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Discrimination of *Panax ginseng* Roots Cultivated in Different Areas in Korea Using HPLC-ELSD and Principal Component Analysis

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In order to distinguish the cultivation area of *Panax ginseng*, principal component analysis (PCA) using quantitative and qualitative data acquired from HPLC was carried out. A new HPLC method coupled with evaporative light scattering detection (HPLC-ELSD) was developed for the simultaneous quantification of ten major ginsenosides, namely Rh_1, Rg_2, Rg_3, Rg_1, Rf, Re, Rd, Rb_2, Rc, and Rb_1 in the root of *P. ginseng* C. A. Meyer. Simultaneous separations of these ten ginsenosides were achieved on a carbohydrate analytical column. The mobile phase consisted of acetonitrile-water-isopropanol, and acetonitrile-water-isopropanol using a gradient elution. Distinct differences in qualitative and quantitative characteristics for ginsenosides were found between the ginseng roots produced in two different Korean cultivation areas, Ganghwa and Punggi. The ginsenoside profiles obtained via HPLC analysis were subjected to PCA. PCA score plots using two principal components (PCs) showed good separation for the ginseng roots cultivated in Ganghwa and Punggi. PC1 influenced the separation, capturing 43.6% of the variance, while PC2 affected differentiation, explaining 18.0% of the variance. The highest contribution components were ginsenoside Rg_3 for PC1 and ginsenoside Rf for PC2. Particularly, the PCA score plot for the small ginseng roots of six-year old, each of which was light than 147 g fresh weight, showed more distinct discrimination. PC1 influenced the separation between different sample sets, capturing 51.8% of the variance, while PC2 affected differentiation, also explaining 28.0% of the variance. The highest contribution component was ginsenoside Rf for PC1 and ginsenoside Rg_2 for PC2. In conclusion, the HPLC-ELSD method using a carbohydrate column allowed for the simultaneous quantification of ten major ginsenosides, and PCA analysis of the ginsenoside peaks shown on the HPLC chromatogram would be a very acceptable strategy for discrimination of the cultivation area of ginseng roots.

Keywords: *Panax ginseng*, Carbohydrate column, Root discrimination, Ginsenosides, HPLC-evaporative light scattering detection, Principal component analysis

INTRODUCTION

Korean ginseng, the root of *Panax ginseng* C. A. Meyer, has been frequently used for various therapeutic purposes in oriental traditional medicine and is now widely used around the world [1]. Because the roots cultivated in different areas show significant variation in pharmacological efficacy and root components, it is necessary to

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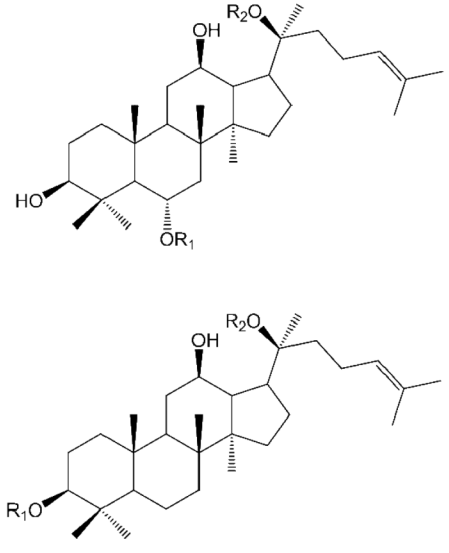
discriminate the origin of cultivation [2]. Ginsenosides, the ginseng saponins, are the principal components (PCs) which manifest a variety of the pharmacological and biological activities of the ginseng root [3-5]. Qualitative and quantitative characteristics of ginsenosides from the roots cultivated in different areas were also significantly different [6]. Therefore, analysis of ginsenosides is very important for the identification of the cultivation origin of ginseng and for the quality control of ginseng products. Many methods for quantifying ginsenosides have been developed using thin layer chromatography [7], HPLC coupled with a UV detector [8] or an evaporative light scattering detector (ELSD) [9], and mass spectrometry (MS) [10] methods. However, the above described methods cannot fully resolve ginsenosides due to their similarities in chemical structures. Most HPLC analysis using reversed phased resin, octadecyl silica gel (ODS), the most widely used for HPLC analysis of ginsenosides, simultaneously separates fewer than ten of the 70 total ginsenosides reported in the literature [11]. In the present study, we attempted to develop a simple and reliable method using HPLC-ELSD attached to a carbohydrate column for the simultaneous quantitative determination of major ginseng saponins, namely ginsenosides Rh_1, Rg_2, Rg_3, Rg_1, Rf, Re, Rd, Rb_2, Rc, and Rb_1 (Fig. 1).

