Research article

C/N/O/S stable isotopic and chemometric analyses for determining the geographical origin of *Panax ginseng* cultivated in Korea

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ABSTRACT

Background: The geographical origin of *Panax ginseng* Meyer, a valuable medicinal plant, is important to both ginseng producers and consumers in the context of economic profit and human health benefits. We, therefore, aimed to discriminate between the cultivation regions of ginseng using the stable isotope ratios of C, N, O, and S, which are abundant bioelements in living organisms.
Methods: Six Korean ginseng cultivars (3-yr-old roots) were collected from five different regions in Korea. The C, N, O, and S stable isotope ratios in ginseng roots were measured by isotope ratio mass spectrometry, and then these isotope ratio profiles were statistically analyzed using chemometrics.
Results: The various isotope ratios found in *P. ginseng* roots were significantly influenced by region, cultivar, and the interactions between these two factors ($p \leq 0.001$). The variation in $\delta^{15}N$ and $\delta^{13}C$ in ginseng roots was significant for discriminating between different ginseng cultivation regions, and $\delta^{18}O$ and $\delta^{34}S$ were also affected by both altitude and proximity to coastal areas. Chemometric model results tested in this study provided discrimination between the majority of different cultivation regions. Based on the external validation, this chemometric model also showed good model performance ($R^2 = 0.853$ and $Q^2 = 0.738$).
Conclusion: Our case study elucidates the variation of C, N, O, and S stable isotope ratios in ginseng root depending on cultivation region. Hence, the analysis of stable isotope ratios is a suitable tool for discrimination between the regional origins of ginseng samples from Korea, with potential application to other countries.

1. Introduction

Because of a growing consumer interest in health, the demand for information relating to food authenticity, origin, growth methods, and processing techniques has also increased. In particular, significant interest has been paid to discriminate between different geographical origins of medicinal herbal plants [1]. For example, ginseng, a perennial plant belonging to the *Panax* genus, is a traditional medicinal plant used to protect against several diseases and illnesses [2,3]. Among the 13 reported species of ginseng in the *Panax* genus, *Panax ginseng* Meyer is usually recognized as the most important, because of its significant health benefits [2].

Indeed, numerous bioactive compounds such as ginsenosides, polysaccharides, polyacetylenes, and vitamins found in ginseng root have been associated with its reported health benefits [4,5]. However, as the composition and content of bioactive compounds in ginseng root are significantly affected by the environment of its cultivation region, the geographical origin of the plant is a key parameter in determining the quality of ginseng root and its corresponding market selling price [6,7]. As such, ginseng root of high pharmacological quality tends to originate from specific origins, thus influencing the inclination of customers to purchase these products [1]. For example, the selling price of Korean ginseng is usually higher than that of Chinese ginseng sold in Korea [2,3].

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Hence, because of the enormous market size of ginseng as an herbal medicine, it has long been the subject of falsification in the context of geographical origin, yielding unfair economic profits for producers and merchants [8]. Therefore, accurate discrimination between different geographical origins of ginseng is very important for both ginseng producers and customers. To date, the majority of studies regarding authentication of the geographical origin of traditional Chinese medicinal plants has focused mainly on the determination of chemical compositions and on morphological inspections (i.e., shape, size, color). However, these methods tend to lack reproducibility, mainly because of variable analyst proficiency, annual variations of the targeted chemicals, and differing environmental or cultivation conditions [8−10].

Thus, to accurately discriminate between the geographical origins of different ginseng roots, an analytical approach based on mass spectrometry or spectroscopy has been reported [1,2,8]. In this approach, the primary metabolites (e.g., amino acids, organic acids, and simple sugars), various abundant elements (e.g., Mg, Fe, Al, and Sc), rare earth elements (e.g., La, Yb, and Ce), and strontium isotope ratios (i.e., $^{87}Sr/^{86}Sr$) were successfully differentiated between Korean and Chinese ginseng roots [1,2,8]. In addition, the stable isotope ratios of common bioelements such as H, C, N, O, or S have also been used to discriminate between different geographical origins of foods, as these ratios are influenced by parameters such as climate, water availability, coastline proximity, altitude, latitude, soil properties, agricultural practice, and anthropogenic activity close to cultivation regions. For example, the H isotope ratio in ginseng root is a potential marker to discriminate between Korean and Chinese ginseng [3], and H, N, and O isotope ratios have also been used to discriminate the geographical origin of raw American ginseng root and its tablet form [11]. Moreover, analysis of the stable isotope ratios of H, C, N, O, or S has been successfully applied to the geographical origin discrimination of various foodstuffs such as cereals [12−15], potatoes [16,17], honey [18], and milk [19].

However, despite the recognized high quality of Korean ginseng and its resulting popularity, few studies have examined the discrimination between different cultivation regions within Korea using stable isotopic compositions present in ginseng roots. As such, we herein report our preliminary investigation into the variation of C, N, O, and S stable isotope compositions in ginseng roots with respect to ginseng cultivars and cultivation regions in Korea, where high-quality ginseng root is typically produced. In addition, multivariate analysis, such as principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA), were performed to allow clear discrimination between cultivation origins based on the stable isotopic compositions present in ginseng. We expect that preliminary results will demonstrate the feasibility of our authentication technique to differentiate between ginseng root obtained from different cultivation regions within Korea, with the ultimate aim of preventing the fraudulent labeling of ginseng products, and potential broader application to ginseng root samples from other countries.

2. Materials and methods

2.1. Ginseng materials

Six Korean ginseng cultivars (cv)—cv. Gopung, cv. Geumpung, cv. Sunwoon, cv. Yeonpung, cv. Cheonpung, and cv. K-1—were cultivated from five different regions in Korea since March 2013. Details regarding the agricultural practices applied for the cultivation of these samples are provided in Table 1. To control the physical and chemical properties of the soil, the field used for ginseng cultivation had been subjected to rye and sudangrass cultivation/decomposition over 2 yr prior to transplantation of the 1-yr-old ginseng seedlings. In addition, an organic press cake fertilizer was applied at a level of 600 kg/1,000 m² prior to transplantation. The experimental field area for ginseng cultivation was 200 m² for each region, and a planting density of 15 cm × 20 cm (i.e., the space between the rows × the space between ginseng plants in a row) was used. No synthetic chemical fertilizers were applied during the ginseng cultivation period. Other agricultural practices, such as soil management, disease/insect/pest control, and physiological disorder control, were used according to the standard Good Agricultural Practice ginseng cultivation method [20]. Following cultivation, 3-yr-old ginseng roots were obtained in triplicate for each cultivation region in September 2014. Each replicate was composed of at least seven ginseng roots and was lyophilized (≤−40°C for 3 d), pulverized, and pooled in preparation for analysis by isotope ratio mass spectrometry (IRMS). The pooled ginseng powder samples were stored at −70°C and were relyophilized prior to IRMS analysis.

2.2. IRMS measurements

The pulverized ginseng samples were weighed into a tin capsule (3.5 mm × 17 mm; IVA Analysentechinik e.K., Dusseldorf, Germany) for measurement of the C, N, and S stable isotope ratios ($\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$, respectively), whereas a silver capsule (3.5 mm × 5.0 mm; Elemental Microanalysis, Okehampton, UK) was used for determination of the O stable isotope ratio ($\delta^{18}O$). The quantities of ginseng powder required for each measurement were as follows: $\delta^{13}C$ and $\delta^{15}N$, 5 mg; $\delta^{18}O$, 0.2 mg; and $\delta^{34}S$, 20 mg. The encapsulated samples were stored in a desiccator prior to measurement [12].

