

## HPLC-based metabolic profiling and quality control of leaves of different *Panax* species

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Leaves from *Panax ginseng* Meyer (Korean origin and Chinese origin of Korean ginseng) and *P. quinquefolius* (American ginseng) were harvested in Haenam province, Korea, and were analyzed to investigate patterns in major metabolites using HPLC-based metabolic profiling. Partial least squares discriminant analysis (PLS-DA) was used to analyze the HPLC chromatogram data. There was a clear separation between *Panax* species and/or origins from different countries in the PLS-DA score plots. The ginsenoside compounds of Rg1, Re, Rg2, Rb2, Rb3, and Rd in Korean leaves were higher than in Chinese and American ginseng leaves, and the Rb1 level in *P. quinquefolius* leaves was higher than in *P. ginseng* (Korean origin or Chinese origin). HPLC chromatogram data coupled with multivariate statistical analysis can be used to profile the metabolite content and undertake quality control of *Panax* products.

**Keywords:** *Panax* leaves, Ginsenosides, HPLC, Metabolic profiling, Quality control

### INTRODUCTION

Ginsengs, which are the root of *Panax ginseng* Meyer (Araliaceae), *P. japonicus* Meyer, *P. quinquefolius*, and *P. notoginseng* are widely used as a medicinal herb around the world. Ginseng roots are reported to contain carbohydrates, fatty acids, amino acids, alkaloids, polysaccharides, sesquiterpenes, polyacetylenes, phenolic compounds, and triterpene saponins [1,2]. Ginsenosides are the major components with biological and pharmacological importance, of described benefits to the immune [3], cardiovascular [4], and central nervous systems [5,6], and anti-diabetic [7-9], anti-tumor [10,11] and antioxidant properties [12,13]. Its main constituents are dammarane-type glycoside, such as triterpenes, protopanaxadiol, and protopanaxatriol. The ginsenosides Rb1, Rb2, Rb3, Rc,

Rg3, Rh2, Rc, and Rd are classified as the protopanaxadiols group, and Re, Rf, Rg1, Rh1, and Rg2 are classified as the protopanaxatriol group [14].

Metabolomics can be used to identify and quantitatively analyze all low molecular weight metabolites in a cell, tissue, organism or biological system [15,16]. Additionally, metabolomics analyses have been applied in several fields, including quality control of crops, plant breeding assessment, food assessment, and food safety [16]. Herbal medicine metabolomics research has developed rapidly as an important field in medicinal plant research. Metabolomics analysis for herbal medicine has increased quality control and allowed to differentiate among samples harvested in different seasons and areas,

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composed of different plant parts, and/or grown in different soil types.

Chromatography, such as LC and GC, is recommended for the quality control of medicinal plants [17,18]. Polar, thermo-stable, and non-derivatization samples can be analyzed by HPLC; it can also be used in plant, clinical diagnosis, and biomarker discovery [19]. Previous studies have conducted fingerprinting or metabolic profiling of ginseng root by the separation of different ages [20,21] or the discrimination of different *Panax* species [22,23]. However, there are no reports on metabolic differentiation and prediction of different *Panax* species using leaf samples from varieties grown in the same region.

In this study, we examine metabolic profiling and quality control of *Panax* species leaves with *P. ginseng* (Korean origin and Chinese origin), and *P. quinquefolius* (American ginseng) cultivated in Haenam in Korea, us-

ing HPLC chromatography and multivariate statistical analysis.