As mentioned previously, a variety of methods for distinguishing the cultivation area of *P. ginseng* were developed, which included the qualitative and quantitative analyses of standard components of the ginseng roots using GC or HPLC or distinction based on the appearance or the smell of the roots. However, those classical methods have limitations with regard to precise discrimination and very often lead to misidentifications, because such botanical and chemical characteristics easily vary according to climate, soil, or cultivation technique. Recently, PC analysis (PCA) using multivariate data obtained from MS, GC, HPLC, IR, or NMR experiments has been developed to discriminate ginseng roots cultivated under different conditions. Because PCA is an unsupervised clustering method that reduces the dimensionality of multivariate data while preserving most of the variance, without requiring any knowledge of the data set [12], it can be used as a fingerprinting tool for authentication of different natural products. As a result, this study was initiated to discriminate the cultivation area of ginseng through a PCA experiment using ginsenoside profile data from a HPLC chromatogram. Ganghwa in Incheon and Punggi in Gyeongsangbuk-do provinces were selected as the representative cultivation areas in the central and southern districts of Korea, respectively. Both of these Korean ginsengs are frequently mistakenly mislabeled and/or confused in the herbal markets. Therefore, it is necessary to establish a reliable technique to distinguish these two herbal drugs in order to indicate cultivation origin. PCA is an unsupervised pattern recognition technique, a data visualization method useful for rapid means of visualizing similarities



20(S)-protopanaxatriol type		
Ginsenoside	R_1	R_2
Rg1	Glc	Glc
Re	Glc^2-Rha	Glc
Rf	Glc^2-Glc	H
Rh1	Glc	H
Rg2	Glc^2-Rha	H

20(S)-protopanaxadiol type		
Ginsenoside	R_1	R_2
Rb1	Glc^2-Glc	Glc^6-Glc
Rc	Glc^2-Glc	Glc^6-Ara(f)
Rb2	Glc^2-Glc	Glc^6-Ara(p)
Rd	Glc^2-Glc	Glc
Rg3	Glc^2-Glc	H

Fig. 1. Chemical structures of the major ginsenosides isolated from the root of *Panax ginseng*. Glc, glucopyranose; Rha, rhamnopyranose; Ara(f), arabinofuranose; Ara(p), arabinopyranose.

or differences within multivariate data [12]. Thus, this paper describes the procedures of the HPLC experiment for the simultaneous quantitative determination of more than ten ginseng saponins and the statistical tool of PCA based on multiple-compound characterization regarding the HPLC analysis data of several ginsenosides to discriminate the origins of ginseng roots cultivated in Ganghwa and Punggi.

MATERIALS AND METHODS

Ginseng samples

The fresh ginseng roots were collected from herbal markets in Ganghwa, Incheon, and Punggi, Gyeongsangbuk-do, Korea in October, 2008. Each sample set included 20 four-year-old roots (G4, 1-10 for Ganghwa; P4, 1-10 for Punggi), and 20 six-year-old roots (G6, 1-10 for Ganghwa; P6, 1-10 for Punggi), all of which were identified by Dr. Woo-Saeng Kwon, Kyung Hee University, Yongin, Korea. The voucher specimens were deposited in the Natural Products Chemistry Lab, Kyung Hee University, Yongin, Korea. The sample's codes, dry weights, size classification, and collecting locations are listed in Table 1.

Standard samples, chemicals, and reagents

Acetonitrile, methanol, isopropanol, and water were of HPLC grade (Merck, Darmstadt, Germany). All solvents were filtered through 0.45 μm membrane filters before being injected into the HPLC. Ten ginsenosides were isolated and purified from the roots of *P. ginseng* through a series of chromatography procedures in our laboratory, and their structures were elucidated by comparison of spectroscopic data (IR, MS, ^{1}H-NMR, and ^{13}C-NMR) with the literature data: ginsenoside Rh_1 (1) [13]; ginsenoside Rg_2 (2) [13]; ginsenoside Rg_3 (3) [14]; ginsenoside Rg_1 (4) [15]; ginsenoside Rf (5) [16]; ginsenoside Re (6) [16]; ginsenoside Rd (7) [17]; ginsenoside Rb_2 (8) [17]; ginsenoside Rc (9) [17] and ginsenoside Rb1 (10) [18]. The purities of these isolated components were determined to be greater than 98% according to normalization of the peak areas detected via HPLC analysis.