The conditions used for IRMS analysis were previously reported in detail [12]. A PDZ Europa 20-20 IRMS (Sercon Ltd., Cheshire, UK) linked to a PDZ Europa ANCA-GSL elemental analyzer (Sercon Ltd.) was used for $\delta^{13}C$ and $\delta^{15}N$ analysis, whereas an Elementar Pyro-Cube (Elementar Analysensysteme GmbH, Hanau, Germany) equipped with a PDZ Europa 20-20 IRMS was used for $\delta^{18}O$ analysis, and an Elementar vario ISOTOPE cube linked to a continuous-flow SerCon 20-22 IRMS (Sercon Ltd.) was used for $\delta^{34}S$ analysis. In addition, two to three laboratory reference materials, which were compositionally related to the ginseng root samples, were analyzed simultaneously to monitor and correct any variations in drift or linearity. The final $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ values in the ginseng samples were reported in parts per thousand (‰) relative to internationally established reference standards, namely, Vienna Pee Dee Belemnite (VPDB) for carbon, atmospheric N_2 (air) for nitrogen, Vienna Standard Mean Ocean Water (VSMOW) for oxygen, and Vienna Canyon Diablo Troilite (VCDT) for sulfur [21]. The analytical precision (± standard deviation) based on the laboratory reference materials used herein was ≤±0.1‰ for $\delta^{13}C$ and $\delta^{15}N$ (bovine liver, nylon 5, peach leaves), ≤±0.4‰ for $\delta^{18}O$ (cellulose, alanine, nylon), and ≤±0.5‰ for $\delta^{34}S$ (hair standard, Mahi-Mahi muscle, whale baleen, taurine).

2.3. Statistical analyses

Fisher's least significant difference test was conducted at the 0.05 probability level using the general linear model of the statistical analysis program SAS (version 9.2; SAS Institute Inc., Cary, NC, USA). Two-way analysis of variance was also performed to examine the interaction (ginseng cultivar × cultivation region) between the main variables on the values of $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$. In addition, chemometric approaches, such as PCA and PLS-DA, were conducted to discriminate between different ginseng geographical origins based on the obtained $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ values. The isotopic data acquired by IRMS were scaled to unit variance prior to multivariate analysis, and were then subjected to PCA (SIMCA-P

Table 1
Brief overview of the ginseng cultivation regions and conditions

Cultivation region	Cheorwon	Eumseong	Jinan	Punggi	Pyeongchang	Geographic origin
Latitude, °N	38°23′	36°94′	35°59′	36°80′	37°70′	Cheorwon, Pyeongchang, Punggi, Eumseong, Jinan (map)
Longitude, °E	127°33′	127°75′	127°16′	128°53′	128°57′	
Altitude, m a.s.l.	306 m	185 m	402 m	212 m	558 m	
Distance from nearest coast, km	~103 km (from East)	~79 km (from West)	~52 km (from West)	~82 km (from East)	~36 km (from East)	
Cultivation area, m²	200 m²	200 m²	200 m²	200 m²	200 m²	
Cultivation density, cm (between rows × between ginseng plants in a row)	15 cm × 20 cm	15 cm × 20 cm	15 cm × 20 cm	15 cm × 20 cm	15 cm × 20 cm	
Cultivation soil type	Upland	Upland	Upland	Upland	Upland	
Other agricultural practice managements	• The ginseng preplanting field management prior to the 1-yr-old ginseng seedling transplantation: rye and sudangrass cultivation/decomposition for 2 yr + a press cake application at the level of 600 kg per 1,000 m². • No microelements application for the ginseng cultivation period. • Disease and insect pest control: pesticides registered (i.e., metalaxyl, polyoxin B, cadusafos, dazomet, azoxystrobin) have been applied with the standard Good Agricultural Practice (GAP) ginseng farming method issued by the Rural Development Affairs, 2007. • Other agricultural managements including some physiological disorders or environmental disaster control occurred for the ginseng cultivation period was followed to the standard GAP ginseng farming method.					
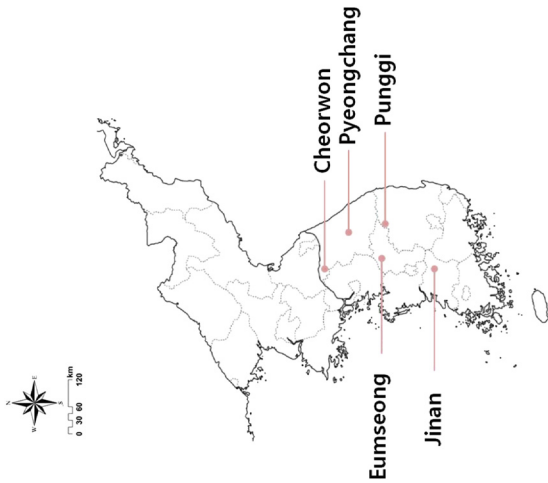
version 13.0; Umetrics, Umeå, Sweden) to evaluate the differences among the groups of multivariate data. The PCA and PLS-DA outputs consisted of score plots to visualize the contrast between different samples and loading plots to account for the cluster separation. Three-dimensional (3D) PCA and PLS-DA score plots were also constructed to improve the discrimination model for the geographical origin of ginseng root.

3. Results and discussion

3.1. Variation of $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ in ginseng root based on cultivation region and ginseng cultivar

We initially examined the different $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ profiles over the various ginseng roots samples according to cultivation regions and ginseng cultivars, and the results are outlined in Table 2. The mean $\delta^{13}C$ value ranged from $-22.0‰$ to $-28.4‰$ as a function of the ginseng cultivation region and cultivar, although higher values were observed in the ginseng samples obtained from the Pyeongchang region ($p < 0.050$). In addition, the mean $\delta^{15}N$ value varied significantly between $0.7‰$ and $9.0‰$, with higher mean $\delta^{15}N$ values being found in ginseng from the Jinan region, and lower mean $\delta^{15}N$ values being found in ginseng from the Eumseong region ($p < 0.050$). Furthermore, the mean $\delta^{18}O$ value of ginseng root ranged from $25.2‰$ to $28.0‰$, and it was found that this value was negatively correlated with the altitude of the ginseng cultivation region ($r = -0.47$, $p < 0.001$). More specifically, higher altitude cultivation regions such as Pyeongchang and Jinan resulted in lower $\delta^{18}O$ values compared to the low altitude regions ($p < 0.050$), likely because of environmental conditions such as precipitation and water irrigation sources. In contrast, higher mean $\delta^{34}S$ values were detected in ginseng root cultivated from higher altitude regions, with the proximity to the coast also appearing to affect the $\delta^{34}S_{VCDT}$ values of the ginseng roots ($r = -0.48$, $p < 0.001$). In this case, the Jinan and Pyeongchang regions yielded higher $\delta^{34}S$ values compared to inland regions ($p < 0.050$). Moreover, the cv. Cheonpung tended to exhibit higher mean $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ values compared to other ginseng cultivars examined herein, and the two-way analysis of variance analysis indicated that the $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ values in ginseng roots were highly influenced by cultivation region, cultivar, and the interactions between the two factors ($p \leq 0.001$, Table 2).

