Discrimination of white ginseng origins using multivariate statistical analysis of data sets

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Abstract

Background: White ginseng (Panax ginseng Meyer) is commonly distributed as a health food in food markets. However, there is no practical method for distinguishing Korean white ginseng (KWG) from Chinese white ginseng (CWG), except for relying on the traceability system in the market.

Methods: Ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry combined with orthogonal partial least squares discrimination analysis (OPLS-DA) was employed to discriminate between KWG and CWG.

Results: The origins of white ginsengs in two test sets (1.0 mL and 0.2 mL injections) could be successfully discriminated by the OPLS-DA analysis. From OPLS-DA S-plots, KWG exhibited tentative markers derived from ginsenoside Rf and notoginsenoside R3 isomer, whereas CWG exhibited tentative markers derived from ginsenoside Ro and chikusetsusaponin Iva.

Conclusion: Results suggest that ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry coupled with OPLS-DA is an efficient tool for identifying the difference between the geographical origins of white ginsengs.

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1. Introduction

Ginseng (Panax ginseng Meyer) is a multifunctional therapeutic herb that is commonly used throughout the world. Primarily in East Asia, ginseng has been used as traditional medicine to enhance the immune system, control blood pressure, and strengthen the cardiovascular system [1]. The ginseng herb is processed using various methods. For example, peeled ginseng root turns white when dried in the sun, which has led to it being called white ginseng, whereas red ginseng is produced by steaming and drying. A wide variety of pharmacological properties have been reported for ginseng, such as anti-oxidant, anti-stress, neuroprotective, hypoglycemic, and anti-tumor effects [2–5]. The ginseng herb and ginseng-derived products include multiple secondary metabolites, such as protopanaxadiol (PPD)-type (e.g., ginsenoside Rb1, Rb2, Rc, Rd, and Rg3), protopanaxatriol (PPT)-type (e.g., ginsenoside Rg1, Re, Rf, and Rg2), and oleanane (OCO)-type ginsenosides (e.g., ginsenoside Ro) [6]. Different ginsenoside ratios have been reported for different species, geographical origins, and processing methods, and such ratios are considered to be responsible for the different bioactivities [7,8].

Metabolomics primarily focuses on comprehensive and quantitative profiling for small-molecule metabolites in a biological system. It has been applied to a variety of areas, such as plant toxicology, nutrition, and systems biology [9–11]. Multiple analytical methods, including nuclear magnetic resonance, gas chromatography-mass spectrometry, and liquid chromatography-mass spectrometry, have been applied in metabolic profiling in...
order to differentiate *Panax* species [12–14]. Among the various analytical methods, ultra-performance liquid chromatography quadrupole time-of-light mass spectrometry (UPLC-QTOF/MS) is used in comprehensive and reliable ginsenoside profiling for various ginseng products [15–17]. In certain studies, morphological and chemical methods were used to discriminate Korean ginseng from other *P. ginseng* sources [14,18]. Recently, metabolomics research has been used to discriminate the origin of ginseng products [19]. Despite this, ginsenosides have not been fully investigated as chemical markers despite their pharmacological importance. In our study, a metabolomics approach, combining a UPLC-QTOF/MS-based analysis with orthogonal partial least squares discrimination analysis (OPLS-DA), is used to determine the geographical origin of white ginsengs. This study manifests that the statistical model (OPLS-DA) would facilitate the discrimination of Korean white ginseng (KWG) and Chinese white ginseng (CWG) origins in concert with the UPLC-QTOF/MS. Furthermore, the prediction model exhibited statistical reliability and could be applied to discriminate samples in the market.