Sample preparation for HPLC analysis

Each root was weighed and dried at 50°C for 12 h after washing. The main roots and lateral roots were subjected to HPLC analysis after removing rhizomes and fine roots. The dried roots were powdered to a homogeneous size using a mill and were sieved through a 40 mesh fil-

Table 1. The ginseng roots collected at Ganghwa and Punggi area

No.	Sample code	Years	Ganghwa area	Fresh weight (g)	Size classification	No.	Sample code	Years	Punggi area	Fresh weight (g)	Size classification
1	G-6-1	6 yr	Gimpo	102	Small	21	P-6-1	6 yr	Anjeong	156	Big
2	G-6-2		Ganghwa	200	Big	22	P-6-2		Anjeong	185	Big
3	G-6-3		Baran	185	Big	23	P-6-3		Sambum	203	Big
4	G-6-4		Ganghwa	211	Big	24	P-6-4		Sambum	62	Small
5	G-6-5		Pochon	147	Small	25	P-6-5		Anjeong	99	Small
6	G-6-6		Gimpo	187	Big	26	P-6-6		Anjeong	110	Small
7	G-6-7		Gimpo	117	Small	27	P-6-7		Anjeong	258	Big
8	G-6-8		Ganghwa	75	Small	28	P-6-8		Sunheung	180	Big
9	G-6-9		Pochon	99	Small	29	P-6-9		Sunheung	75	Small
10	G-6-10		Ganghwa	189	Big	30	P-6-10		Baekdong	85	Small
11	G-4-1	4 yr	Pochon	56	Big	31	P-4-1	4 yr	Anjeong	53	Big
12	G-4-2		Baran	52	Big	32	P-4-2		Anjeong	46	Big
13	G-4-3		Baran	32	Small	33	P-4-3		Buseok	25	Small
14	G-4-4		Baran	69	Big	34	P-4-4		Sunheung	13	Small
15	G-4-5		Gimpo	60	Big	35	P-4-5		Dansan	64	Big
16	G-4-6		Gimpo	25	Small	36	P-4-6		Dansan	23	Small
17	G-4-7		Pochon	32	Small	37	P-4-7		Anjeong	63	Big
18	G-4-8		Pochon	14	Small	38	P-4-8		Anjeong	60	Big
19	G-4-9		Ganghwa	14	Small	39	P-4-9		Sunheung	15	Small
20	G-4-10		Pochon	57	Big	40	P4-10		Baekdong	15	Small

ter. The accurately weighed powder (100 mg) was suspended in 1.5 mL of 80% methanol and was ultrasonically extracted for 1 h at 30°C. The extracted solution was centrifuged at 8,000 rpm for 10 min. One milliliter of the supernatant was evaporated under reduced pressure. Then the concentrated extract was dissolved in 0.6 mL of *n*-butanol and 0.6 mL water. The mixture solution was ultrasonicated at 30°C for 10 min and then centrifuged at 8,000 rpm for 5 min. Then, 0.5 mL of supernatant was filtered through a 0.45 μm syringe filter, and the filtrate was stored at 4°C until analysis.

HPLC- evaporative light scattering detector analysis

The Shimadzu HPLC LC-20A was used for analysis, equipped with a G-1312 binary pump, a 1313 CBM, a CTO-15 column oven, an 1111 UV lamp (Shimadzu, Tokyo, Japan). The detector connected to the HPLC was a Shimadzu ELSD 2000 instrument, and the system was operated using Shimadzu LC-solution software ver. 2.0. Chromatography was performed on a Prevail Carbohydrates ES column (4.6×250 mm, 5 μm; Alltech, Deerfield, IL, USA) set at 50°C. The mobile phases consisted of (A) acetonitrile-water-isopropanol (80:5:15) and (B) acetonitrile-water-isopropanol (60:25:15) using a gradient system. The drift tube temperature of the ELSD was set at 80°C with a nitrogen flow-rate of 30 psi. Aliquots of 15 μL of each sample solution were injected and eluted according to the following program at a flow rate of 0.8 mL/min: 25% of B from 0 to 10 min, 25→50% of B from 10 to 35 min, 51→75 % of B from 35 to 42 min, 75→80% of B from 42 to 60 min, and 80% of B from 60 to 70 min.

