Metabolomic Studies on Geographical Grapes and Their Wines Using ^{1}H NMR Analysis Coupled with Multivariate Statistics

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Environmental vineyard conditions can affect the chemical composition or metabolites of grapes and their wines. Grapes grown in three different regions of South Korea were collected and separated into pulp, skin, and seed. The grapes were also vinified after crushing. ^{1}H NMR spectroscopy with pattern recognition (PR) methods was used to investigate the metabolic differences in pulp, skin, seed, and wines from the different regions. Discriminatory compounds among the grapes were Na, Ca, K, malate, citrate, threonine, alanine, proline, and trigonelline according to PR methods of principal component analysis (PCA) or partial least-squares discriminant analysis (PLS-DA). Grapes grown in regions with high sun exposure and low rainfall showed higher levels of sugar, proline, Na, and Ca together with lower levels of malate, citrate, alanine, threonine, and trigonelline than those grown in regions with relatively low sun exposure and high rainfall. Environmental effects were also observed in the complementary wines. This study demonstrates that ^{1}H NMR-based metabolomics coupled with multivariate statistical data sets can be useful for determining grape and wine quality.

KEYWORDS: ^{1}H NMR; metabolomics; geographical grapes; wine

INTRODUCTION

Many factors affect wine quality, including grapes, yeast strains, winemaking technologies, and human practices. The grape is the most basic and important factor for making good-quality wine; thus, many studies on the relationships between grapes and wine quality have been published (*1−3*).

Genetic and environmental factors are essential to grape and wine quality (*4*). In particular, it is well-known that "terroir" and "vintage" are related to environmental factors. The term "terroir" has been used to specify the origin of the grape and can be defined as an interactive ecosystem in a given place, including soil, climate, and cultivar (*5*). The term "vintage" refers to variations in the composition or metabolites of wines (*6*). Climatic conditions change with each year, resulting in differences in the chemical composition or metabolites of the grape. Although terroir and vintage are important concepts affecting the qualities of grape and wine, they are difficult to study on a scientific basis because they involve many factors. To understand how terroir and vintage function, more detailed studies or new analytical techniques are necessary to characterize the factors that contribute to the metabolites of grape and wine.

Metabolites are the intermediates and end products of cellular regulatory processes, and their levels can be regarded as the ultimate response of biological systems to environmental changes (*4*). In other words, many metabolites in grapes, such as sugars, organic acids, phenols, and amino acids, may be affected by the environment. It is impossible to define the ideal climate for fine grapes and wines in terms of temperature, rainfall, or solar energy because high-quality grapes are grown in various climates (*1*). However, it is meaningful to investigate the relationship between metabolites and environmental parameters such as temperature, rainfall, light exposure, and soil.

Metabolomics is a promising new approach aimed at improving our understanding of metabolic perturbations in drug toxicity (*7−9*), disease status (*10*), dietary intervention (*11, 12*), and plant primary and secondary metabolism (*13, 14*). Recently, metabolomic studies using ^{1}H NMR have also been applied in the food sciences, especially in the analysis of grapes (*4, 5, 15*) and wines (*16−18*). However, there are few studies on me-

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tabolomic approaches for evaluating the quality of grapes harvested in different regions and of their wines.

^{1}H NMR analysis coupled with multivariate statistics for the purpose of latent-information extraction and sample classification offers a powerful new approach for assessing metabolic function. Principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) are often used to separate sample groups and to identify the biochemical compounds responsible for the separation (*19*). PCA and PLS-DA were therefore applied to a ^{1}H NMR data set in this study to visualize correlation patterns among metabolites of grapes and wines from different regions.

The aim of the present work was to describe the effects of environmental factors on the chemical composition or metabolites of grapes harvested in three different regions and of their wines.

MATERIALS AND METHODS

Origin of the Samples. Muscat Bailey A (*Vitis* hybrid) cultivars harvested from three regions in Korea in October 2007 were purchased in the market. Origins and producers of grapes in the market were certified according to system for traceability of farm products by the Korean Rural Development Administration, and thus grapes could be obtained from five different vineyards or five different producers in the same geographical regions. Fifteen grapes from Yeongdong, Yeongcheon, and Chochiwon regions were obtained.