The distribution and variation of $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ values across the examined ginseng roots obtained from the different cultivation regions are outlined in Fig. 1. As shown, the $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ values were statistically different over the majority of cultivation regions ($p < 0.050$); however, the distribution patterns of these values differed significantly. For example, the $\delta^{13}C$ and $\delta^{18}O$ values for ginseng root cultivated in the Jinan region showed a normal Gaussian distribution, despite a few outlier values. However, the $\delta^{15}N$ and $\delta^{34}S$ values for the same region exhibited a nonnormal distribution and wide variations. In general, the $\delta^{18}O$ values showed relatively normal distribution patterns for all regions, whereas the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values were prone to larger variation, in addition to the nonnormal distribution in the Eumseong, Punggi, Jinan, and Pyeongchang regions. These results suggest that the differences in the distributions and variations of the $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ values as a function of cultivation region are strongly associated with the cultivation environments, including soil type, soil nutrition history, light supply, and water source and availability.

Fig. 2 shows the 2D plots of the $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ values of the ginseng root samples obtained from the different cultivation regions, with the aim of discriminating between the various regions. With the exception of the $\delta^{18}O$ and $\delta^{34}S$ combinations, the 2D

plots served as a suitable discriminative tool for the ginseng cultivated in the Jinan and Eumseong regions; however, such clear association was not apparent for the other regions. In addition, it was not possible to distinguish between different ginseng roots as a function of altitude or coastal proximity using these 2D plots.

In general, the measurement of H, C, O, N, and S stable isotope ratios is considered a promising tool for the discrimination of different geographical origins of various agricultural products, owing to natural differences in physical, chemical, or microbial isotopic fractionation processes [22,23]. For example, the metabolic processes taking place in plants (e.g., C3, C4, and Crassulacean acid metabolism (CAM) photosynthesis) can result in differences in $\delta^{13}C$ values. Variation can also occur because of environmental factors, such as water availability, drought stress, nutrient availability, and anthropogenic effects. However, in plants, metabolic activity has a greater effect on the variation of $\delta^{13}C$ than environmental factors [24,25]. For example, ~2‰ variation in $\delta^{13}C$ is normal for agricultural products grown in the same area owing to slight variations in the nutrient and water levels available for plant growth [24]. However, the large $\delta^{13}C$ variation of >2‰ observed for the ginseng

root samples likely results from significant differences in environmental factors, in addition to anthropogenic effects around the cultivation regions [24,26]. In particular, the extremely low $\delta^{13}C$ value of $<-30‰$ could be associated with an increase in the anthropogenic exhaust of CO_2 [3,27].

According to previous studies [3,11,26], the $\delta^{13}C$ values of *P. ginseng* and *Panax quinquefolius* roots obtained from Canada, China, Korea, and the United States varied between $-31.2‰$ and $-22.2‰$, which is similar to the $\delta^{13}C$ variation ($-28.4‰$ to $-22.0‰$) observed for the 3-yr-old *P. ginseng* roots reported herein. These $\delta^{13}C$ distributions also correspond with the known $\delta^{13}C$ range ($-30‰$ to $-22‰$) of C3 plants [24]. Interestingly, the $\delta^{13}C$ value alone was unable to clearly discriminate between the different geographical origins of *P. ginseng* and *P. quinquefolius* [3,11], whereas in our study, the $\delta^{13}C$ values of the various *P. ginseng* root samples did allow the successful discrimination of the geographical origins of ginseng from five different regions in Korea ($p < 0.050$, Table 1). We expect that this may be attributable to the certain differences in cultivation environments associated with ginseng growth in each cultivation region.

Table 2
Comparison of $\delta^{13}C_{VPDB}$, $\delta^{15}N_{AIR}$, $\delta^{18}O_{VSMOW}$, and $\delta^{34}S_{VCDT}$ in ginseng roots depending on ginseng cultivar and cultivation region

Region	Cultivar	$\delta^{13}C_{VPDB}$, ‰	Mean	$\delta^{15}N_{AIR}$, ‰	Mean	$\delta^{18}O_{VSMOW}$, ‰	Mean	$\delta^{34}S_{VCDT}$, ‰	Mean
		$n = 3$ for each cultivar	$n = 18$	$n = 3$ for each cultivar	$n = 18$	$n = 3$ for each cultivar	$n = 18$	$n = 3$ for each cultivar	$n = 18$
Eumseong	Gopung	-27.6 ± 0.03^e	-26.0 ± 1.63^e	2.0 ± 0.06^a	1.5 ± 0.49^e	27.4 ± 0.39^b	27.1 ± 0.65^b	4.7 ± 0.7^b	4.3 ± 1.5^b
	Geumpung	-25.4 ± 0.04^c		0.7 ± 0.01^e		28.0 ± 0.21^a		3.7 ± 0.2^b	
	Sunwoon	-25.7 ± 0.04^d		1.3 ± 0.08^d		27.4 ± 0.21^b		3.4 ± 0.1^b	
	Yeonpung	-24.9 ± 0.16^b		2.1 ± 0.07^a		26.3 ± 0.34^c		3.1 ± 0.1^b	
	Cheonpung	-23.7 ± 0.11^a		1.8 ± 0.03^b		27.1 ± 0.31^b		6.7 ± 2.4^a	
	K-1	-28.4 ± 0.05^f		1.5 ± 0.10^c		26.4 ± 0.28^c		4.2 ± 0.2^b	
LSD$_{0.05 \text{ for cultivar}}$		0.15	—	0.12	—	0.55	—	1.83	—
Jinan	Gopung	-25.7 ± 0.07^d	-25.6 ± 0.46^d	7.5 ± 0.08^d	8.4 ± 0.66^a	25.3 ± 0.42^a	25.5 ± 0.38^e	5.6 ± 0.1^b	5.4 ± 1.0^a
	Geumpung	-26.3 ± 0.04^f		8.9 ± 0.04^b		25.5 ± 0.33^a		5.8 ± 0.1^b	
	Sunwoon	-25.2 ± 0.01^b		8.6 ± 0.07^c		25.7 ± 0.68^a		6.3 ± 0.6^a	
	Yeonpung	-25.9 ± 0.08^e		7.5 ± 0.03^d		25.6 ± 0.03^a		3.4 ± 0.1^c	
	Cheonpung	-25.0 ± 0.10^a		8.9 ± 0.04^{ab}		25.2 ± 0.03^a		5.6 ± 0.1^b	
	K-1	-25.6 ± 0.03^c		9.0 ± 0.05^a		25.8 ± 0.37^a		5.8 ± 0.1^b	
LSD$_{0.05 \text{ for cultivar}}$		0.11	—	0.10	—	0.69	—	0.44	—
Cheorwon	Gopung	-24.7 ± 0.02^d	-24.9 ± 1.06^c	3.7 ± 0.12^a	3.2 ± 0.68^d	26.9 ± 0.38^d	27.4 ± 0.44^a	3.7 ± 0.8^a	3.3 ± 0.6^d
	Geumpung	-24.6 ± 0.04^c		2.7 ± 0.04^d		27.5 ± 0.39^{bc}		3.6 ± 0.1^{ab}	
	Sunwoon	-25.4 ± 0.00^e		3.6 ± 0.02^b		27.1 ± 0.19^{cd}		2.9 ± 0.1^{bc}	
	Yeonpung	-26.9 ± 0.07^f		1.9 ± 0.03^e		27.7 ± 0.04^{ab}		2.6 ± 0.3^c	
	Cheonpung	-24.4 ± 0.01^b		3.7 ± 0.04^a		28.0 ± 0.27^a		3.9 ± 0.5^a	
	K-1	-23.6 ± 0.00^a		3.3 ± 0.01^c		27.5 ± 0.19^{bc}		3.4 ± 0.2^{ab}	
LSD$_{0.05 \text{ for cultivar}}$		0.06	—	0.10	—	0.47	—	0.73	—
Pyeongchang	Gopung	-23.3 ± 0.05^c	-23.9 ± 1.26^a	4.6 ± 0.08^a	3.3 ± 0.74^c	25.3 ± 0.19^c	26.1 ± 0.61^d	5.6 ± 0.0^b	5.2 ± 2.2^a
	Geumpung	-24.7 ± 0.04^d		2.4 ± 0.10^f		26.4 ± 0.12^b		5.3 ± 0.1^b	
	Sunwoon	-23.1 ± 0.04^b		3.7 ± 0.12^b		26.9 ± 0.24^a		8.5 ± 1.7^a	
	Yeonpung	-22.0 ± 0.06^a		3.3 ± 0.07^c		26.0 ± 0.15^b		1.4 ± 0.2^c	
	Cheonpung	-24.7 ± 0.05^d		3.0 ± 0.04^d		26.4 ± 0.22^b		4.7 ± 0.1^b	
	K-1	-25.6 ± 0.01^e		2.7 ± 0.03^e		25.5 ± 0.36^c		5.5 ± 0.1^b	
LSD$_{0.05 \text{ for cultivar}}$		0.08	—	0.14	—	0.40	—	1.25	—
Punggi	Gopung	-23.6 ± 0.02^b	-24.3 ± 0.96^b	3.1 ± 0.07^d	3.6 ± 1.13^b	27.4 ± 0.24^a	26.5 ± 0.55^c	4.4 ± 0.5^a	3.9 ± 0.4^c
	Geumpung	-25.8 ± 0.01^f		2.7 ± 0.04^e		26.7 ± 0.10^b		3.8 ± 0.1^{bc}	
	Sunwoon	-24.9 ± 0.01^e		3.6 ± 0.05^c		26.4 ± 0.21^b		3.5 ± 0.1^c	
	Yeonpung	-24.6 ± 0.03^d		2.5 ± 0.02^f		26.4 ± 0.53^b		3.7 ± 0.2^{bc}	
	Cheonpung	-24.2 ± 0.05^c		4.2 ± 0.03^b		26.3 ± 0.46^b		4.1 ± 0.4^{ab}	
	K-1	-22.8 ± 0.05^a		5.7 ± 0.04^a		26.1 ± 0.58^b		3.7 ± 0.0^{bc}	
LSD$_{0.05 \text{ for cultivar}}$		0.06	—	0.08	—	0.73	—	0.51	—
LSD$_{0.05 \text{ for region}}$			0.04		0.04		0.22		0.41
ANOVA analysis									
Region		$p < 0.001$		$p < 0.001$		$p < 0.001$		$p < 0.001$	
Cultivar		$p < 0.001$		$p < 0.001$		$p < 0.001$		$p < 0.001$	
Region × cultivar		$p < 0.001$		$p < 0.001$		$p < 0.001$		$p < 0.001$	