Principal component analysis

Recorded peak data were exported to an Excel spreadsheet and analyzed using PCA. The PCA was performed using the S-plus ver. 6.0.3 (TIBCO Software Inc., Somerville, MA, USA) with mean-center preprocessing. Data were visualized using the PCs score and loading plots. Each point on the score plot represented an individual sample, and each point on the loading plot represented the contribution of an individual peak to the score.

RESULTS AND DISCUSSION

The purpose of this study was to establish a method for identifying ginseng roots cultivated in different areas. Several methods for distinguishing the cultivation area of *P. ginseng* have been developed through component analysis using GC or HPLC. Ginsenosides, the ginseng saponins, are the PCs which manifest several of the pharmacological activities of ginseng root [2-4]. The analyses of ginsenosides have been mainly performed using HPLC experiments with an ODS column equipped with UV or RI detectors. However, several factors affecting cultivation such as climate, soil, or cultivation method lead to qualitative as well as quantitative variations in ginsenosides. Therefore, such classical methods using single-dimensional data might produce incorrect information. PCA is an unsupervised pattern recognition tool which uses an *n*-dimensional vector approach to separate samples on the basis of the cumulative correlation of all component data and then identifies the vector that yields the greatest separation between samples [19]. The multivariate data for PCA can be obtained from a variety of spectroscopic experiments such as MS, NMR, HPLC, GC, LC/MS, GC/MS, and IR. In this study, we first attempted to develop a simple and reliable method for the simultaneous quantitative determination of more than ten ginsenosides through HPLC experiments. Next, we aimed to confirm the finger printing data obtained from PCA analysis using the HPLC chromatogram to enable the discrimination of ginseng roots cultivated in different area of Korea.

Ginseng roots were collected from herbal markets in Punggi, Gyungsangbuk-do and in Ganghwa, Incheon. Even though Geumsan, Chungcheongnam-do has the largest ginseng market in Korea, it was excluded for collection of ginseng roots, because few of the ginseng roots sold at the Geumsan market are cultivated in the area, most are imported from numerous regions of Korea. Ganghwa was classified as a representative of the central districts of Korea, while Punggi is one of the southern districts.

Thus far, ODS and SiO_2 columns have mainly been used for HPLC analysis of ginsenosides. However, use of a SiO_2 column usually fails to separate non-polar ginsenosides, that is, ginsenosides Rh_1 or Rh_2, while use of an ODS column fails to separate polar ginsenosides, that is, ginsenosides Ra analogues or Rb_1. The weaknesses of the columns led to the search for more efficient HPLC analysis methods including column resin, solvent-elution system, and peak separation [20,21].

The previously described method using HPLC-ELSD equipped with a carbohydrate column was applied for the simultaneous determination of ten major ginsenosides from the 40 ginseng root samples cultivated in Ganghwa (20 samples) and Punggi (20 samples). This method more clearly separated ginsenosides Rg_1 and Re than when using an ODS column. To analyze the amounts of the ten major ginsenosides, the elution sol-

vent for HPLC was optimized by changing the elution gradient. An acetonitrile-water-isopropanol mixture showed a more powerful separation for all components than did methanol-water or acetonitrile-water mixtures [8,10]. Additionally, the column temperature also significantly affected the chromatographic behavior. The column temperature was tested from 30°C to 60°C, and we found that all of the components were distinctly separated under a column temperature of 50°C. As a result, acetonitrile-water-isopropanol was optimized as the eluting solvent, and the solvent gradient system was determined as described in Materials and Methods. ELSD was used for the detection of ginsenosides due to their poor UV absorption. The operating conditions for ELSD were optimized according to the data computed with the ELSD software: the nebulizing gas flow rate was set at 30 psi, the drift tube temperature was 80°C, and a gain value of 5 were used as the optimum parameters. Under these HPLC conditions, each ginsenoside was so clearly resolved and separated that this method could be used for the analysis of ginsenosides in ginseng root samples. The identity of each peak in the Ganghwa and Punggi samples was confirmed by comparison of retention times and ELSD spectra of each peak with those of the ten major ginsenosides (Fig. 2).