2. Materials and methods

2.1. Chemicals and materials

High-performance liquid chromatography-grade acetonitrile and methanol were obtained from SK Chemicals Co. (Seongnam, Korea). The aqueous solutions were prepared using ultrapure water from a Milli-Q system (18.2 MΩ, Millipore, Bedford, MA, USA). Leucine-enkephalin and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). The white ginseng samples were provided by the Experiment Research Institute of National Agricultural Products Quality Management Service. KWG (53 samples) was obtained from several Korean markets in 2008–2009. CWG (10 samples from China and eight samples from Korea) was purchased from several vendors in China and Korea during 2006–2009 (Table 1). All samples were verified by the National Agricultural Products Quality Management Service and were used for origin identification. Reference standards of ginsenosides Rg1 (5), ginsenoside Re (6), ginsenoside Rf (9), 20(R)-ginsenoside Rh1 (11), ginsenoside Ra2 (14), ginsenoside Rb1 (15), ginsenoside Rc (17), ginsenoside Rb1 (18), ginsenoside Rb3 (22), ginsenoside Rb2 (23), and ginsenoside Rd (28) were provided from Floten Natural Products Co., Ltd. (Chengdu, China). The standards were dissolved in methanol to obtain stock solutions at approximately 1.0 mg/mL and were stored at 4°C.

2.2. Sample preparation

The ginseng samples were dried and pulverized to powder using a mill and passed through a 40-mesh sieve. The fine ginseng powder was weighed (0.4 g) and extracted with 5 mL of 70% methanol in an ultrasonic waterbath for 60 min [13]. The extract was filtered through a syringe filter (0.22 μm) and injected directly into the UPLC system.

2.3. UPLC-QTOF/MS analysis

Ginseng metabolite profiling was performed using the ACQUITY UPLC system (Waters Corporation, Milford, MA, USA), which was equipped with a binary solvent delivery manager and a sample manager coupled to a Micromass Q-TOF Premier mass spectrometer (Waters Corporation, Milford, MA, USA) with an electrospray interface. Chromatographic separation was performed using an ACQUITY BEH C18 chromatography column (Waters Corporation; 2.1 mm × 100 mm, 1.7 μm). The column temperature was maintained at 35°C, and the mobile Phases A and B were water with 0.1% formic acid and acetonitrile with 0.1% formic acid, respectively. The gradient elution program to get the ginsenoside profile was as follows: 0 min, 10% B; 0–7 min, 10–33% B; 7–14 min, 33–56% B; 14–21 min, 56–100% B; wash for 23.5 min with 100% B; and a 1.5 min recycle time. The injection volumes were 1.0 μL and 0.2 μL for each test set, and the flow rate was 0.4 mL/min. The mass spectrometer was operated in positive ion mode. N2 was used as the desolvent gas. The desolvation temperature was 350°C, the flow rate was 500 L/h, and the source temperature was 100°C. The capillary and cone voltages were 2700V and 27V, respectively. The data were collected for each test sample from 200 Da to 1,500 Da with 0.25-s scan time and 0.01-s interscan delay over a 25-min analysis time. Leucine-enkephalin was used as the reference compound (m/z 556.2771 in the positive mode).

2.4. Chemometric data analysis

The raw mass data were normalized to total intensity (area) and analyzed using the MarkerLynx Applications Manager version 4.1 (Waters, Manchester, UK). The parameters included a retention time range of 4.0–19.0 min, a mass range from 200 Da to 1,500 Da, and a mass tolerance of 0.04 Da. The isotopic data were excluded, the noise elimination level was 10, and the mass and retention time windows were 0.04 min and 0.1 min, respectively. After creating a suitable processing method, the dataset was processed through the Create Dataset window. The resulting two-dimensional matrix for the measured mass values and intensities for each sample was further exported to SIMCA-P+ software 12.0 (Umetrics, Umeå, Sweden) using both unsupervised principal component analysis and supervised OPLS-DA.

