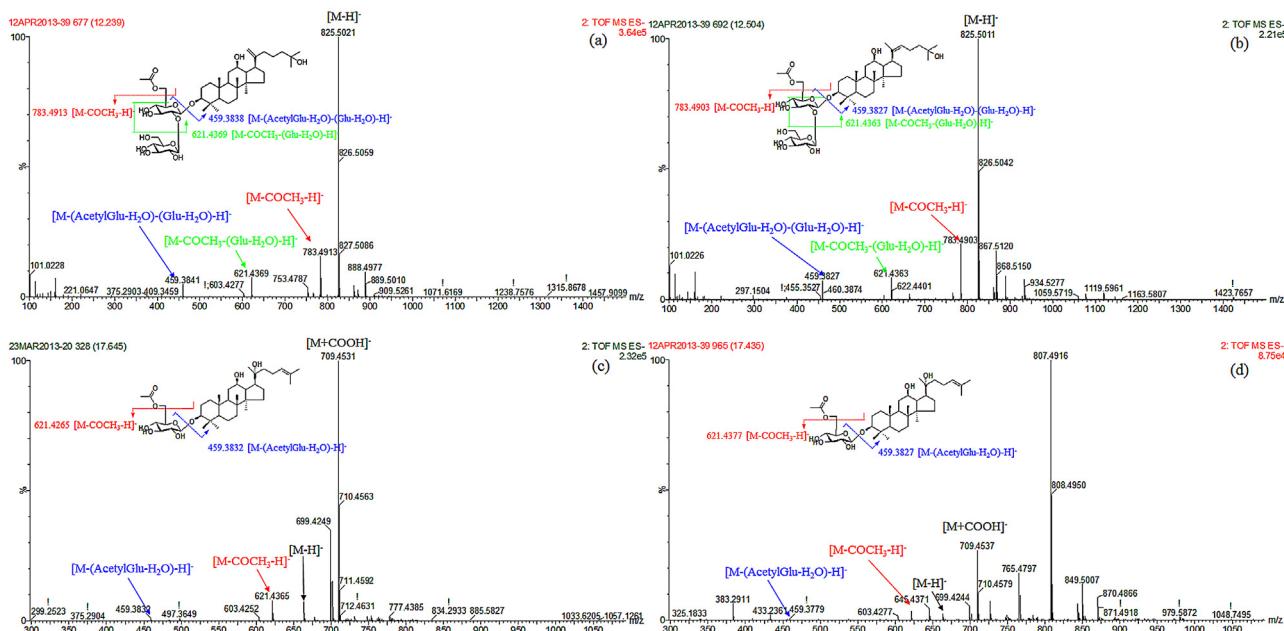
<sup>a</sup> Glu: β-D-glucose.

<sup>b</sup> AcetylGlu: β-D-6-O-acetyl-glucose; the peaks were collected from a UPLC-QTOF/MS chromatogram of a sample from 11 batches of PEG.

amino acid and polysaccharides in the intermediate step between DGE and I-1, the chemical profile of the mixture of ginsenosides were not significantly changed. However, the less polar ginsenosides were produced during sequential manufacturing processes from I-1. All samples from the PEG group were distinguishable from other samples via PC1 (*t*(1)) (the greatest variance of data), whereas the I-2 group (the second greatest variance of data, orthogonal with PC1) was clearly separated from others by PC2 (*t*(2)). This approach might be useful for performing similarity evaluations from batch

to batch during the manufacturing process if crude ginsengs were cultivated or collected from other circumstances.

Furthermore, we performed an OPLS-DA analysis to find metabolic markers that contributed to the discrimination of each step (Fig. 4). Each step of DGE vs. I-1 ( $R^2 = 0.734$ ,  $Q^2 = 0.985$ , Fig. 4a), I-1 vs. I-2 ( $R^2 = 0.592$ ,  $Q^2 = 0.956$ , Fig. 4b), and I-2 vs. PEG ( $R^2 = 0.685$ ,  $Q^2 = 0.977$ , Fig. 4c) was clearly discriminated by *t*(1). All of the  $R^2$  (the goodness-of-fit parameter) and  $Q^2$  (the predictive ability parameter) values were greater than 0.5, and these values



**Fig. 2.** MS spectral data and fragmentation patterns of three unreported compounds (1–3): (3β,12β)-12,25-dihydroxydammara-20-en-3-O-β-6'-O-acetyl-d-glucosyl-(1→2)-O-β-D-glucose (1) (a), (3β,12β,20E)-12,25-dihydroxydammara-20(22)-en-3-O-β-6'-O-acetyl-d-glucosyl-(1→2)-O-β-D-glucose (2) (b), and (3β,12,20R)-12,20-dihydroxydammara-24-en-3-O-β-6'-O-acetyl-d-glucose (3) (c); isolated compound, and d; peak detected in PEG).

**Table 4**

Metabolic markers contributing to discrimination between DGE and I-1.