Next, the HPLC-ELSD profiles were used for the assessment of substantial compositional similarity between the ginseng roots collected at Ganghwa (4 and 6 years old) and Punggi (4 and 6 years old) (Fig. 3). Ten major ginsenoside peaks were identified in the chromatogram by comparing the individual ELSD spectra and retention times. Accordingly, the profiles of ten ginsenosides identified from ginseng roots were subjected to PCA analysis to identify the production area of the ginseng root. The results are indicated by the PCs score plotting (Fig. 4). The first PC1 contains the most variance in the data. The second PC2 is orthogonal to PC1 and represents the maximum amount of variance not explained by PC1. The two highest ranking PCs, PC1 and PC2, described 43.6% and 18.0% of the total variability in the original observations, respectively, and consequently both PCs accounted for 61.6% of the total variance. These values were acknowledged as acceptable and contained the chemically relevant variance which was informative for cluster samples. Examination of the scores and loading plots for PC1 versus PC2 (Fig. 4) showed the ability to distinguish between the ginseng roots collected at Ganghwa and Punggi. The scores plot (Fig. 4A) demonstrated that PCA of HPLC-ELSD data for ginsenosides obtained from Ganghwa and Punggi cultivated ginseng roots sepa-

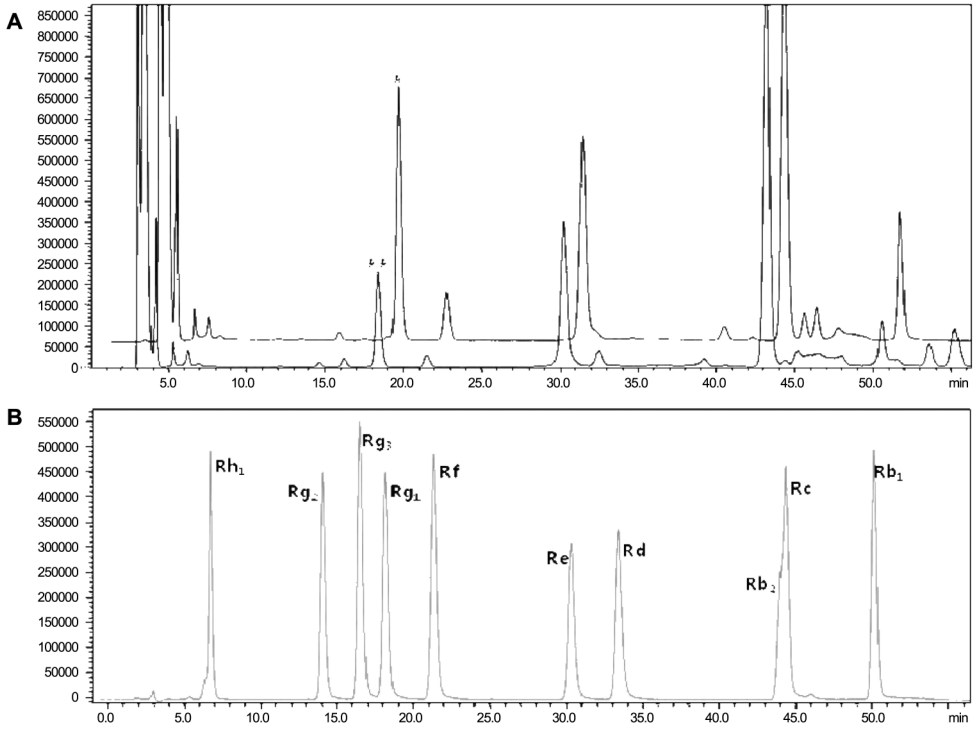


Fig. 2. HPLC chromatograms for alcohol extracts from the ginseng roots cultivated in Ganghwa (*) and Punggi (**) (A), and the mixture of ginsenosides isolated from the ginseng roots (B). Column, Carbohydrate ES column (4.6 mm×250 mm, 5 μm); detector, evaporative light scattering detector.