3. Results and discussion

3.1. Mass spectrometry data analysis of white ginseng ginsenosides

As shown in previous articles [13,16], the ACQUITY BEH C18 column (Waters Corporation) has frequently been used to separate ginsenosides from various *Panax* herbs. As presented in Fig. 1A (CWG) and Fig. 1B (KWG), 11 compounds were assigned by comparing them to standard ginsenosides and 19 ginsenosides were identified by comparing their retention time and mass spectra with the reference compounds. The compounds were further
Fig. 1. Total ion current chromatograms of white ginseng extract (1.0 μL) using UPLC-QTOF/MS. (A) Chinese White Ginseng and (B) Korean White Ginseng. [1–4, notoginsenoside R3 isomer; 5, ginsenoside Rg1; 6, ginsenoside Re; 7, malonyl ginsenoside Rg1; 8, unknown; 9, ginsenoside Rf; 10, notoginsenoside R2; 11, 20(E)-ginsenoside Rb1; 12, notoginsenoside R4/Fa; 13, ginsenoside Rb1; 14, ginsenoside Ra2; 15, ginsenoside Rb1; 16, malonyl ginsenoside Rb1; 17, ginsenoside Rc; 18, ginsenoside Ra1; 19, ginsenoside Ro; 20, malonyl ginsenoside Rc; 21, malonyl ginsenoside Rb1 isomer; 22, ginsenoside Rb3; 23, ginsenoside Rb2; 24, malonyl ginsenoside Rb2; 25, malonyl ginsenoside Rb2; 26, quinquenoside R1; 27, chikusetsusaponin Iva; 28, ginsenoside Rb; 29, malonyl ginsenoside Rb; 30, gypenoside XV.]
confirmed through ion fragmentation patterns [20,21]. As illustrated in Table 2, white ginseng saponins were detected as protonated ions [M+H]+, sodium adduct ions [M+Na]+, and/or ammonium adduct ions [M+NH4]+ in the positive ion mode. The pathway for the specific fragmentation pattern supports the classification of 30 ginsenosides into three groups according to the following structures: (1) 11 compounds (peak 1–11) were identified as protopanaxatriol (PPT) type with sugar moieties attached to the C-6 and/or C-20; (2) two ginsenosides (peaks 19 and 27) were identified as OCO-type ginsenosides; and (3) the rest of compounds were identified as PPD-type with sugar moieties attached to the C-3 and/or C-20. Three types showed their own diagnostic ions in fragmentation. PPT- and PPD-type ginsenosides showed characteristic fragment ions at m/z 441.37 and m/z 425.37, respectively, indicating the losses of sugar moieties, whereas OCO-type ginsenosides showed fragment ion at m/z 439.36 corresponding to their aglycone. The cleaved pathways of three types were reported in previous researches [21,22].

3.2. Discrimination of white ginsengs' origin

The extracts from KWG (53 samples) and CWG (18 samples) were continuously and randomly injected into the UPLC-QTOF/MS system with a 25-min run time. Given the peaks’ complexity in the UPLC chromatograms, it was difficult to distinguish between KWG and CWG through visual chromatogram observation, which indicated that the major components in the ginseng from the two origins were similar.

In this case, an effective approach for discerning differences is multivariate statistical analysis. Multivariate analysis has been widely used in the metabolomics field in recent years for extremely complex samples [23]. First, we performed principal component analysis, which is widely used as a metabolomics profiling technique for plant metabolites [24,25]. After Pareto (Par) scaling with mean-centering, the data were displayed as a score plot in a coordinate system with latent variables, “principal components” (data not shown).

Recently, supervised OPLS-DA has been widely used to study the differences between two similar groups [26]. OPLS-DA model quality can be estimated using the cross-validation parameters Q2 (model predictability) and R2 (total explained variation for the X matrix). OPLS-DA for the samples produced one predictive as well as one orthogonal (1 + 3) component and showed that the cross-validated predictive ability Q2 was 0.877, and the variance related to the differences between the two origins R2(y) was 0.992 (Fig. 2A) and cross validated analysis of variation (CV-ANOVA) p = 2.52 × 10⁻25.

Validation of an analysis model is critical for statistical multivariate analyses. We validated the analysis model by excluding certain data (a test data set) and reconstructing a new model with the remaining data (a training data set). The Y-predicted score plot indicated a confident prediction between two groups through the first predicted score (rPS), which summarized the X variation orthogonal to Y for the prediction set. The predicted assignment for each sample was compared to the original value, and thereby the model was evaluated for prediction accuracy and reliability. This method has been used to predict drug toxicity and geographical origin in recent metabolomics studies [27,28]. For the prediction test confidence, one-third of the samples (18 Korean and six Chinese samples) were randomly excluded and reanalyzed using the OPLS-DA model. The model for predicting their origins was established using one predictive component and one orthogonal component with R2(y) = 0.930 and Q2 = 0.796. The samples from the blind test were correctly assigned to their origin cluster, and the 24 analyzed samples were well predicted as shown in Fig. 2B, which indicates that the OPLS-DA model can discriminate between KWG and CWG. A variety of concentrations of ginsenosides in the sample, however, can cause difficulty in generating quantitative ion intensity for a compound in the UPLC-QTOF/MS system. As major peaks of ginsenosides were frequently saturated at a high concentration, we applied two sample sets (0.2 μL and 1.0 μL) for optimal analysis. The 0.2 μL test set model produced similar results to the 1.0 μL test set with R2(y) = 0.954, Q2 = 0.792, and CV-ANOVA p = 5.37 × 10⁻20 (Fig. 2C). The OPLS-DA model for predicting the ginseng origins was established using one predictive and two orthogonal components with R2(y) = 0.973 and Q2 = 0.775. In addition, the blind test samples were correctly assigned to their origin’s cluster (Fig. 2D).