Extraction of Pulp, Skin, and Seed. Extraction procedures were performed according to the method of Pereira et al. (*4*). To prepare the pulps, the skins and seeds from 10 berries were separated manually. Pulps were directly extracted with 95% ethanol for 15 min. Skins were ground in a blender for 2 min and then extracted with 95% ethanol for 1 h. Seeds were crushed manually using a mortar and then extracted with 95% ethanol for 4 h. All extractions were conducted with agitation at 4 °C, and 80 mL of ethanol was used to extract each sample of 10 berries. After centrifugation (3000*g* for 10 min), an aliquot was removed for other analyses (organic acid, metal, and total phenol), and the remaining supernatants were dried under vacuum for NMR analysis.

Vinification. Muscat Bailey A grapes cultivated in three regions of South Korea, Yeongdong, Yeongcheon, and Chochiwon (**Figure 1**), were used to prepare five batches (10 kg) of must for each of the three regions. After washing, the grapes were crushed manually, and sugar was added to adjust the must to 21.2−23.4 °Brix. The must was distributed into 15 25-L plastic tanks. After the addition of 100 ppm of $K_2S_2O_5$ to the must, yeast culture was inoculated at 2×10^6 cells/mL. *Saccharomyces cerevisiae* Bourgovin RC-212 (Lalvin, Canada) was used for alcoholic fermentation. The alcoholic fermentation was carried out in 15 plastic tanks at room temperature for 8 days. After completion of alcoholic fermentation, the musts were transferred into 15 4-L glass carboys at day 9 and then racked at 3 and 6 months.

Analyses of Grape Extracts. For analysis of organic acids, extracts of pulps and skins were filtered through a 0.2 μm membrane filter and analyzed by HPLC (Gilson, France). The injection volume was 20 μL. Samples were eluted with a 25 mM KH_2PO_4 solution at pH 2.5 with phosphoric acid and detected with a UV−vis detector at 210 nm. A Prevail Organic Acid column (150 × 4.6 mm i.d., 5 μm particle size) was used, and the flow rate was 1.0 mL/min. Acid concentration was determined according to external standard solution calibrations. Na, K, Ca, Mg, Fe, and Cu metals in the extracts (pulps, skins, and seeds) were measured by atomic absorption spectrophotometer (AA-6701F, Shimadzu) with an air/acetylene flame (*20*). Phosphorus interference was overcome by adding lanthanum to about 0.1% into Ca standards and samples (*21*). The total phenol contents of extracts (pulps, skins, and seeds) were determined using the Folin−Ciocalteu method (*22*) with gallic acid as a standard, and the results were expressed as milligrams of gallic acid equivalents per liter (mg of GAE L^{-1}).