No.	<i>t</i> <sub>R</sub>	Measured mass	Theoretical mass	ΔmDa	<i>m/z</i> ion	Molecular formula	MS/MS fragmentation (ΔmDa)	ID
1	1.68	503.1758	n.d. <sup>a</sup>					n.d.
2	5.48	945.5435	945.5423	1.2	C <sub>48</sub> H <sub>81</sub> O <sub>18</sub> [M-H] <sup>-</sup>	C <sub>48</sub> H <sub>82</sub> O <sub>18</sub>	799.4846 [M-(Rha <sup>b</sup> -H <sub>2</sub> O)-H] <sup>-</sup> (0.2), 783.4886 [M-(Glu <sup>c</sup> -H <sub>2</sub> O)-H] <sup>-</sup> (-0.9), 637.4312 [M-(Rha-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.4), 475.3781 [M-(Rha-H <sub>2</sub> O)-2(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.6)	Re
3	5.50	991.5493	991.5478	1.5	C <sub>49</sub> H <sub>83</sub> O <sub>20</sub> [M+COOH] <sup>-</sup>	C <sub>48</sub> H <sub>82</sub> O <sub>18</sub>	945.5435 [M-H] <sup>-</sup> (1.2), 799.4886 [M-(Rha-H <sub>2</sub> O)-H] <sup>-</sup> (-0.9), 783.4886 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.9), 637.4312 [M-(Rha-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.4), 475.3781 [M-(Rha-H <sub>2</sub> O)-2(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.6)	Re
4	5.98	987.5544	987.5529	1.5	C <sub>50</sub> H <sub>83</sub> O <sub>19</sub> [M-CO <sub>2</sub> -H] <sup>-</sup>	C <sub>51</sub> H <sub>86</sub> O <sub>21</sub>	945.5417 [M-Mal <sup>d</sup> -H] <sup>-</sup> (-0.6), 799.4821 [M-Mal-(Rha-H <sub>2</sub> O)-H] <sup>-</sup> (-1.8), 783.4872 [M-Mal-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-2.3), 637.4308 [M-Mal-(Rha-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.8), 475.3782 [M-Mal-(Rha-H <sub>2</sub> O)-2(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.5)	Mal-Re
5	7.34	799.4847	799.4879	-3.2	C <sub>42</sub> H <sub>71</sub> O <sub>14</sub> [M-H] <sup>-</sup>	C <sub>42</sub> H <sub>72</sub> O <sub>14</sub>	637.4316 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.0), 475.3787 [M-2(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.2)	Rg1 or Rf
6	7.67	1107.5981	1107.5951	3.0	C <sub>54</sub> H <sub>91</sub> O <sub>23</sub> [M-H] <sup>-</sup>	C <sub>54</sub> H <sub>90</sub> O <sub>23</sub>	945.5421 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.2), 783.4893 [M-2(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.0), 621.4377 [M-3(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (1.1), 459.3837 [M-4(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.1)	Rb1
7	7.76	1193.5989	1193.5955	3.4	C <sub>57</sub> H <sub>93</sub> O <sub>26</sub> [M-H] <sup>-</sup>	C <sub>57</sub> H <sub>94</sub> O <sub>26</sub>	1149.6075 [M-CO <sub>2</sub> -H] <sup>-</sup> (1.8), 1107.5963 [M-Mal-H] <sup>-</sup> (1.2), 945.5459 [M-Mal-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (3.6), 783.4908 [M-Mal-2(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (1.5), 621.4364 [M-Mal-3(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.2), 459.3877 [M-Mal-4(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (4.1)	Mal-Rb1
8	7.76	1149.6083	1149.6057	2.6	C <sub>56</sub> H <sub>93</sub> O <sub>24</sub> [M-CO <sub>2</sub> -H] <sup>-</sup>	C <sub>57</sub> H <sub>94</sub> O <sub>26</sub>	1107.5963 [M-Mal-H] <sup>-</sup> (1.2), 945.5459 [M-Mal-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (3.6), 783.4908 [M-Mal-2(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (1.5), 621.4364 [M-Mal-3(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.2), 459.3877 [M-Mal-4(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (4.1)	Mal-Rb1
9	7.88	1077.5870	1077.5845	2.5	C <sub>53</sub> H <sub>89</sub> O <sub>22</sub> [M-H] <sup>-</sup>	C <sub>53</sub> H <sub>90</sub> O <sub>22</sub>	945.5453 [M-(Ara(p) <sup>e</sup> -H <sub>2</sub> O)-H] <sup>-</sup> (3.2), 783.4897 [M-(Ara(p)-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.2), 621.4375 [M-(Ara(p)-H <sub>2</sub> O)-2(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (1.8), 459.3854 [M-(Ara(p)-H <sub>2</sub> O)-(Rha-H <sub>2</sub> O)-2(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (1.6)	Rc
10	7.95	1119.5979	1119.5951	2.8	C <sub>55</sub> H <sub>91</sub> O <sub>23</sub> [M-H] <sup>-</sup>	C <sub>55</sub> H <sub>92</sub> O <sub>23</sub>	1077.5864 [M-COCH <sub>3</sub> -H] <sup>-</sup> (1.9), 945.5433 [M-(Pen <sup>f</sup> -H <sub>2</sub> O)-H] <sup>-</sup> (1.2), 783.4897 [M-(Pen-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.2), 621.4368 [M-(Pen-H <sub>2</sub> O)-2(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (1.1)	Rs1 or Rs2
11	7.99	955.4922	955.4903	1.9	C <sub>48</sub> H <sub>75</sub> O <sub>19</sub> [M-H] <sup>-</sup>	C <sub>48</sub> H <sub>76</sub> O <sub>19</sub>	793.4386 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (1.2), 455.3500 [M-2(Glu-H <sub>2</sub> O)-(GlcA <sup>g</sup> -H <sub>2</sub> O)-H] <sup>-</sup> (-2.5)	Ro
12	8.13	1077.5869	1077.5845	2.4	C <sub>53</sub> H <sub>83</sub> O <sub>22</sub> [M-H] <sup>-</sup>	C <sub>53</sub> H <sub>84</sub> O <sub>22</sub>	945.5432 [M-(Xyl <sup>h</sup> -H <sub>2</sub> O)-H] <sup>-</sup> (0.9), 915.5332 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (3.2), 783.4895 [M-(Xyl-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.0), 621.4360 [M-(Xyl-H <sub>2</sub> O)-2(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.6), 459.3843 [M-(Xyl-H <sub>2</sub> O)-3(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.6)	Rb2
13	8.20	1163.588	1163.5849	3.1	C <sub>56</sub> H <sub>91</sub> O <sub>25</sub> [M-H] <sup>-</sup>	C <sub>56</sub> H <sub>92</sub> O <sub>25</sub>	1119.5988 [M-CO <sub>2</sub> -H] <sup>-</sup> (3.7), 1077.5874 [M-Mal-H] <sup>-</sup> (2.9), 945.5439 [M-(Pen-H <sub>2</sub> O)-H] <sup>-</sup> (1.8), 783.4901 [M-(Pen-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.6), 621.4375 [M-(Pen-H <sub>2</sub> O)-2(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (1.8), 459.3829 [M-(Pen-H <sub>2</sub> O)-3(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.9)	Mal-Rb2