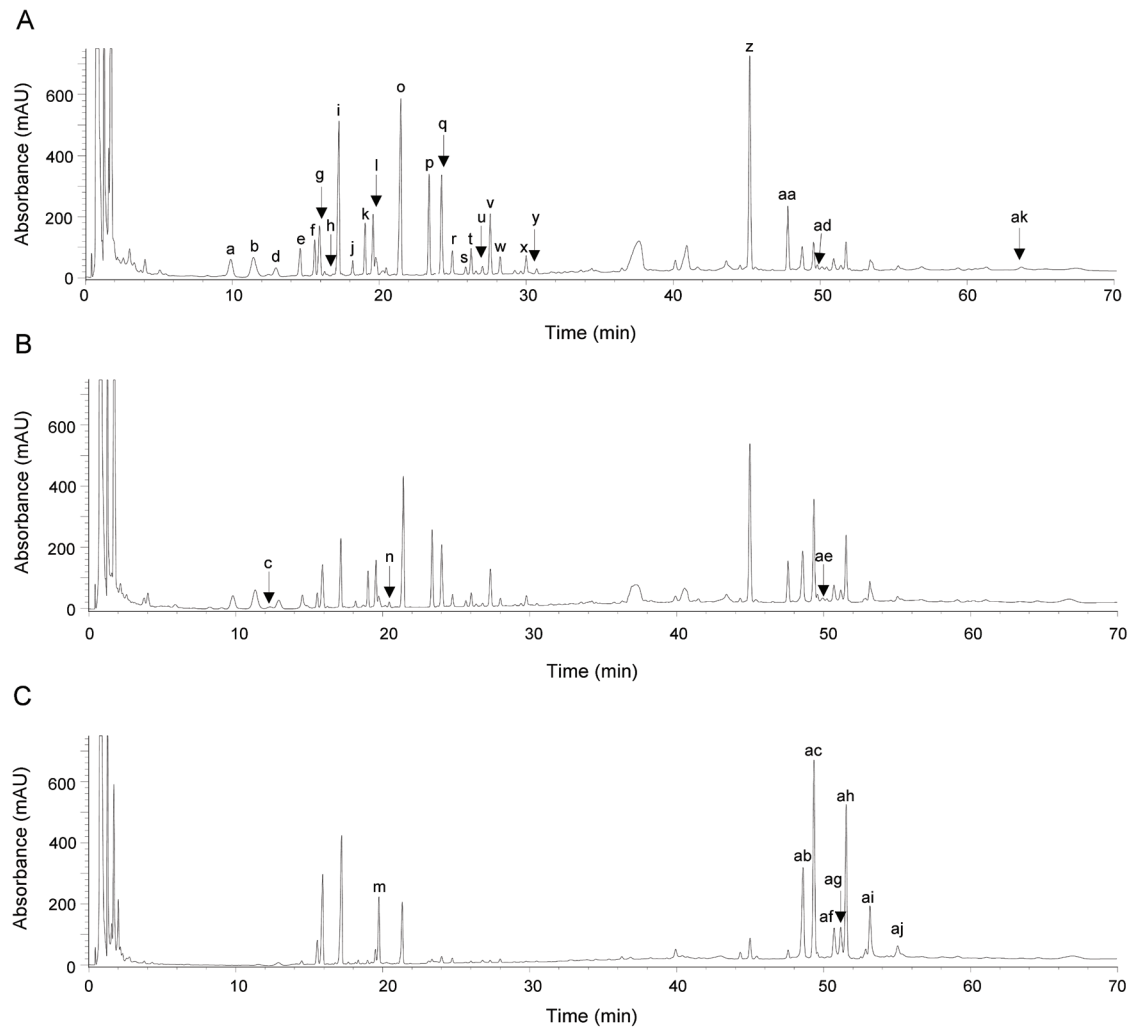
## MATERIALS AND METHODS

### Solvents and chemicals

The standards of ginsenoside Rg1, Re, Rf, Rg2, Rb1, Rc, Rb2, Rb3, and Rd were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile and methanol were obtained from JT Baker (Phillipsburg, NJ, USA) and SK Chemicals (Ulsan, Korea), respectively.

### Plant materials

One-year-old roots of *P. ginseng* Meyer (Korean origin and Chinese origin) and *P. quinquefolius* (American ginseng) were planted and allowed to grow for 4 yr.