Analyses of Musts. Before inoculation of yeast, total soluble solids (°Brix) of musts were measured using a digital refractometer (PR-32, Atago) with temperature compensation. Total titratable acidity and pH were determined with a pH-meter (Orion 3 Star, Thermo Fisher
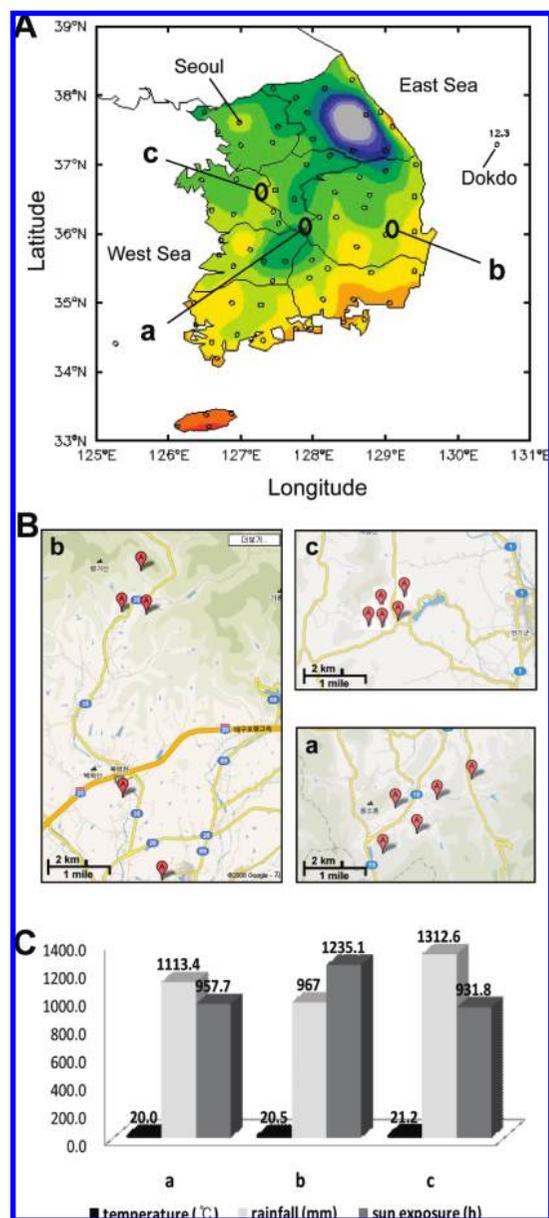
Figure 1. Geographical regions (**A**), precise local vineyards (**B**), and climatic conditions with average temperature (°C), total rainfall (mm), and total sun-exposed time (h) (**C**) of three regions of South Korea from April to September 2007. The distance between each degree of latitude is approximately 111 km, and the distance between the degrees of longitude is approximately 89 km at 37° N. a, b, and c denote Yeongdong, Yeongcheon, and Chochiwon, respectively. "A" in panel **B** represents localization of vineyards within three geographical regions. Data were obtained from Korea Meteorological Administration.

Scientific Inc.). Color measurements were taken directly in a 1 cm cell after the must was diluted 1:5 with water (*23*). Before the reading was taken, the samples were filtered through 0.45 μm filters, and summation of absorbance readings at 420, 520, and 620 nm using a UV spectrophotometer (UV-1700, Shimadzu) provided a measure of color intensity. Hue was measured as the ratio of absorbance readings at 420 and 520 nm (*21*).

^{1}H NMR Spectroscopic Analysis of Grape Extracts and Wines. Dried samples (extracts of pulps, skins, and seeds) were dissolved in 2 mL of 400 mM oxalate buffer, pH 4, and dried again under vacuum. Each extract was dissolved in 2 mL of 99.9% deuterium oxide and lyophilized (*4*). Skin, pulp, and seed extracts and wines aged for 3 and 6 months were dried, lyophilized, and dissolved in 99.9% deuterium oxide (400 μL, D$_2$O). The samples were then mixed with 400 mM oxalate buffer (140 μL, pH 4.0) and 5 mM sodium 2,2-dimethyl-2-

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Table 1. Mineral and Organic Acid Content (Parts per Million)[a] of Pulp, Skin, and Seed of Mature Grape Berries from Three Regions