Table 4 (Continued)

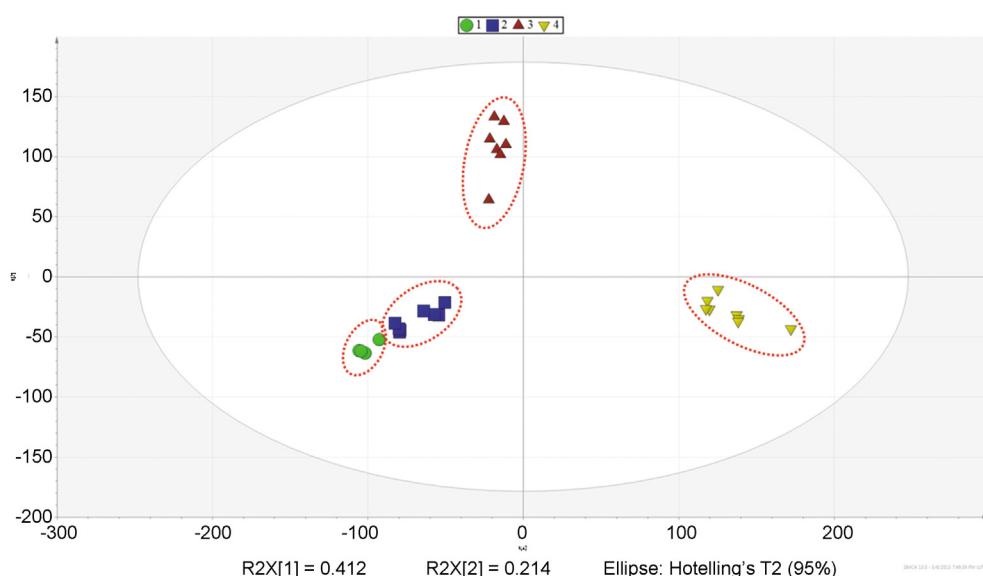
No.	$t_R$	Measured mass	Theoretical mass	$\Delta mDa$	$m/z$ ion	Molecular formula	MS/MS fragmentation ( $\Delta mDa$ )	ID
14	8.21	1119.5978	1119.5988	-1	$C_{56}H_{91}O_{25}$ [M+COOH] $^-$	$C_{56}H_{92}O_{25}$	1077.5874 [M-Mal-H] $^-$ (2.9), 945.5439 [M-(Pen-H <sub>2</sub> O)-H] $^-$ (1.8), 783.4901 [M-(Pen-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] $^-$ (0.6), 621.4375 [M-(Pen-H <sub>2</sub> O)-2(Glu-H <sub>2</sub> O)-H] $^-$ (1.8), 459.3829 [M-(Pen-H <sub>2</sub> O)-3(Glu-H <sub>2</sub> O)-H] $^-$ (-0.9)	Mal-Rb2
15	8.67	991.5495	991.5536	-4.1	$C_{48}H_{81}O_{18}$ [M-H] $^-$	$C_{48}H_{82}O_{18}$	783.4885 [M-(Glu-H <sub>2</sub> O)-H] $^-$ (-1.0), 621.4346 [M-2(Glu-H <sub>2</sub> O)-H] $^-$ (-2.0), 459.3813 [M-3(Glu-H <sub>2</sub> O)-H] $^-$ (-2.5)	Rd
16	8.67	945.5432	945.5423	0.9	$C_{48}H_{81}O_{18}$ [M-H] $^-$	$C_{48}H_{82}O_{18}$	783.4885 [M-(Glu-H <sub>2</sub> O)-H] $^-$ (-1.0), 621.4346 [M-2(Glu-H <sub>2</sub> O)-H] $^-$ (-2.0), 459.3813 [M-3(Glu-H <sub>2</sub> O)-H] $^-$ (-2.5)	Rd
17	8.75	1031.5445	1031.5427	1.8	$C_{51}H_{83}O_{21}$ [M-H] $^-$	$C_{51}H_{84}O_{21}$	987.5547 [M-CO <sub>2</sub> -H] $^-$ (1.8), 945.5436 [M-Mal-H] $^-$ (1.3), 783.4905 [M-Mal-(Glu-H <sub>2</sub> O)-H] $^-$ (1.0), 621.4376 [M-Mal-2(Glu-H <sub>2</sub> O)-H] $^-$ (1.0), 459.3847 [M-Mal-3(Glu-H <sub>2</sub> O)-H] $^-$ (-3.1)	Mal-Rd
18	8.76	987.5542	987.5577	-3.5	$C_{50}H_{83}O_{19}$ [M-CO <sub>2</sub> -H] $^-$	$C_{51}H_{84}O_{21}$	945.5436 [M-Mal-H] $^-$ (1.3), 783.4905 [M-Mal-(Glu-H <sub>2</sub> O)-H] $^-$ (1.0), 621.4376 [M-Mal-2(Glu-H <sub>2</sub> O)-H] $^-$ (1.0), 459.3847 [M-Mal-3(Glu-H <sub>2</sub> O)-H] $^-$ (-3.1)	Mal-Rd
19	14.61	765.4791	765.4789	0.2	$C_{42}H_{69}O_{12}$ [M-H] $^-$	$C_{42}H_{69}O_{12}$	603.4276 [M-(Glu-H <sub>2</sub> O)-H] $^-$ (1.5)	Rg5
20	21.01	698.4167	n.d.					n.d.