Data represent the mean ($\pm$ SD) $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ values of ginseng roots. [a–f] Values with different superscripts differ significantly from other ginseng cultivars and/or from other ginseng cultivation sites ($p < 0.050$)
ANOVA, analysis of variance; LSD, least significant difference; SD, standard deviation; VCDT, Vienna Canyon Diablo Troilite; VPDB, Vienna Pee Dee Belemnite; VSMOW, Vienna Standard Mean Ocean Water

Moreover, the N stable isotope composition in plants is generally influenced by the soil environment, in addition to the local nitrogen-based fertilizer regime, where the type, brand, chemical form, intensity, and timing of the different fertilizers used can affect plant growth [28–31]. In general, organic fertilizers result in higher $\delta^{15}N$ values (1‰ to 37‰, typically >5‰) compared to synthetic fertilizers (−4‰ to +4‰). However, green manures that exhibit atmospheric N_2-fixation provide $\delta^{15}N$ values close to 0‰ [32,33].

According to previous studies [3,11,26], the geographical origin of ginseng root can significantly affect the $\delta^{15}N$ values, with variations between −2.46‰ and 11.81‰ being recorded. Indeed, these results were similar to our finding, where a $\delta^{15}N$ range of 0.7−9.0‰ was observed. In addition, unlike the lower $\delta^{15}N$ variation (∼1‰) in vegetables such as carrots, tomatoes, and lettuce, the $\delta^{15}N$ value in ginseng root tends to vary between roots, even when the same type and quantity of fertilizer is used in the same farm or region. This is because ginseng is a perennial plant, which is cultivated for 4−6 yr in the same location [26]. Moreover, negative $\delta^{15}N$ values can be assumed to arise from the use of synthetic chemical nitrogen-based fertilizers during ginseng cultivation, despite an ongoing ban of such synthetic fertilizers by the Korean Ginseng Industry Act [3,26,34].

Although in this study the same organic fertilizer type and level were used in each cultivation region prior to transplantation of the ginseng seedlings, the ginseng roots grown in the Eumseong region exhibited lower $\delta^{15}N$ values compared to those grown in other regions. Moreover, these $\delta^{15}N$ values were comparable to the $\delta^{15}N$ ranges found in chemical fertilizers. Interestingly, it was previously reported [29] that the $\delta^{15}N$ values of the same nitrogen-based fertilizers could vary significantly because of the different synthetic processes used by different manufacturers. Furthermore, the nitrate levels in irrigation water, N-isotopic fractionation in soil, and fertilizer availability were also found to influence the $\delta^{15}N$ levels of various agricultural products [31,35]. Thus, although we did not examine the $\delta^{15}N$ values of the organic fertilizer or of the cultivation soil used, the low $\delta^{15}N$ values in Eumseong ginseng roots were expected to result from the differences in N-isotopic fractionation and fertilizer availability, which in turn are influenced by the physical, chemical, and microbial properties of the soil, rather than by the specific use of a chemical nitrogen-based fertilizer.