3.3. Assignment of tentative markers of white ginseng origins

A useful tool for comparing a variables’ magnitude and reliability is the S-plot from the OPLS-DA model. Each point on the S-plot represents the exact mass retention time (tR-m/z) pair. As a result, the white ginseng’s differential variables (markers) associated with KWG and CWG are based on the threshold of variable importance in the projection (VIP) value (VIP > 1.0) from the S-plot [29]. The VIP value represents the importance of a variable in modeling both X (the projections) and Y (its correlation to all the responses). The VIP values of selected ions are enumerated in Table 3.

From the 1.0 μL injection test set, ions 1A, 1B, and 1C in Fig. 2E were the characteristics of KWG, and ions 2A–2G and 3A–3D were the characteristics of CWG. The fold values were obtained by dividing the mean value of mass intensity of KWG by the mean value of mass intensity of CWG. Ions 2A–2G, having fold values of 0.38–0.48 at tR 9.06 min, imply that these ions originated from only one compound, which was identified as NG R2. This result is well matched with the fragmentation ion patterns of NG R2 in the MassFragment tool of MassLynx 4.1 (Waters, Manchester, UK) (Fig. 3A). It was found that ions 1A–1C, which were highly detected in KWG (fold values: 3.13–4.66) at tR 9.05 min, were not from NG R2, although they had retention times similar to NG R2 (tR 9.06 min). The structures of the ions could not be confirmed, but it was determined that the molecular weights were different from NG R2. Ions 3A–3D at tR 11.36 min were assigned to chikusetusaponin Iva, and were found by matching the molecular formula and fragment ion patterns [30]. Those ions were significant in CWG, with fold values of 0.30–0.37.

From the 0.2 μL injection test set, several ginsenoside ions were also detected in the S-plot (Fig. 2F). The fragment ion of 5A (765.4810 at tR 8.86 min), which was assigned to ginsenoside RF by matching the molecular formula and retention time with a standard compound, was postulated to be a tentative marker of KWG (VIP value >1.0). The ions 4A and 4B (985.5287 and 783.4919, respectively, at tR 5.20 min) could be assigned to one of the NG R3 isomers, including 20-gluco-ginsenoside RF, NG R6, NG M, or NG N. These isomers showed the same molecular ions and same fragmentation patterns at different retention times (peaks 1–4 in Table 2) [30,31]. From the results, ions 5A, 4A, and 4B can be postulated as tentative markers for KWG, ions 6A–6F at tR 10.28 min, which were assigned to ions derived from ginsenoside Ro (Fig. 3B), could be tentative markers for CWG by VIP value and fold values [32].

4. Conclusion

Two sample sets (0.2 μL and 1.0 μL) were applied in the UPLC-QTOF/MS with OPLS-DA and several ginsenosides were postulated for discriminating markers between the white ginseng sample sets
Fig. 2. Multivariate statistical analysis for Korean white ginseng (KWS) and Chinese white ginseng (CWG). (A) Orthogonal partial least squares discrimination analysis (OPLS-DA) score plots and (B) predicted score plot for the 1.0 μL injection data set, (C) OPLS-DA score plots and (D) predicted score plot for the 0.2 μL injection data set, (E) S-plot of OPLS-DA model for the 1.0 μL injection data set, and (F) S-plot of OPLS-DA model for the 0.2 μL injection data set.