<sup>a</sup> n.d.: no decision.<sup>b</sup> Rha:  $\alpha$ -L-rhamnose.<sup>c</sup> Glu:  $\beta$ -D-glucose.<sup>d</sup> Mal: malonyl.<sup>e</sup> Ara(p):  $\alpha$ -L-arabinopyranose.<sup>f</sup> Pen: pentose.<sup>g</sup> GlcA:  $\beta$ -D-glucuronic acid.<sup>h</sup> Xyl:  $\beta$ -D-xylose.

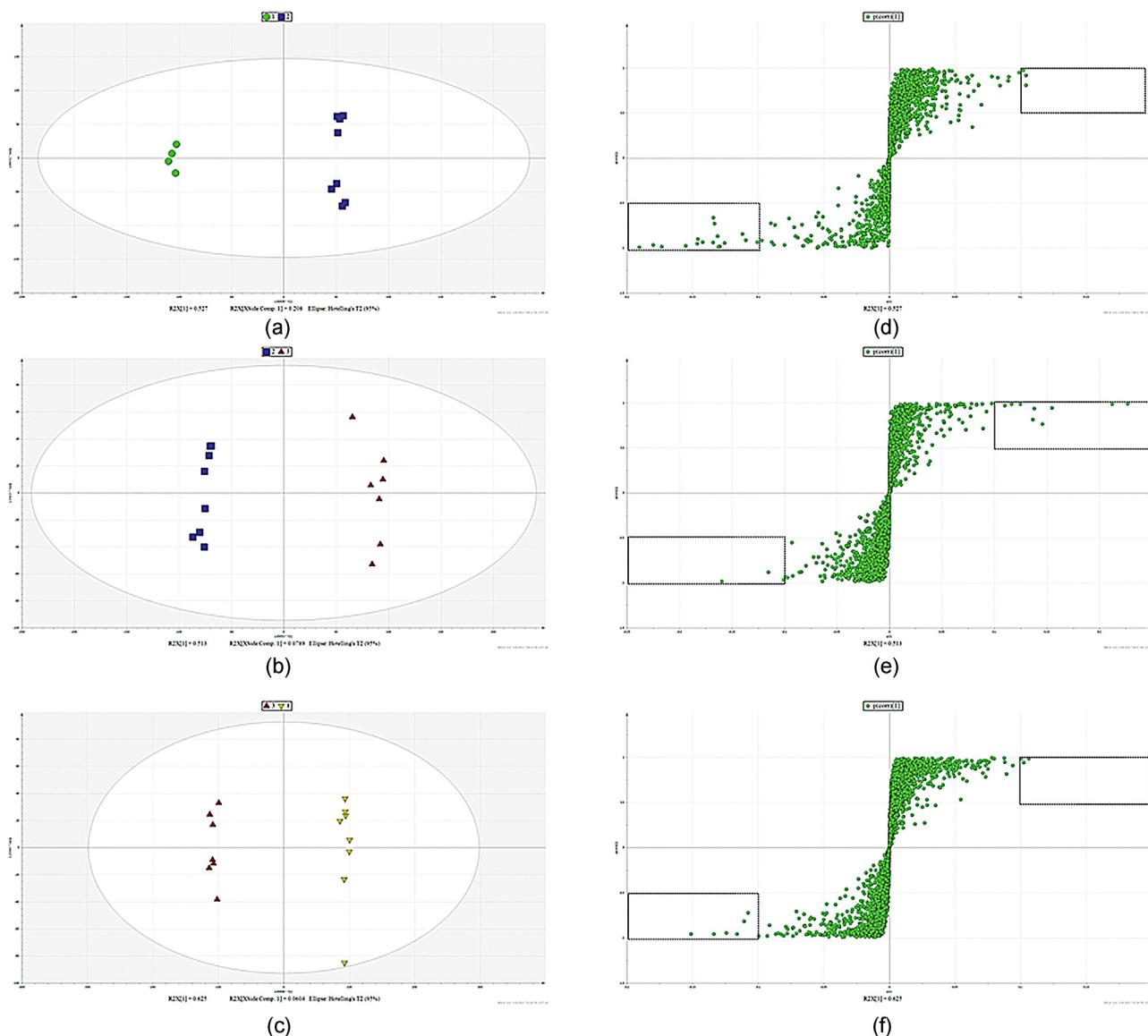
indicated the good quality of the OPLS-DA models. The OPLS-DA loading S-plots were used to statistically obtain the variables that significantly and strongly contributed to the difference between each step (Fig. 4d-f) [33]. We used cutoff values for the covariance of  $p[1] < |0.1|$  and for the correlation of  $p(\text{corr})[1] < |0.5|$  to select the characteristic metabolic markers at the top or bottom of S-shaped plots (Tables 4–6).