compound		pulp			skin			seed		
		region a[b]	region b	region c	region a	region b	region c	region a	region b	region c
minerals	Na	nd[c]	nd	nd	4.33 ± 0.49ab[d]	5.20 ± 1.07a	3.81 ± 0.46b	nd	nd	nd
	K	0.44 ± 0.22	0.82 ± 0.68	0.60 ± 0.32	7.96 ± 2.26b	13.78 ± 2.91a	12.32 ± 2.76a	3.64 ± 0.72	4.36 ± 0.91	4.55 ± 0.80
	Mg	nd	nd	nd	0.39 ± 0.09	0.37 ± 0.18	0.35 ± 0.16	nd	nd	nd
	Ca	0.21 ± 0.04	0.23 ± 0.05	0.23 ± 0.07	0.29 ± 0.07c	0.77 ± 0 0.10a	0.59 ± 0.06b	1.05 ± 0.10c	8.83 ± 0.31a	7.97 ± 0.49b
organic acids	Oxa[e]	5.00 ± 0.90	6.94 ± 2.48	6.77 ± 1.26	17.00 ± 1.87	17.68 ± 5.93	14.51 ± 3.69			
	Tar	33.67 ± 12.83	50.52 ± 14.15	40.09 ± 6.72	81.81 ± 6.81ab	100.75 ± 26.93a	74.39 ± 5.80b			
	Mal	53.98 ± 17.57b	70.84 ± 18.72ab	93.28 ± 34.34a	44.56 ± 14.76b	81.28 ± 38.98ab	98.94 ± 38.78a			
	Cit	5.72 ± 1.61b	5.77 ± 1.32b	9.18 ± 3.08a	3.83 ± 2.29	4.35 ± 0.21	4.26 ± 1.27			

[a] Mineral and organic acid contents are expressed as ppm per g of extract. Fe and Cu were not detected in any samples. [b] a, b, and c denote Yeongdong, Yeongcheon, and Chochiwon, respectively. [c] nd, not detected. [d] Duncan's multiple-range test ($p = 0.05$). Different letters indicate significant difference between samples. [e] Oxa, oxalate; Tar, tartarate; Mal, malate; Cit, citrate.

Table 2. Total Phenol Content (Parts per Million)[a] of Pulp, Skin, and Seed of Mature Grape Berries and Physicochemical Analysis of Musts from Three Regions

		region		
		a[b]	b	c
extracts	total phenol (pulp)	0.19 ± 0.05	0.22 ± 0.10	0.26 ± 0.11
	total phenol (skin)	28.18 ± 7.71	32.30 ± 10.32	22.78 ± 1.42
	total phenol (seed)	217.29 ± 36.41	210.00 ± 60.72	212.92 ± 36.41
musts	soluble solid (°Brix)	17.62 ± 0.42a[c]	16.84 ± 0.80a	15.68 ± 0.40b
	total acidity[d] (g/L)	7.25 ± 0.13a	7.41 ± 0.44a	6.71 ± 0.27b
	pH	3.44 ± 0.03	3.49 ± 0.06	3.47 ± 0.01
	hue	1.32 ± 0.17	1.34 ± 0.16	1.31 ± 0.27
	color intensity	0.94 ± 0.15a	0.71 ± 0.08b	0.60 ± 0.06b

[a] Total phenol contents are expressed as ppm per g of extract. [b] a, b, and c denote Yeongdong, Yeongcheon, and Chochiwon, respectively. [c] Duncan's multiple-range test ($p = 0.05$). Different letters indicate significant difference between samples. [d] Total acidity is expressed as tartaric acid.

silapentane-5-sulfonate (60 μL, DSS, 97%) and centrifuged at 13000 rpm for 10 min. Supernatants (550 μL) were transferred into 5 mm NMR tubes. D_2O and DSS provided a field frequency lock and chemical shift reference (^1H, δ 0.00), respectively. ^1H NMR spectra were recorded on a Varian Inova-600 MHz NMR spectrometer, operating at 599.84 MHz ^1H frequency and a temperature of 298 K, using a triple-resonance 5 mm HCN salt tolerant cold probe. The noesypresat pulse sequence was applied to suppress the residual water signal. For each sample, 16 transients were collected into 32K data points using a spectral width of 9615.4 Hz with a relaxation delay of 1.5 s, an acquisition time of 4.00 s, and a mixing time of 400 ms. A line-broadening function of 0.3 Hz was applied to all spectra prior to Fourier transformation (FT).