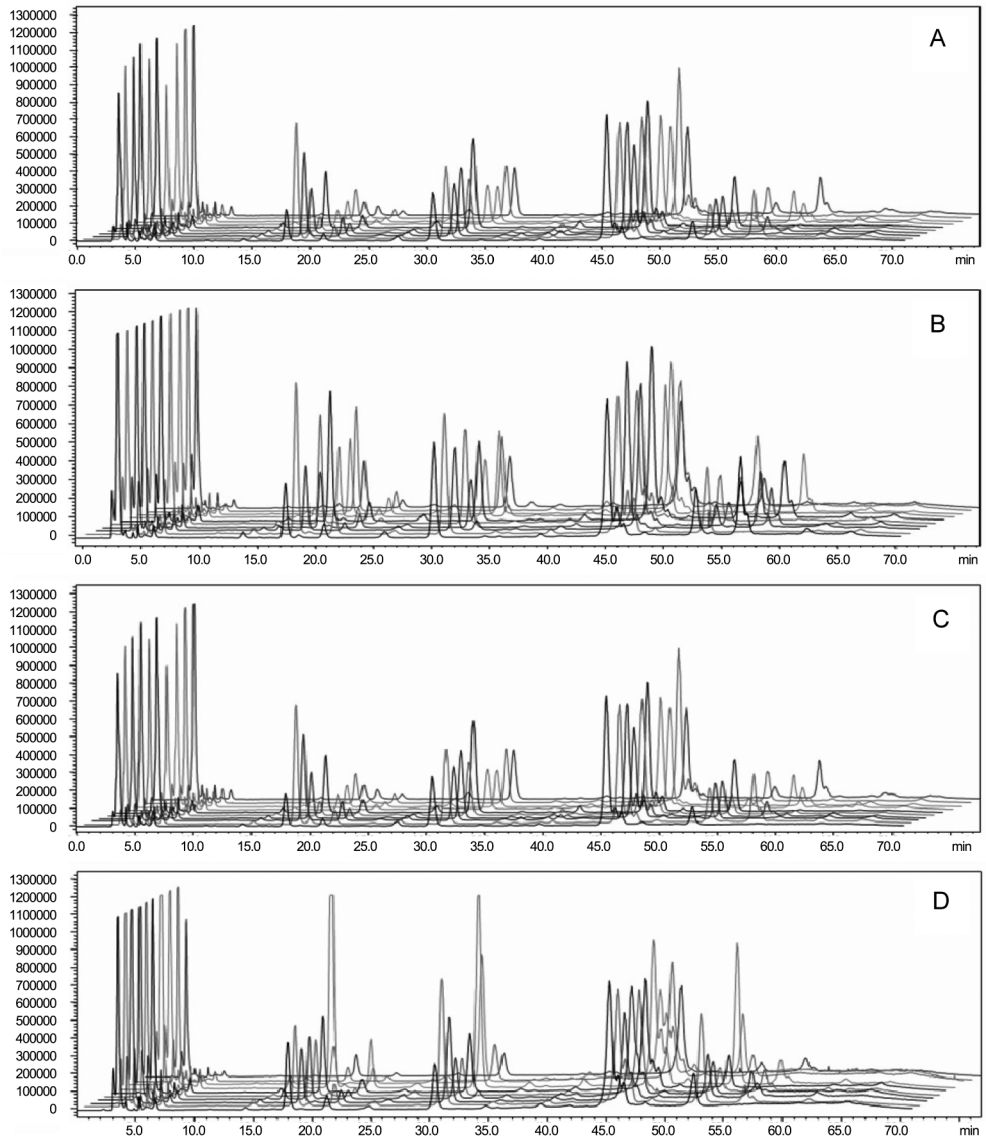


Fig. 3. HPLC chromatograms for alcohol extracts of the ginseng roots collected at Ganghwa and Punggi. (A) Extracts of Ganghwa ginseng (6-years roots; G6, 1-10). (B) Extracts of Punggi ginseng (6-years roots; P6, 1-10). (C) Extracts of Ganghwa ginseng (4-years roots; G4, 1-10). (D) Extracts of Punggi ginseng (4-years roots; P4, 1-10). All HPLC data were duplicated experiments.