### 3.4. Identification of metabolic markers

The development of the UPLC-QTOF/MS system effectively enabled the elucidation and characterization of the ginsenoside structures without the laborious process of isolation and purification from ginseng [34]. Specifically, the TOF analyzer can provide an accurate measurement (mass accuracy; <5 ppm) of molecular



**Fig. 3.** Principal component analysis (PCA) score plots from four steps: DGE (circle), I-1 (quadrangle), I-2 (triangle), and PEG (inverse triangle). Results are expressed as the mean of three independent experiments.



**Fig. 4.** Orthogonal partial least squares discriminant analysis (OPLS-DA) score plots from four steps: DGE vs. I-1 (a), I-1 vs. I-2 (b), and I-2 vs. PEG (c) and S-plots of DGE vs. I-1 (d), I-1 vs. I-2 (e), and I-2 vs. PEG (f). Cutoff values for the covariance of  $p[1] < |0.1|$  and for the correlation of  $p(\text{corr})[1] < |0.5|$  were used to select the characteristic metabolic markers. The selected variables are boxed in the S-plot with retention time and are listed in Tables 4–6.

weight and is useful for determination of the molecular formula of a ginsenoside [35]. In the study, the accurate mass acquired from the QTOF/MS analysis and a dereplication process described in Section 3.2 were used for the structural elucidation of the unidentified peaks in the MS chromatograms. Additionally, we performed MS/MS experiments for determination of the type of aglycone and the sequence of sugar chains attached to the backbone. The characteristic metabolic markers acquired from the multivariate analysis could be identified by interpretation of MS/MS spectra and comparison with the MS spectral data of ginsenosides in the on-line chemical database (Scifinder® and Reaxys®).

As shown in Fig. 4d and Table 4, the metabolic markers that contribute the most to the differences between DGE and I-1 were the more polar ginsenosides that include three or four sugar chains, i.e., Rb1, Rb2, Rc, Rd, Re, malonyl-ginsenoside Rb1 (mRb1), mRb2, and mRd in the S-plot. However, the less polar ginsenosides (except for Rh4) were not found as metabolic markers in this step. These results showed that the amount of these polar ginsenosides significantly changed during the production of I-1 from DGE. Experimentally,

I-1 represented a concentrated extract of the more polar ginsenosides obtained by column chromatography from DGE. When the markers were extracted by MarkerLynx® software, a subset of the adduct ions or the fragmented ions of a single compound generated by decarboxylation or deacetylation was recognized as different markers. For example, although the ions of  $m/z$  945.5439 at 5.48 min and  $m/z$  991.5504 at 5.50 min were characteristic markers with a significance level of less than 0.05 in the S plot, both of them could be assigned as Re due to the presence of the same MS/MS fragmented ions (799.4847 [ $M-(\text{Rha}-\text{H}_2\text{O})-\text{H}$ ] $^-$ , 783.4902 [ $M-(\text{Glu}-\text{H}_2\text{O})-\text{H}$ ] $^-$ , 637.4319 [ $M-(\text{Rha}-\text{H}_2\text{O})-(\text{Glu}-\text{H}_2\text{O})-\text{H}$ ] $^-$ , 475.3780 [ $M-(\text{Rha}-\text{H}_2\text{O})-2(\text{Glu}-\text{H}_2\text{O})-\text{H}$ ] $^-$ ). In the study, if the daughter ions were selected as the markers for  $\pm 0.02$  min and showed the identical MS/MS fragmentation pattern, they were assigned as the same compounds. This criterion was also applied to the identification of the markers in all steps. Thus, we could identify the characteristic metabolic markers between I-1 and I-2 (Table 5) and between I-2 and PEG (Table 6). Certain metabolic markers (Rb1, Rb2, Rc, Rd, Re, and compound K) were assigned

**Table 5**

Metabolic markers contributing to discrimination between I-1 and I-2.