Although metabolic differences in agricultural products can result in different $\delta^{18}O$ values in sap or plant tissues, the $\delta^{18}O$ values of such products are more closely associated with the climatic conditions of the respective cultivation regions, with examples including precipitation, irrigation/ground water source, altitude, and distance from the coast [13,28]. In general, the increased quantities of rainfall containing heavier water isotopomers (i.e., $H_2^{18}O$, $D_2^{16}O$, or $D_2^{18}O$) near coastal regions result in lower quantities of heavy H and O isotopes in precipitation and groundwater at
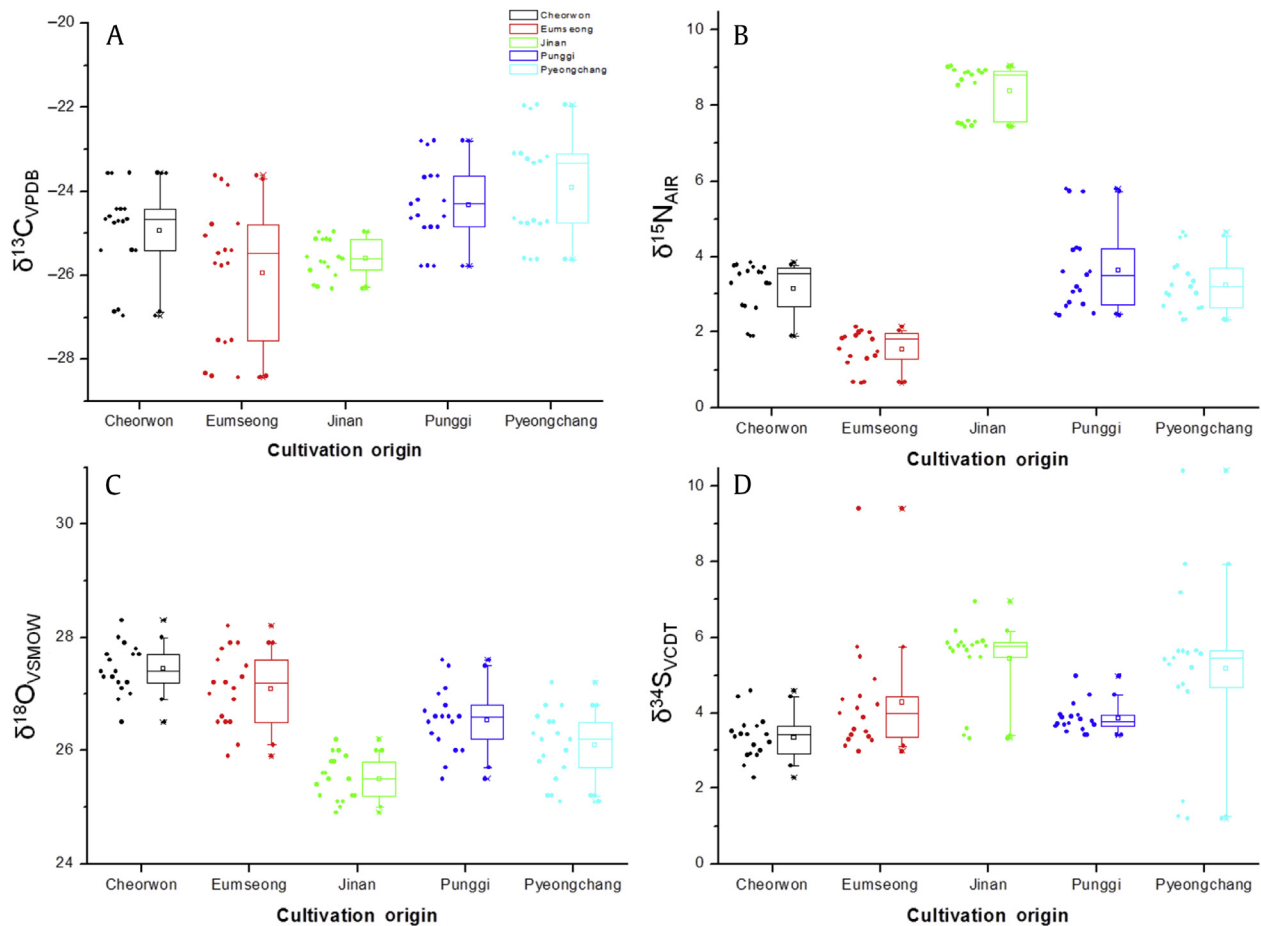
Fig. 1. Box/whisker charts. The charts show the distribution of all data summarizing the variation in (A) $\delta^{13}C_{VPDB}$, (B) $\delta^{15}N_{AIR}$, (C) $\delta^{18}O_{VSMOW}$, and (D) $\delta^{34}S_{VCDT}$ values in the ginseng root samples according to cultivation regions. The boxes correspond to the interquartile range containing the middle 50% of data, whereas the whiskers indicate the highest and lowest values (95% and 5%, respectively) over the entire data range. The squares inside the boxes represent the mean values, whereas the lines across each box and the filled circles on the box/whisker charts indicate the median values and outlier values, respectively. The (×) symbol represents the 99% and 1% values of the whole data range, and the (−) symbol indicates the maximum and minimum values. VCDT, Vienna Canyon Diablo Troilite; VPDB, Vienna Pee Dee Belemnite; VSMOW, Vienna Standard Mean Ocean Water.
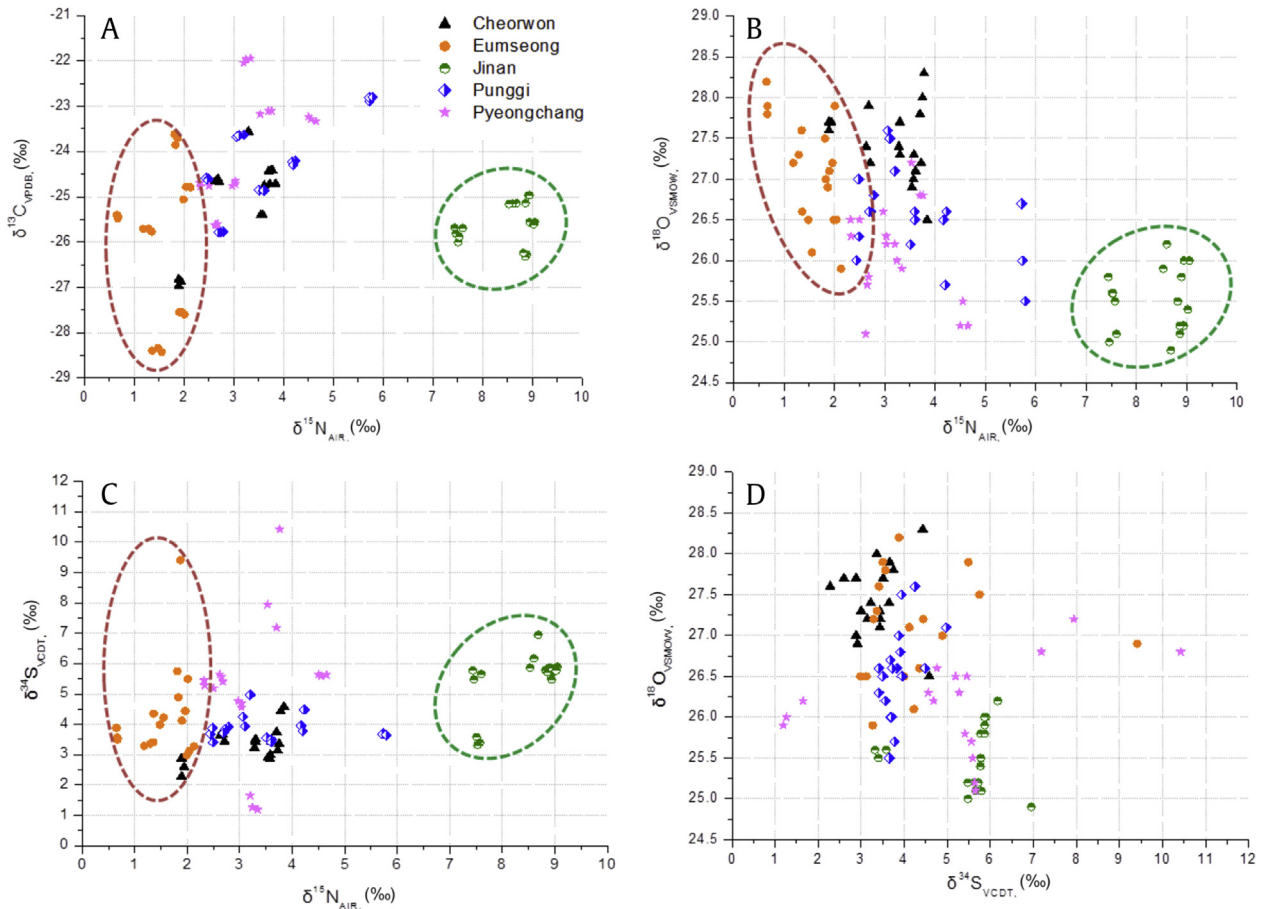
Fig. 2. Two-dimensional (2D) plots. (A) $\delta^{13}C_{VPDB}−\delta^{15}N_{AIR}$, (B) $\delta^{15}N_{AIR}−\delta^{18}O_{VSMOW}$, (C) $\delta^{15}N_{AIR}−\delta^{34}S_{VCDT}$, and (D) $\delta^{18}O_{VSMOW}−\delta^{34}S_{VCDT}$ values are plotted for discrimination between the different ginseng cultivation regions. The highlighted areas are for illustrative purposes only and do not indicate statistical analysis. VCDT, Vienna Canyon Diablo Troilite; VPDB, Vienna Pee Dee Belemnite; VSMOW, Vienna Standard Mean Ocean Water.