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| Data set | Marker | tR_m/z | VIP | Formation of fragment ions | Parent compound | Average mass intensity | Fold
|----------|--------|--------|-----|-----------------------------|-----------------|----------------------|------|
| 1.0 µL   | 1A     | 9.05_1379.6535 1.81 | [M+Na]^+ | Unknown | 1.14 | 0.37 | 3.13 |
|          | 1B     | 9.05_1357.6732 2.48 | [M+H]^+ | unknown | 3.14 | 0.67 | 4.66 |
|          | 1C     | 9.05_875.4757 3.39 |             |         | 6.15 | 1.85 | 3.33 |
|          | 2A     | 9.06_771.4917 2.19 | [M+H]^+ | Notoginsenoside R2 | 1.70 | 4.52 | 0.38 |
|          | 2B     | 9.06_753.4822 5.23 | [M+H-H2O]^+ |         | 11.08 | 27.15 | 0.41 |
|          | 2C     | 9.06_735.4808 4.28 | [M+H-2H2O]^+ |         | 8.33 | 19.01 | 0.44 |
|          | 2D     | 9.06_621.4376 1.57 | [M+H-Xyl]^+ |         | 1.25 | 2.77 | 0.45 |
|          | 2E     | 9.06_441.3727 4.26 | [M+H-Glc-Xyl-H2O]^+ |         | 8.63 | 19.13 | 0.45 |
|          | 2F     | 9.06_423.3617 6.50 | [M+H-Glc-Xyl-2H2O]^+ |         | 23.24 | 48.32 | 0.48 |
|          | 2G     | 9.06_405.3452 2.93 | [M+H-Glc-Xyl-3H2O]^+ |         | 4.54 | 9.66 | 0.47 |
|          | 3A     | 11.36_817.4389 3.07 | [M+Na]^+ | Chikusetsusaponin Iva | 3.07 | 8.31 | 0.37 |
|          | 3B     | 11.36_812.4812 3.07 | [M+NH4]^+ |         | 3.56 | 10.83 | 0.33 |
|          | 3C     | 11.36_633.4013 3.42 | [M+H-Glc]^+ |         | 1.32 | 4.34 | 0.30 |
|          | 3D     | 11.36_439.3546 3.56 | [M+H-Glc-U-Glc-2H2O]^+ |         | 13.62 | 6.24 | 2.18 |
| 0.2 µL   | 4A     | 5.20_985.5287 2.62 | [M+Na]^+ | Notoginsenoside R3 isomer | 7.63 | 2.48 | 3.08 |
|          | 4B     | 5.20_783.4919 1.62 | [M+H-Glc-H2O]^+ |         | 7.63 | 2.48 | 3.08 |
|          | 5A     | 8.86_765.4810 2.06 | [M+H-2H2O]^+ | Ginsenoside Rf | 36.00 | 27.76 | 1.30 |
|          | 6A     | 10.28_979.4910 3.52 | [M+Na]^+ | Ginsenoside Ro | 65.06 | 83.65 | 0.78 |
|          | 6B     | 10.28_974.5358 2.45 | [M+NH4]^+ |         | 28.86 | 37.52 | 0.77 |
|          | 6C     | 10.28_957.6210 2.60 | [M+H]^+ |         | 16.16 | 26.45 | 0.61 |
|          | 6D     | 10.28_795.5720 2.90 | [M+H-Glc]^+ |         | 22.58 | 35.02 | 0.64 |
|          | 6E     | 10.28_633.5164 2.28 | [M+H-2Glc]^+ |         | 9.00 | 14.72 | 0.61 |
|          | 6F     | 10.28_439.3555 5.12 | [M+H-Glc-U-2(Glc-H2O)]^+ |         | 84.38 | 121.26 | 0.70 |

1) Variable importance in the projection.
2) Fold value was calculated by dividing the mean value of ion mass intensity of KWG by that of CWG.

Fig. 3. Fragmentation ions patterns of tentative markers. (A) notoginsenoside R2 (793.4822; [M+Na]^+; tR; 9.06 min) and (B) ginsenoside Ro (957.6210; [M+H]^+; tR; 10.29 min).
originated from Korea and China. Blind tests with arbitrarily selected samples comprising one-third of the total were performed to validate the OPLS-DA model, and all of the samples were correctly assigned to their origins. Furthermore, profiling the details of the samples enabled the observation of the differences of ginsenosides between KGW and CWG. Our results suggest that the approach in the present study could be effectively applied to discriminate the geographical origins between KGW and CWG in the markets.

Conflicts of interest

All authors declare no conflicts of interest.

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