No.	<i>t</i> <sub>R</sub>	Measured mass	Theoretical mass	ΔmDa	<i>m/z</i> ion	Molecular formula	MS/MS fragmentation	ID
1	1.68	503.1758						n.d. <sup>a</sup>
2	9.68	961.5378	961.5372	0.6	C <sub>48</sub> H <sub>81</sub> O <sub>19</sub> [M+COOH] <sup>-</sup>	C <sub>47</sub> H <sub>80</sub> O <sub>17</sub>	915.5322 [M-COOH-H] <sup>-</sup> (0.5), 783.4894 [M-(pentose-H <sub>2</sub> O)-H] <sup>-</sup> (-0.1), 621.4358 [M-(pentose-H <sub>2</sub> O)-(Glu <sup>b</sup> -H <sub>2</sub> O)-H] <sup>-</sup> (-0.8), 459.3830 [M-(pentose-H <sub>2</sub> O)-2(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.7)	Rd2 or notogisenoside Fd or C-Mc1
3	10.48	829.4964	829.4949	1.5	C <sub>43</sub> H <sub>73</sub> O <sub>15</sub> [M+COOH] <sup>-</sup>	C <sub>42</sub> H <sub>72</sub> O <sub>13</sub>	783.4913 [M-H] <sup>-</sup> (1.8), 621.4376 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.6), 459.3838 [M-2(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.0)	F2
4	10.60	793.4398	793.4374	2.4	C <sub>42</sub> H <sub>65</sub> O <sub>14</sub> [M-H] <sup>-</sup>	C <sub>42</sub> H <sub>66</sub> O <sub>14</sub>	613.3755 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (1.5), 455.3519 [M-(Glu-H <sub>2</sub> O)-(GlcA <sup>c</sup> -H <sub>2</sub> O)-H] <sup>-</sup> (-0.6)	Silphioside G
5	11.01	885.5225	885.5212	1.3	C <sub>46</sub> H <sub>77</sub> O <sub>16</sub> [M-H] <sup>-</sup>	C <sub>46</sub> H <sub>78</sub> O <sub>16</sub>	753.4803 [M-(pentose-H <sub>2</sub> O)-H] <sup>-</sup> (1.4), 621.4374 [M-2(pentose-H <sub>2</sub> O)-H] <sup>-</sup> (0.8), 459.3826 [M-2(pentose-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-1.1)	Chikusetsusaponin LM2
6	11.04	931.5278	931.5266	1.2	C <sub>47</sub> H <sub>79</sub> O <sub>18</sub> [M+COOH] <sup>-</sup>	C <sub>46</sub> H <sub>78</sub> O <sub>16</sub>	885.5225 [M-H] <sup>-</sup> (1.3), 753.4803 [M-(pentose-H <sub>2</sub> O)-H] <sup>-</sup> (1.4), 621.4374 [M-2(pentose-H <sub>2</sub> O)-H] <sup>-</sup> (0.8), 459.3826 [M-2(pentose-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-1.1)	Chikusetsusaponin LM2
7	11.82	799.4846	799.4844	0.2	C <sub>42</sub> H <sub>71</sub> O <sub>14</sub> [M+COOH] <sup>-</sup>	C <sub>41</sub> H <sub>70</sub> O <sub>12</sub>	753.4788 [M-H] <sup>-</sup> (-0.1), 621.4372 [M-(pentose-H <sub>2</sub> O)-H] <sup>-</sup> (0.6), 459.3830 [M-(pentose-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-1.7)	C-Mc or C-Y or Mx
8	12.18	799.4870	799.4844	2.6	C <sub>42</sub> H <sub>71</sub> O <sub>14</sub> [M+COOH] <sup>-</sup>	C <sub>41</sub> H <sub>70</sub> O <sub>12</sub>	753.4806 [M-H] <sup>-</sup> (1.7), 621.4400 [M-(pentose-H <sub>2</sub> O)-H] <sup>-</sup> (3.4), 459.3842 [M-(pentose-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.5)	C-Mc or C-Y or Mx
9	12.19	753.4806	753.4789	1.7	C <sub>41</sub> H <sub>69</sub> O <sub>12</sub> [M-H] <sup>-</sup>	C <sub>41</sub> H <sub>70</sub> O <sub>12</sub>	621.4400 [M-(pentose-H <sub>2</sub> O)-H] <sup>-</sup> (3.4), 459.3842 [M-(pentose-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.5)	C-Mc or C-Y or Mx
10	12.80	663.4109	663.4108	0.1	C <sub>37</sub> H <sub>59</sub> O <sub>10</sub> [M+COOH] <sup>-</sup>	C <sub>36</sub> H <sub>58</sub> O <sub>8</sub>	617.4064 [M-H] <sup>-</sup> (1.1), 455.3522 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.3)	Oleanolic acid 28-O-b-glu
11	13.43	595.2882						n.d.
12	14.04	667.4421	667.4427	0.4	C <sub>37</sub> H <sub>63</sub> O <sub>10</sub> [M+COOH] <sup>-</sup>	C <sub>36</sub> H <sub>62</sub> O <sub>8</sub>	621.4369 [M-H] <sup>-</sup> (0.3), 459.3844 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.6)	Compound K

<sup>a</sup> n.d.: no decision.<sup>b</sup> Glu: β-D-glucose.<sup>c</sup> GlcA: β-D-glucuronic acid.