rated the samples into two groups according to PC2. A group belonging to Ganghwa-cultivated ginseng roots was clustered by positive values of PC2, while another group belonging to Punggi-cultivated ginseng roots was separated by negative values of PC2. To investigate the contribution of the PCs of the PCA, the metabolic loading bar plot of PC1 and PC2 was evaluated and showed the distribution of metabolites in the form of retention time and ELSD chromatogram relative to the PCs. As is clearly shown in Fig. 4B, ginsenoside Rg_3 contributed the most to PC1, and ginsenoside Rf was the most inversely correlated with PC2. In particular, PCA scores plot for the small ginseng roots of six-years, each of which was light than 147 g in fresh weight, showed more distinct discrimination. PC1 influenced the separation between different sample sets, explaining 51.8% of the variance, while PC2 affected differentiation, also capturing 28.0% of the variance. A group belonging to Ganghwa-cultivated ginseng roots was also clustered according to the positive values of PC2, while another group belonging to Punggi-cultivated ginseng roots was separated based on the negative values of PC2. The highest contribution components were ginsenoside Rf for PC1 and RG_2 for PC2, respectively.

In conclusion, the newly established HPLC analysis methods including the use of HPLC-ELSD using a

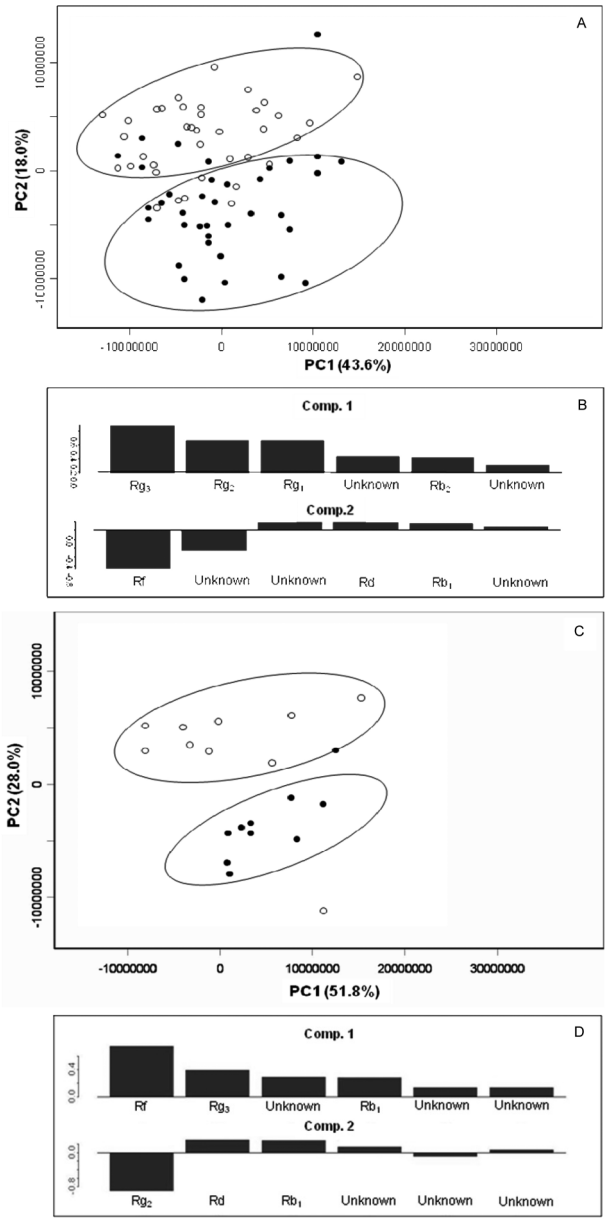


Fig. 4. Score plot (A, all ginseng roots; C, small 6-years ginseng roots lighter than 147 g of fresh weight) and loading bar plot (B, all ginseng roots; D, small 6-years ginseng roots lighter than 147 g of fresh weight) for principal components (PCs) analysis using HPLC profiles data obtained from the ginseng roots of Ganghwa (empty circle) and Punggi (black circle) area. The percentages of PC1 and PC2 in score plot represent the respectively capturing fractions of total variance. The height of each bar in loading bar plot show contributes of each metabolite to PC1 or PC2 components.

carbohydrate column method had for a more powerful separation to evaluate the quality and quantity of ginsenosides in *P. ginseng*. PCA was proven to be a useful statistical tool for the characterization of ginseng roots cultivated in different areas based on their metabolites. These results are probably due to the different influence resulting from a varied geographical origins, climate and cultivation. In summary, HPLC-ELSD coupled with PCA analysis could be a well-accepted strategy for differentiating ginseng roots produced in different areas of Korea.

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