**Fig. 1.** Representative HPLC chromatogram of the methanol extracts of fresh leaves of the three different *Panax* varieties. (A) *P. ginseng* (Korean origin), (B) *P. ginseng* (Chinese origin), and (C) *P. quinquefolius*. a, Rg1; b, Re; d, Rf; e, Rg2; g, Rb1; i, Rc; k, Rb2; l, Rb3; o, Rd.

The 5-year-old roots grown in Haenam province (GPS: E 126°45'74" N 34°62'34"), Korea were then studied. *Panax* origin and/or species were kindly obtained from the Department of Medicinal Crop Research of Rural Development Administration. The fresh leaves (10 leaves per species) of 5-year-old roots of *P. ginseng* (Korean origin and Chinese origin), and *P. quinquefolius* were collected from Haenam in Korea during the growing season of July 2007. The center leaflet of the five part compound leaf was selected. The leaf samples were powdered and stored in a -70°C freezer until they were used for analysis.

### Sample preparation and extraction

The selected center-part leaves were put in 15 mL centrifuge tubes (Corning, Corning, NY, USA), and extraction was conducted with 25 mL of 99.8% methanol at room temperature for 24 h. After shaking each tube, 5 mL from each tube was filtered by polyvinylidenedifluoride syringe filter (0.45 µm; Whatman, Piscataway, NJ, USA) in preparation for HPLC.

### HPLC analysis

Methanol extracts of the leaves were separated and identified using HPLC equipped with an ultraviolet detector (Agilent 1100 series; Agilent Technologies, Santa Clara, CA, USA). Separation of the samples was achieved using a 5-µm Capcell Pak C<sub>18</sub> MGII column (150 mm×3.0 mm ID; Shiseido, Tokyo, Japan). The injection volume was 20 µL, and the leaf samples were detected at 203 nm. The mobile phase was composed of 100% acetonitrile (solvent A) and 100% water (solvent B). Linear gradient elution was performed from 30% solvent A and 70% solvent B (*t*=0 min) to 100% solvent A at *t*=70 min. The mobile phase was achieved at 0.8 mL/min<sup>-1</sup> and the column oven temperature was 30°C.

### Statistical analysis

The variables were normalized to 37 variables for partial least squares discriminant analysis (PLS-DA). The PLS-DA was performed with SIMCA-P software ver. 12.0 (Umetrics, Umea, Sweden). Data were analyzed statistically with an ANOVA and Duncan's multiple range test using SPSS ver.18 (SPSS Inc., Chicago, IL, USA). A *p*-value <0.05 was considered significant.

## RESULTS AND DISCUSSION

### HPLC chromatogram and assignment of the peaks in *Panax* leaf samples

Fig. 1 shows the representative HPLC chromatogram

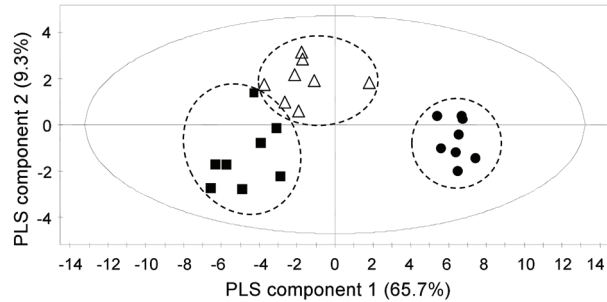
**Table 1.** Assignment of the HPLC data peaks of methanol extracts from fresh *Panax* leaves

No.	Retention time (min)	Factor	Assignment
1	9.895	a	Rg1
2	11.438	b	Re
3	12.456	c	Unknown
4	12.907	d	Rf
5	14.573	e	Rg2
6	15.647	f	Unknown
7	16.072	g	Rb1
8	16.279	h	Unknown
9	17.197	i	Rc
10	18.188	j	Unknown
11	18.981	k	Rb2
12	19.523	l	Rb3
13	19.780	m	Unknown
14	20.460	n	Unknown
15	21.309	o	Rd
16	23.364	p	Unknown
17	24.009	q	Unknown
18	24.745	r	Unknown
19	25.685	s	Unknown
20	26.054	t	Unknown
21	26.825	u	Unknown
22	27.356	v	Unknown
23	28.032	w	Unknown
24	29.809	x	Unknown
25	30.526	y	Unknown
26	44.988	z	Unknown
27	47.583	aa	Unknown
28	48.626	ab	Unknown
29	49.353	ac	Unknown
30	49.574	ad	Unknown
31	49.931	ae	Unknown
32	50.725	af	Unknown
33	51.158	ag	Unknown
34	51.529	ah	Unknown
35	53.130	ai	Unknown
36	55.008	aj	Unknown
37	63.497	ak	Unknown