higher altitudes or in inland regions [13,30,36]. According to prior studies [13,16,17], rice grains and potato tubers cultivated at high altitude regions exhibited lower $\delta^{18}O$ values compared to those cultivated at low altitude regions, which is consistent with our finding regarding the negative correlation ($r = -0.47$, $p < 0.001$) between the $\delta^{18}O$ value in ginseng root and the altitude of the cultivation region.

In addition, because of the continental effect, ginseng roots grown in coastal areas exhibited higher $\delta^{18}O$ and δD values ($\sim 10‰$ higher) than those from inland areas, resulting in a clear discrimination of ginseng samples between Korea and China, as well as among different cultivation regions within Korea itself [3,26,37]. Thus, in addition to determining that the $\delta^{18}O$ value alone allowed discrimination between the above areas, we also observed that the $\delta^{18}O$ value of ginseng root was positively correlated ($r = 0.59$, $p < 0.001$) with the distance of the cultivation regions from coastal areas, which differs from previous reports. This discrepancy may be attributable to $\delta^{18}O$ levels being subject to local climates in addition to topographical characteristics (i.e., altitude and proximity from the coast). However, it remained unclear which parameter was most important for determining the $\delta^{18}O$ levels in ginseng roots. Similar to our current findings, a previous study described that the $\delta^{18}O$ level in potato tubers was also lower at increasing cultivation altitudes, although it was not influenced by the distance from the coast [16]. Further studies are therefore required to clearly elucidate the combined effects of topographical and local climatic
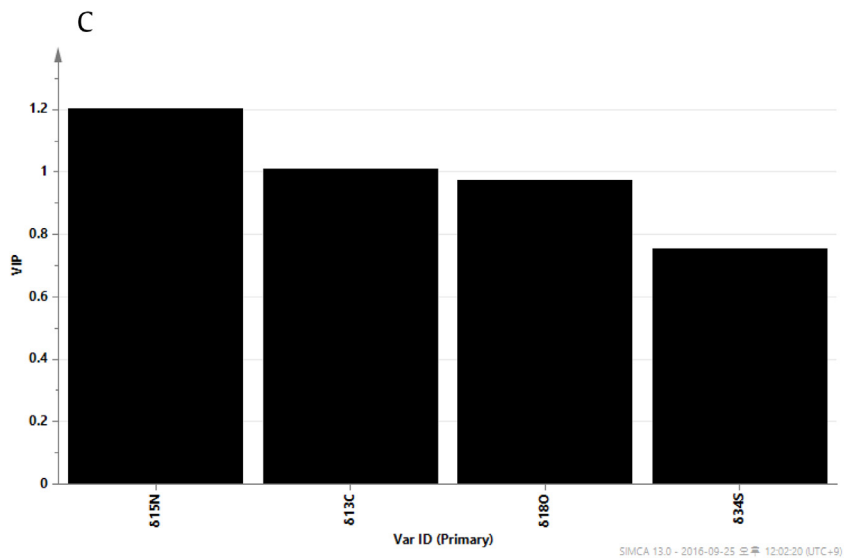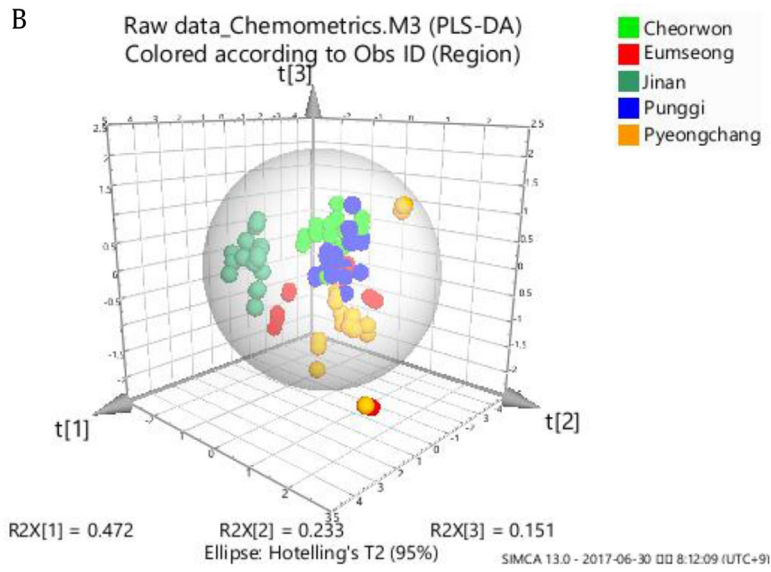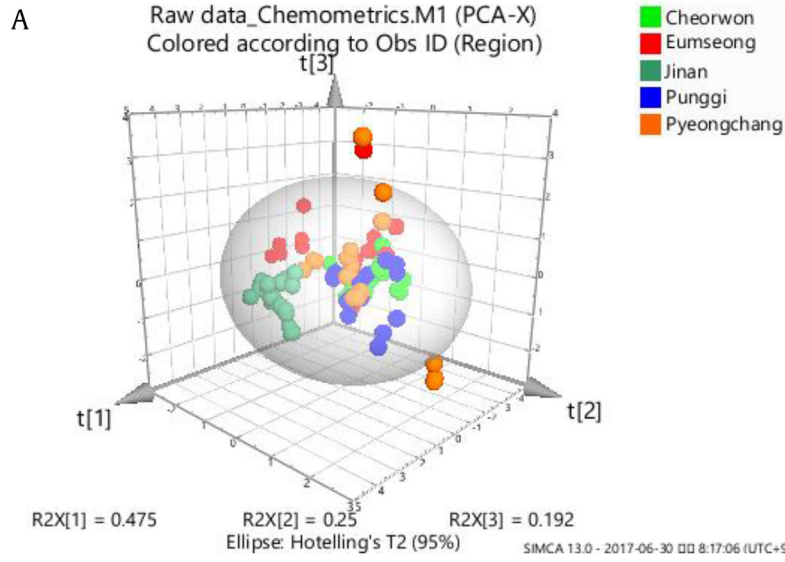
parameters on $\delta^{18}O$ variability in ginseng roots obtained from different cultivation regions.