NMR Data Reduction and Preprocessing. All NMR spectra were phased and baseline corrected by Chenomx NMR suite 4.6 software, professional edition (Chenomx Inc.). The NMR spectral data were reduced into 0.001 ppm spectral buckets, whereas the region corresponding to water (4.6−4.8) was removed. In addition, the regions of residual ethanol (1.15−1.20 and 3.59−3.72 ppm), which resulted from incomplete removal during lyophilization, and of DSS (−0.5 to 0.5, 1.70−1.80, and 2.89−2.94 ppm) were also removed. The spectra were then normalized to total spectral area and converted to ASCII format. The ASCII format files were imported into MATLAB (R2006a, Mathworks, Inc., 2006), and all spectra were aligned using the Correlation Optimized Warping (COW) method (17). The resultant data sets were then imported into SIMCA-P version 12.0 (Umetrics) for multivariate statistical analysis.

Multivariate Data Analysis. The mean center was applied for all multivariate analysis by SIMCA-P version 12.0. PCA, an unsupervised pattern recognition method, was performed to examine the intrinsic variation in the data set. To maximize the separation between samples, PLS-DA was applied. PLS-DA can be described as the regression extension of PCA that gives the maximum covariance between measured data (X variable, metabolites in NMR spectra) and the response variable (Y variable, NMR spectral intensities). The Hotelling $T2$ region, shown as an ellipse in score plots of the models, defines the 95% confidence interval of the modeled variation (24). The quality of the models was described by R_X^2 and Q^2 values. R_X^2 is defined as the proportion of variance in the data explained by the models and indicates goodness of fit. Q^2 is defined as the proportion of variance in the data predictable by the model and indicates predictability (25).

Chemicals. For analysis of grapes, musts, and wines, all chemical reagents were of analytical grade. D_2O (99.9%) and DSS (97%) were purchased from Sigma.

RESULTS AND DISCUSSION

Climates of the Three Different Regions. Three factors have major roles in grape maturation dynamics: light, temperature, and water availability (26). In general, these factors affect not only the growth but also the metabolic activity of the grapevine. The three principal climatic parameters of light, temperature, and rainfall vary considerably by year and region, thereby giving the enological notions of vintage and terroir. **Figure 1A** shows the location of three regions in South Korea: Yeongdong, Yeongcheon, and Chochiwon. **Figure 1B** represents the precise locations of five vineyards within three geographical regions. **Figure 1C** shows the comparisons of climatic conditions in the three regions from April to September 2007, which is the flowering, ripening, and maturation period for grapes. Yeongcheon had the most sun exposure and the least rainfall of the three regions, whereas Chochiwon had the most rainfall and the least sun exposure among the three regions. These differences in climatic conditions would be sufficient to affect the chemical composition or metabolites of grapes.