of the methanol extracts of fresh leaves obtained from the different ginseng species and/or origins. The chromatograms of *P. ginseng* are different from those of *P. quinquefolius*. The 37 peaks selected from the leaf samples include the ginsenosides Rg1, Re, Rf, Rg2, Rb1, Rc, Rb2, Rb3, and Rd that were identified using ginsenoside standards by HPLC. The various peaks of HPLC chromatogram were divided by factor/retention time/assignment (Table 1). Ligor et al. [24] and Shi et al. [25] isolated ginsenosides such as Rg1, Re, Rb1, Rc, Rb2, Rb3, and Rd from ginseng leaves of *Panax* species.

**Metabolic profiling of *Panax ginseng* and *Panax quinquefolius* by HPLC**

To investigate the differences between *P. ginseng* (Korean origin and Chinese origin) and *P. quinquefolius* leaf metabolites, samples were analyzed with the HPLC-based metabolomics technique. PLS-DA is a supervised

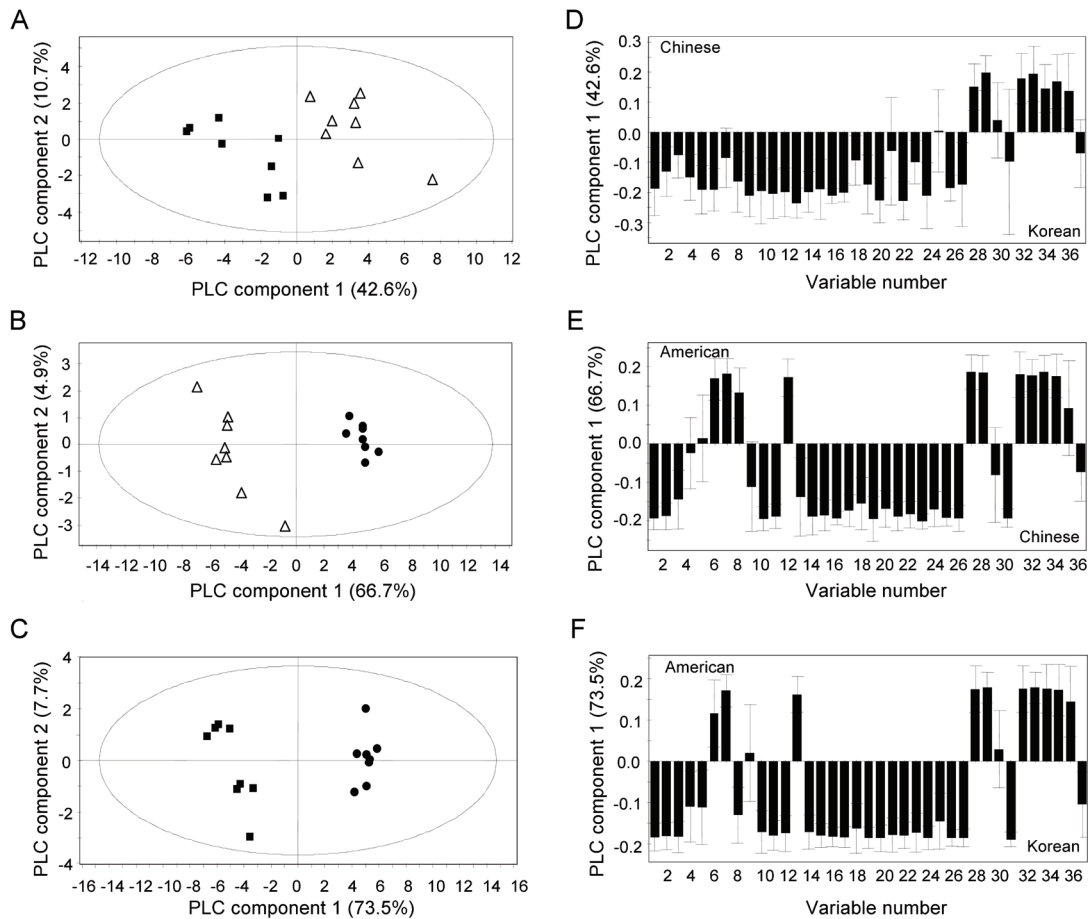


**Fig. 2.** Partial least squares-discriminant analysis (PLS-DA)-derived score plots obtained using HPLC data of methanol extracts from leaf samples. ● *Panax quinquefolius*, △ *P. ginseng* (Chinese origin), ■ *P. ginseng* (Korean origin).

extension of a principal component analysis (PCA) and uses class information to maximize the separation between groups of observations [26]. We conducted the PLS-DA using the processed liquid chromatography data to differentiate samples by metabolic profiles. The chromatography data were mean-centered and scaled to Pareto by SIMCA-P 12.0.

We excluded two outliers from the preliminary PCA of the 10 samples of each species and/or origins (data not shown); therefore PLS-DA was performed using the 8 remaining samples for each species. Fig. 2 shows the PLS-DA-derived score plots of *P. ginseng* and *P. quinquefolius* leaf samples. Korean origin, Chinese origin of *P. ginseng*, and *P. quinquefolius* samples were clearly separated by PLS components 1 and 2. The two PLS components accounted for 74% of the total variance.

Fig. 3A to 3C shows the PLS-DA score plots (PLS components 1 and 2) derived from the HPLC data. Model fit was evaluated using the  $R^2$  and  $Q^2$  parameters,