We then examined the variation in $\delta^{34}S$ values of the ginseng root samples. Although this factor is less well understood than the corresponding $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values, it is more likely to be affected by geological issues than by the biological properties of the cultivation soil [38]. Furthermore, the $\delta^{34}S$ values of agricultural products are also influenced by anthropogenic activities (i.e., use of sulfur-containing fertilizers, industrial SO_2 emissions), as well as the sea-spray effect associated with the distance between coastal and cultivation regions [28,39−41].
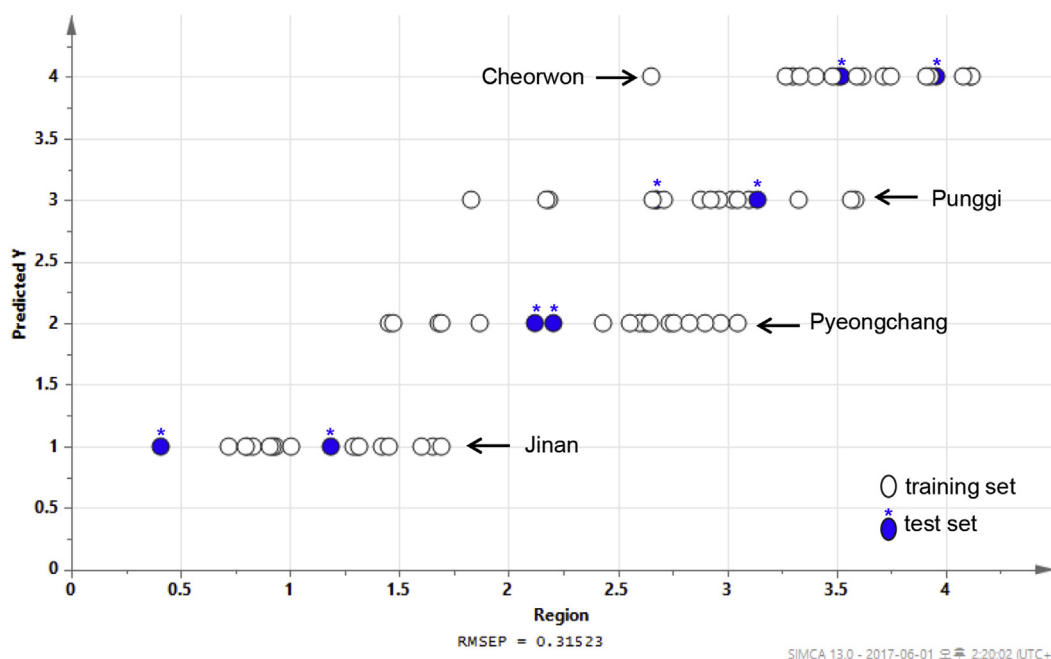
In this context, a previous study reported variations in $\delta^{34}S$ values between ginseng roots cultivated in different types of soil (upland vs. paddy-converted field) using a range of organic fertilizers, with high $\delta^{34}S$ values being reported for ginseng grown in upland areas using rice straw compost as fertilizer [42]. In our study, we found a slight positive correlation between the $\delta^{34}S$ and $\delta^{15}N$ values in ginseng roots ($r = 0.34$, $p < 0.001$), whereas a slight negative correlation was observed for the $\delta^{18}O$ values ($r = -0.30$, $p < 0.010$). In addition, the $\delta^{34}S$ level in ginseng roots exhibited a negative correlation with the distance from coastal areas ($r = -0.48$, $p < 0.001$), which was likely related to the sea-spray effect. This finding was consistent with a previous study, which showed that milk samples in Australia exhibited higher $\delta^{34}S$ values ($9−15‰$) than European samples ($3−8‰$) as a consequence of the sea-spray effect [22]. Hence, the $\delta^{34}S$ values in ginseng root can act
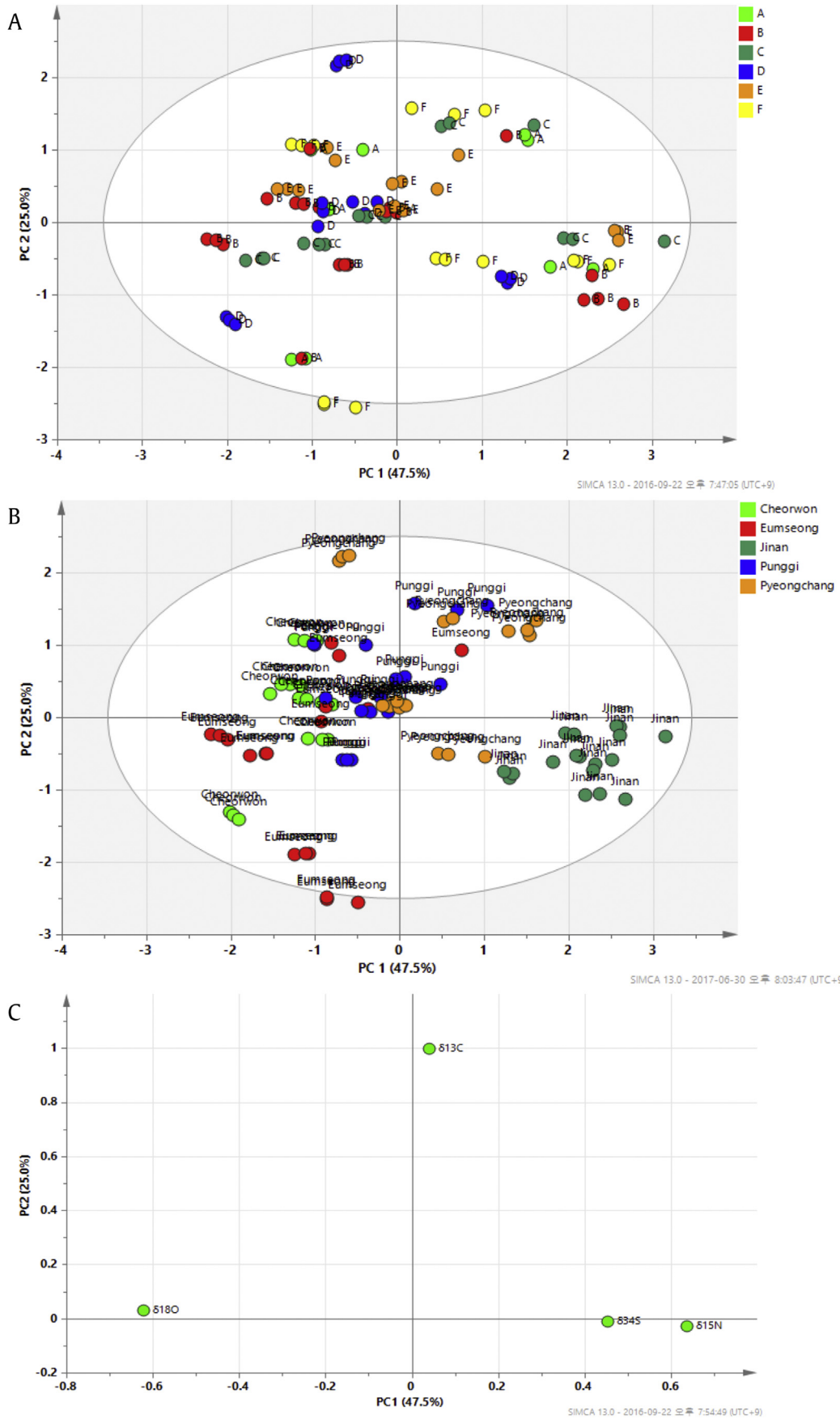
Fig. 3. (A, B) Principal component analysis (PCA) scores and (C) loading plots derived from the isotope levels present in the ginseng root samples. (A) Differentiation between ginseng cultivars, and (B) differentiation between cultivation regions.