by comparing the retention time and the accurate mass with the commercial reference compounds (see Supporting information). Except for these markers, the other markers could be tentatively assigned using the MS/MS experimental data as well as through a comparison with the literature values in the on-line database. However, this approach was limited to determining the structures of the unknown peaks. The MS/MS analysis did not provide substantial information for sugar chains that have the same molecular formula—i.e., arabinofuranose (C<sub>5</sub>H<sub>10</sub>O<sub>5</sub>), arabinopyranose (C<sub>5</sub>H<sub>10</sub>O<sub>5</sub>), and xylose (C<sub>5</sub>H<sub>10</sub>O<sub>5</sub>)—or location (C-3 or C-20) and only suggested possible candidates. In the second step, twelve markers (3 of I-1 > I-2 and 9 of I-1 < I-2) were extracted from the S

plot. Among these, eight ginsenoside markers were collected, along with non-ginsenoside markers and duplicates. The markers could be identified as ginsenosides generated by the deglycosylation of the more polar ginsenosides that contributed to the discrimination between DGE and I-1. Interestingly, certain markers still included pentose residues in their structures. Although these data could not be exactly elucidated due to the lack of information on the type of pentose, the MS/MS data showed that the deglycosylation between I-1 and I-2 selectively occurred at C-3. Ginsenosides with less than one sugar residue at C-3 and pentose residues at C-20 are not common in nature, and these compounds were found to have been artificially generated in the fermentation process [36]. The

**Table 6**

Metabolic markers contributing to discrimination between I-2 and PEG.

No.	<i>t</i> <sub>R</sub>	Measured mass	Theoretical mass	ΔmDa	<i>m/z</i> ion	Molecular formula	MS/MS fragmentation	ID
1	9.68	961.5378	961.5372	0.6	C <sub>48</sub> H <sub>81</sub> O <sub>19</sub> [M+COOH] <sup>-</sup>	C <sub>47</sub> H <sub>80</sub> O <sub>17</sub>	915.5322 [M-COOH-H] <sup>-</sup> (0.5), 783.4894 [M-(pentose-H <sub>2</sub> O)-H] <sup>-</sup> (-0.1), 621.4358 [M-(pentose-H <sub>2</sub> O)-(Glu <sup>a</sup> -H <sub>2</sub> O)-H] <sup>-</sup> (-0.8), 459.3830 [M-(pentose-H <sub>2</sub> O)-2(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.7)	Rd2 or notoginsenoside Fd or C-Mc1
2	10.48	829.4964	829.4949	1.5	C <sub>43</sub> H <sub>73</sub> O <sub>15</sub> [M+COOH] <sup>-</sup>	C <sub>42</sub> H <sub>72</sub> O <sub>13</sub>	783.4913 [M-H] <sup>-</sup> (1.8), 621.4376 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.6), 459.3838 [M-2(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.0)	F2
3	10.69	665.4265	665.4265	0.0	C <sub>37</sub> H <sub>61</sub> O <sub>10</sub> [M+COOH] <sup>-</sup>	C <sub>36</sub> H <sub>60</sub> O <sub>8</sub>	619.4219 [M-H] <sup>-</sup> (0.9)	Rh4
4	11.04	931.5278	931.5266	1.2	C <sub>47</sub> H <sub>79</sub> O <sub>18</sub> [M+COOH] <sup>-</sup>	C <sub>46</sub> H <sub>78</sub> O <sub>16</sub>	885.5225 [M-H] <sup>-</sup> (1.3), 753.4803 [M-(pentose-H <sub>2</sub> O)-H] <sup>-</sup> (1.4), 621.4374 [M-2(pentose-H <sub>2</sub> O)-H] <sup>-</sup> (0.8), 459.3826 [M-2(pentose-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-1.1)	Chikusetsusaponin LM2
5	11.82	799.4846	799.4844	0.2	C <sub>42</sub> H <sub>71</sub> O <sub>14</sub> [M+COOH] <sup>-</sup>	C <sub>41</sub> H <sub>70</sub> O <sub>12</sub>	753.4788 [M-H] <sup>-</sup> (-0.1), 621.4372 [M-(pentose-H <sub>2</sub> O)-H] <sup>-</sup> (0.6), 459.3830 [M-(pentose-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-1.7)	C-Mc or C-Y or Mx
6	12.18	799.4870	799.4844	2.6	C <sub>42</sub> H <sub>71</sub> O <sub>14</sub> [M+COOH] <sup>-</sup>	C <sub>41</sub> H <sub>70</sub> O <sub>12</sub>	753.4806 [M-H] <sup>-</sup> (1.7), 621.4400 [M-(pentose-H <sub>2</sub> O)-H] <sup>-</sup> (3.4), 459.3842 [M-(pentose-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.5)	C-Mc or C-Y or Mx
7	12.80	663.4109	663.4108	0.1	C <sub>37</sub> H <sub>59</sub> O <sub>10</sub> [M+COOH] <sup>-</sup>	C <sub>36</sub> H <sub>58</sub> O <sub>8</sub>	617.4064 [M-H] <sup>-</sup> (1.1), 455.3522 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.3)	Oleanane acid 28-O-β-D-glucopyranoside
8	13.00	825.4993	825.5000	-0.7	C <sub>44</sub> H <sub>73</sub> O <sub>14</sub> [M-H] <sup>-</sup>	C <sub>44</sub> H <sub>74</sub> O <sub>14</sub>	783.4890 [M-COCH <sub>3</sub> -H] <sup>-</sup> (-0.5), 621.4357 [M-COCH <sub>3</sub> -(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.9), 459.3342 [M-(AcetylGlu <sup>b</sup> -H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.6)	Rs3
9	14.04	667.4421	667.4427	0.4	C <sub>37</sub> H <sub>63</sub> O <sub>10</sub> [M+COOH] <sup>-</sup>	C <sub>36</sub> H <sub>62</sub> O <sub>8</sub>	621.4369 [M-H] <sup>-</sup> (0.3), 459.3844 [M-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (0.6)	Compound K
10	17.02	807.4893	807.4895	-0.2	C <sub>44</sub> H <sub>71</sub> O <sub>13</sub> [M-H] <sup>-</sup>	C <sub>44</sub> H <sub>72</sub> O <sub>13</sub>	765.4787 [M-COCH <sub>3</sub> -H] <sup>-</sup> (-0.2), 603.4257 [M-COCH <sub>3</sub> -(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-0.4), 441.3707 [M-(AcetylGlu-H <sub>2</sub> O)-(Glu-H <sub>2</sub> O)-H] <sup>-</sup> (-2.6)	Rs4