**Fig. 3.** Partial least squares-discriminant analysis (PLS-DA) score (A-C) and loading (D-F) plots derived from the HPLC data, demonstrating the separation between leaves from *Panax ginseng* (Chinese origin) and *P. ginseng* (Korean origin) (A,D), *P. quinquefolius* and *P. ginseng* (Chinese origin) (B,E), and *P. quinquefolius* and *P. ginseng* (Korean origin) (C,F). ● *P. quinquefolius*, △ *P. ginseng* (Chinese origin), ■ *P. ginseng* (Korean origin).

both of which vary between 0 and 1.  $R^2$  provides an indication of goodness of fit of the model, and  $Q^2$  provides an indication of predictive ability [26]. The PLS-DA models comparing *P. ginseng* (Chinese origin) and *P. ginseng* (Korean origin), *P. quinquefolius* and *P. ginseng* (Chinese origin), and *P. quinquefolius* and *P. ginseng* (Korean origin) had  $R_x^2$ ,  $R_y^2$ , and  $Q^2$  values of 0.62, 0.93 and 0.65; 0.74, 0.99, and 0.93; and 0.85, 0.99, and 0.96, respectively. The PLS-DA score plot comparing Chinese origin and Korean origin showed clear separation along component 1 (Fig. 3A). Loading scores provide the correlation between the original variables and the new component variables [26]. The loading plot of leaf extracts from *P. ginseng* (Chinese and Korean origins) showed that Korean origin leaves contained more Rg1, Re, Rf, Rg2, Rb1, Rc, Rb2, Rb3, and Rd compounds (Fig. 3D). Clear separation between leaf extracts of *P. quinquefolius* and *P. ginseng* (the Chinese) was observed (Fig. 3B). Comparison of leaf extracts from *P. quinquefolius* and *P. ginseng* (the Chinese) revealed that *P. ginseng* (the Chinese) had higher levels of Rg2, Rb1, and Rc and lower levels of Rg1, Re, Rf, Rc, Rb2, Rb3, and Rd (Fig. 3E). The PLS-DA score plot between leaf extracts of *P. quinquefolius* and *P. ginseng* (the Korean) also showed a clear separation (Fig. 3C). The PLS-DA loading plot showed that Korean origin had higher levels of Rb1 and Rc but lower levels of Rg1, Re, Rf, Rg2, Rb2, Rb3, and Rd in comparison to *P. quinquefolius* (Fig. 3F).