A   Raw data_Chemometrics.M1 (PCA-X)
     Colored according to Obs ID (Region)

R2X[1] = 0.475   R2X[2] = 0.25   R2X[3] = 0.192
              Ellipse: Hotelling's T2 (95%)
                                    SIMCA 13.0 - 2017-06-30 □□ 8:17:06 (UTC+9)

B   Raw data_Chemometrics.M3 (PLS-DA)
     Colored according to Obs ID (Region)

R2X[1] = 0.472   R2X[2] = 0.233   R2X[3] = 0.151
              Ellipse: Hotelling's T2 (95%)
                                    SIMCA 13.0 - 2017-06-30 □□ 8:12:09 (UTC+9)

C

SIMCA 13.0 - 2016-09-25 오후 12:02:20 (UTC+9)

Fig. 5. External validation test of PLS-DA model derived from the isotope levels present in the ginseng root samples. PLS-DA, partial least squares discriminant analysis; RMSEP, root mean square error of prediction.

as a potential isotope marker to discriminate between different ginseng cultivation regions.

3.2. Classification of ginseng cultivation regions using multivariate analysis models

To further determine reliable isotope ratio markers for discrimination between the different geographical origins of ginseng roots, multivariate analyses (i.e., PCA and PLS-DA) were performed. Fig. 3 shows the PCA score and the loading plots obtained using the δ^{13}C, δ^{15}N, δ^{18}O, and δ^{34}S values of the ginseng roots from different ginseng cultivars and cultivation regions. PCA is typically used to analyze data in an unbiased and unsupervised manner, and it is an important tool to reduce the dimensionality of a multivariate dataset while preserving most of the variance within it as a preliminary inspection of the data distribution [2]. As shown in Fig. 3A, the two highest-ranking components showed no clear correlation to the six ginseng cultivars examined in this study, although some differences were found among the various cultivation regions and the two components of the PCA. This accounted for 72.5% of the total variance, with the Jinan region being clearly differentiated from the other four regions based on these isotope ratio profiles of the ginseng roots (Fig. 3B). In addition, the loading plot indicated that the higher δ^{34}S and δ^{15}N values of the ginseng root samples rendered them key components for defining the Jinan cultivation region (Fig. 3C). However, PCA could not clearly discriminate between the ginseng root samples cultivated in the Cheorwon, Eumseong, Punggi, and Pyeongchang regions. As the quality of the PCA models can be expressed in terms of the goodness of the fit (R^2) and the predictive ability (Q^2), the R_X^2 and Q^2 values were calculated to be 0.725 and 0.07, respectively, using the PCA model established using the two higher components. As a model is usually considered to have a good predictive ability if

$Q^2 > 0.5$, it was clear that this PCA result exhibited poor predictive ability when using the multivariate data based on cultivation regions.

To create a better separation model for differentiation between the different cultivation regions, we then conducted PLS-DA, which is a well-established chemometric approach for supervised analyses based on a PLS model (Fig. 4). Initially, the PCA 3D score plots indicated poor separation of the different ginseng root samples according to their cultivation regions ($R^2 = 0.917$ and $Q^2 = -0.026$; Fig. 4A), whereas the PLS-DA 3D score plots (Fig. 4B) exhibited a clearer clustering, where $R^2 = 0.856$ and $Q^2 = 0.393$. At this point, the variables important in the projection (VIP) value should also be considered, as this factor explains the extent to which a variable contributes in the projection. In general, a VIP value of >1 is used as a criterion to identify the variables important to the model [43]. Thus, as shown in Fig. 4C, the variation in δ^{15}N and δ^{13}C values could be considered significant for discriminating between different ginseng cultivation regions. In addition, for an external validation, the total data were divided into a training set and a test set (Fig. 5). The test set was used to test the model performance, and the PLS-DA model suggested in this study had a relatively good model performance ($R^2 = 0.853$ and $Q^2 = 0.738$).

Finally, as previously reported [1,8,44], PCA has a relatively weak discriminative power when a number of different parameters are involved. In contrast, the orthogonal projection to latent structures-discriminant analysis (OPLS-DA), which is an extended version of PLS-DA, is more suitable for the classification of multiparameter samples. As a result, the OPLS-DA method provided a clearer separation of ginseng roots grown in China and Korea [8], as well as between six different cultivation regions within Korea itself [44]. Indeed, these prior reports yielded similar conclusions to our findings, in which an improved discrimination between the five ginseng cultivation regions in Korea was achieved using a PLS-DA

Fig. 4. (A) Three-dimensional (3D) plot of the principal component analysis (PCA) score. (B) 3D plot of the partial least squares-discriminant analysis (PLS-DA) score. (C) Influence of variables used to create a discrimination model for the cultivation regions of ginseng root samples. Variable importance in the projection (VIP) values were obtained from the PLS-DA model.

method. We could therefore conclude that stable isotope ratio analysis combined with various multivariate analysis tools can be used to discriminate between the various geographical origins of ginseng root samples.

4. Conclusion

Analysis of the stable isotope ratios of C, N, O, and S (i.e., $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$) in *P. ginseng* roots expands our current knowledge regarding how these values vary with respect to cultivation region and ginseng cultivar, and allows the discrimination between the various regional origins of ginseng samples. More specifically, the $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ values of *P. ginseng* root samples were significantly influenced by region, cultivar, and interactions between these two factors ($p < 0.001$). Overall, the Cheonpung cultivar exhibited higher mean $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ values compared to the other ginseng cultivars. In addition, we found that the $\delta^{18}O$ value was affected by the altitude of the cultivation region ($r = -0.47$, $p < 0.001$), with higher altitude cultivation regions such as Pyeongchang and Jinan giving lower $\delta^{18}O$ values compared to those of low altitude regions ($p < 0.050$). Furthermore, $\delta^{34}S$ values were higher in ginseng roots grown in the Jinan and Pyeongchang regions, which are located close to coastal areas ($r = -0.48$, $p < 0.001$). However, although the 2D plots served as a relatively good discriminative tool for identifying ginseng grown in the Jinan and Eumseong regions (with the exception of $\delta^{18}O$ and $\delta^{34}S$ combinations), those produced in other regions proved difficult to distinguish. In terms of multivariate analysis tools, the two highest-ranking components of PCA resolved the ginseng roots grown in the Jinan and other regions, whereas the results from PLS-DA allowed classification based on cultivation regions, with the exception of the Eumseong region. As such, the differences between the $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ patterns as a function of cultivation region were concluded to be associated with variations in cultivation environments including soil type, soil nutrition history, light supply, and water source and availability. However, as this preliminary study is limited to 3-yr-old ginseng root grown in Korea, future studies are required to further elucidate how the $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ values of ginseng root samples vary with respect to age. We could, therefore, conclude that our findings represent a feasible tool to discriminate between different geographical origins of Korean *P. ginseng*, and we expect that they will aid in protecting consumers and ginseng producers from fraudulent labeling regarding geographical origins. We also expect that following additional validation, this tool will also be applicable to ginseng root grown in other countries.

Conflicts of interest

All authors have no conflicts of interest to declare.

Acknowledgments

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