<sup>a</sup> Glu: β-D-glucose.<sup>b</sup> AcetylGlu: β-D-6-O-acetyl-glucose.

sugar residues at C-3 may have been selectively cleaved during the reaction with enzymes developed by the manufacturers. The peak (961.5372 [M+COOH]<sup>-</sup>) at 9.68 min was assumed to be Rd2, notoginsenoside Fd, or notoginsenoside Fe, which were generated by the decarboxylation at C-3 from Rb2 or Rc. In the last step, the less polar ginsenosides, i.e., Rh4, Rs3, and Rs4, were identified as metabolic markers that contributed to the discrimination of PEG from the I-2 located in the top right in the S plot. These compounds had a Δ20 and Δ20(22) dehydrated aglycone instead of a free hydroxyl group at C-20. In terms of Rs3 and Rs4, these compounds had a β-D-6-O-acetyl-glucose at the end of the sugar chains at C-3. These results indicate that the metabolic markers in the final process for this stage might be produced by deglycosylation and dehydration at C-20, decarboxylation of the malonylated ginsenosides at C-3,

or acetylation of hydroxyl moiety at C-6 of the end of D-glucose by acetic acid during the mild acidic hydrolysis. In terms of Ro, which had an oleanane-type triterpene in DGE, it was assumed that this compound was subsequently altered to silphioside G, and further, to oleanane acid 28-O-β-D-glucopyranoside, which might have resulted from the faster hydrolysis of the sugar chains at C-3 rather than at C-28.

### 3.5. Metabolic pathway of ginsenosides from DGE to PEG

From the results described above, we proposed a possible metabolic pathway for the manufacturing process from DGE to PEG based on a comparison with the literature (see Supporting Information) [29,30]. Although DGE and I-1 were not easily

discriminated in the PCA plot and with the unaided eye, the more polar ginsenosides were generated as the metabolic markers in the S plot of the OPLS-DA analysis. In the next step, the enzyme reaction used to produce I-2 from I-1 primarily eliminated the sugar residue at C-3 or C-20 without the changes of the aglycone (20-hydroxy-dammar-24-ene type). In the step between I-2 and PEG, the less polar ginsenosides were mainly generated, as shown in Table 6. These results suggested that the terminal sugar residue at C-3 or C-6 might be removed to generate low-molecular-weight ginsenosides, i.e., Rh1 and Rh2, which displayed one or two sugar chains in mild acidic or heat-steaming conditions. The sugar chains at C-20 were eliminated more easily than those at C-3, and ginsenosides with sugar chains at C-20 were not isolated in PEG [29,37]. The type of aglycone produced by dehydration at C-20 was changed to dammar-20, 24-diene type or dammar-20(22), 24-diene type from the 20-hydroxy-dammar-24-ene type [38]. Furthermore, the aglycones of these ginsenosides could be transformed into three sub-groups of hydrated ginsenosides at C-20, C-25 and C-20 and C-25. Among these reactions, the addition reaction with the hydroxyl moiety at C-20 could produce 20R-ginsenosides, i.e., 20R-Rh1, 20R-Rh2, 20R-Rg2, and 20R-Rg3, as well as their 20S-isomers. The 20R-ginsenosides have rarely been found in natural crude ginseng thus far and were assumed to be generated by the addition reaction after elimination of the sugar chains at C-20 [30,39].

#### 4. Conclusion

About 300 of ginsenosides from ginseng preparation have been reported so far [40]. The structural diversity of ginsenoside derivatives was resulted from chemical modifications, i.e., hydroxylation, dehydroxylation, decarboxylation, acetylation and deglycosylation. A dereplication approach in the study aimed that ginsenosides as the metabolic markers, which are produced in the intermediate steps and present in a small amount, can be identified by the chromatographic and spectrometric data of 22 ginsenoside standards. Further, multivariate analysis of the data matrix acquired from a UPLC-QTOF/MS analysis can suggest the metabolic markers that contribute to the discrimination of the manufacturing process from DGE to PEG and that were assumed to be helpful for quality control of this ginseng preparation. The chemical profiles and structural information for known ginsenosides will be useful not only for the prediction of biotransformation and generation during the manufacturing process but also in a dereplication step for the selective purification and isolation of desirable ginsenosides from other ginseng extract or preparation.

#### Acknowledgements

This research was equally supported by the Global Leading Technology Program of the Office of Strategic R&D Planning (OSP grant 10039320) funded by the Ministry of Knowledge Economy, Korea.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jpba.2015.02.034>.

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