The variable influence on projection (VIP) parameters was used to identify the components that play important roles in separation. Previous studies recommend a cut-off for VIP values of approximately 0.7 to 0.8 for variable selection, even though the variables with VIP values greater than 1 were the most influential for the model [26]. As shown in Table 2, the VIP values of the major contributing compounds for the separation found in the score plots from PLS-DA (Fig. 2) were as follows: Rc, 1.522; Rg2, 1.336; Rf, 1.156; Rb1, 1.134; Rg1, 0.921; Re, 0.918; Rb2, 0.876; Rd, 0.869; and Rb3, 0.867.

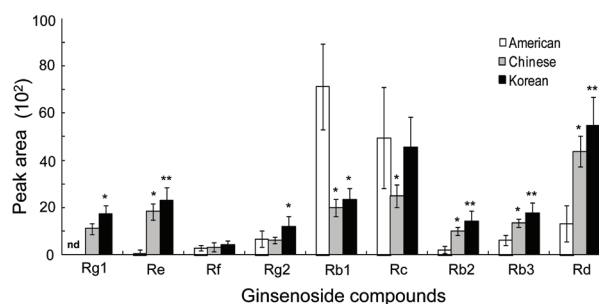
To obtain clear information on the relative levels of each compound from the VIP analysis, we performed an ANOVA as shown in Fig. 4. The levels of Rg1, Re, Rg2, Rb2, Rb3, and Rd were significantly higher ( $p < 0.05$  in all cases) in *P. ginseng* leaves (the Korean), and Rb1 was significantly higher in *P. quinquefolius* leaves.

Xie et al. [22] analyzed root samples from various species of *Panax* for ginsenoside distribution using HPTLC; in this study, Rg1 in *P. ginseng* was higher than it in *P. quinquefolius*, whereas Rb1 was higher in *P. quinquefolius* than in *P. ginseng*. The results of the present

**Table 2.** The VIP values of the major contributing compounds to the separation as shown by the score plots from the PLS-DA model

VIP values	Compounds	Retention time (min)
1.522	Rc	17.197
1.336	Rg2	14.573
1.156	Rf	12.907
1.134	Rb1	16.072
0.921	Rg1	9.895
0.918	Re	11.438
0.876	Rb2	18.981
0.869	Rd	21.309
0.867	Rb3	19.523

VIP, variable influence on projection; PLS-DA, partial least squares discriminant analysis.



**Fig. 4.** Peak areas of Rg1, Re, Rf, Rg2, Rb1, Rc, Rb2, Rb3, and Rd in methanol extracts of leaf samples. ANOVA was performed to compare values, and the statistical significance was assessed with Duncan's post hoc test ( $p < 0.05$ ). The error bars show standard deviation ( $n=8$ ). Each superscript letter (\* and \*\*) in the same row represents significant difference at  $p < 0.05$ . nd, not detected.

study on leaves are similar to the findings of Xie et al. for ginseng root and are expected to provide the indicator identifying the species as analyzing leaves of species.

In the present study, the chemical metabolites of leaves from different species and/or origins grown in the same region (Haenam, Korea) were analyzed with HPLC coupled with multivariate data analysis. Metabolomics approaches were used to develop predictive models to identify the species and/or origins of samples from various *Panax* species. In addition, metabolomic approaches can be used to separate *Panax* species using leaves to avoid damaging the roots. This approach will be useful in understanding the metabolic profile of leaves in *Panax* species and can be used to differentiate between species of *Panax* based on location of origin and cultivation environment.

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