Mineral Analysis of Grape Extracts. The concentrations of Na, K, Mg, and Ca in the pulp, skin, and seed extracts of grapes from the three different regions are shown in **Table 1**. Significantly different levels of Na, K, and Ca in skin extracts were observed among the three regions. No significant differ-
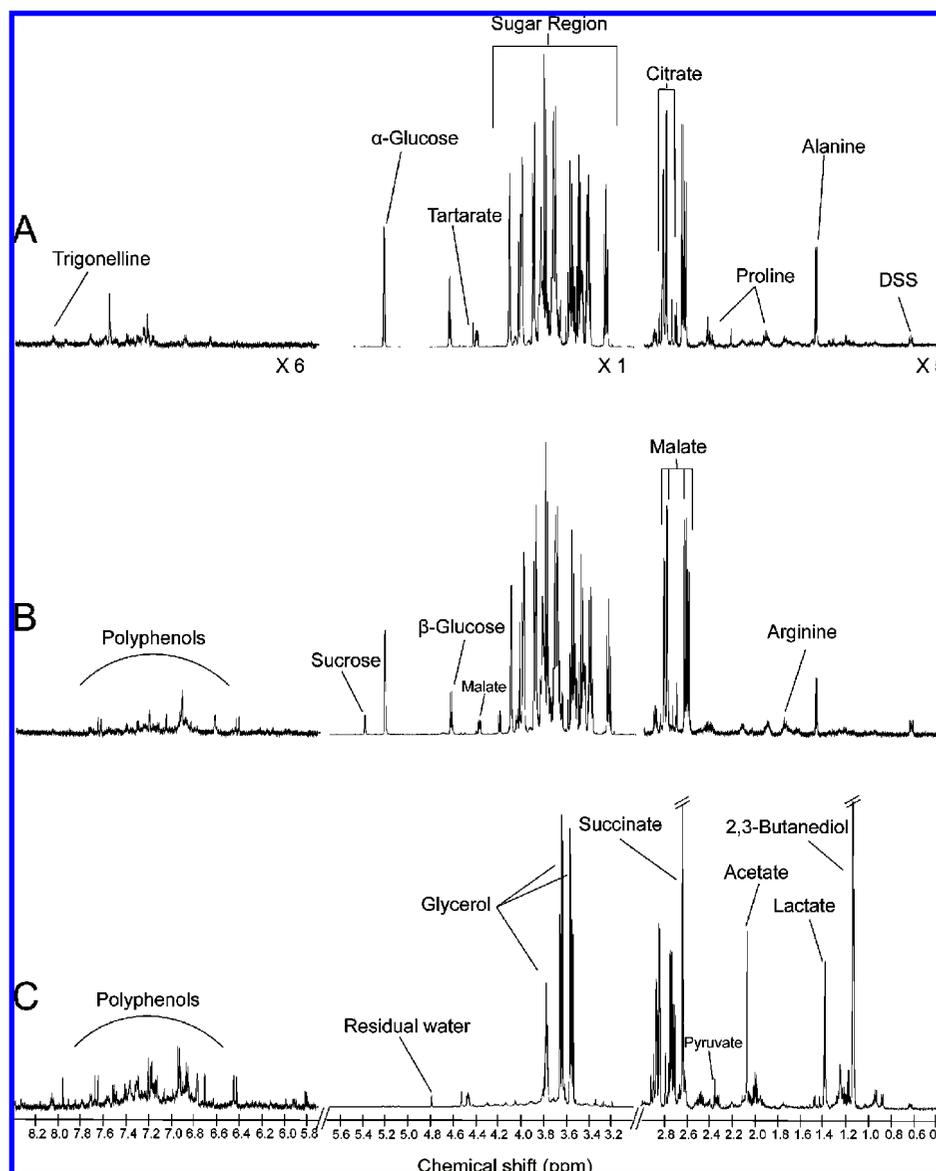
Figure 2. Representative ^1H NMR spectra of pulp (**A**) and skin (**B**) extracts and wine (**C**) of Muscat Bailey A grapes harvested in Yeongdong.

ences in minerals of pulp and seed extracts were observed with the exception of Ca in seed extracts. Two elements, Cu and Zn, were not detected in any samples, presumably due to very low concentrations of these minerals in the extracts.

The concentrations of Na, K, and Ca were highest in the skin extracts from Yeongcheon compared to those from Yeongdong and Chochiwon. In some cases, grapes grown near the sea have more sodium content (*20, 27, 28*). Potassium is located in the pulp cell vacuoles, but the skin cells sometimes contain significant amounts. Potassium plays an important role in the synthesis and translocation of carbohydrates (*29*). In years with favorable climatic conditions, the ripe grape imports large amounts of potassium (*26, 30*). Excessive soil richness or fertilization is another factor responsible for high potassium supply (*31*). Skin extracts contained higher amounts of minerals in the three regions than pulp and seed extract, indicating that soil or climate could affect the chemical composition of grape skin more strongly than that of grape pulp and seed.

Organic Acids in Grape Extracts. The organic acid contents in pulp and skin extracts are shown in **Table 1**. Significantly higher malate contents were observed in both skin and pulp extracts of grapes from Chochiwon than in those from other regions. The imported sugars are the precursors of the malate

found in grape, and malate is a very active intermediary product of grape metabolism. Malate contents were highest in pulp and skin extracts from grapes grown in Chochiwon, which was the region with the least light exposure and the most rainfall in 2007. This result was in good agreement with reports that malate content was significantly related to light exposure (*15, 26*). The malate concentration in mature berries is dependent on the malic enzyme and malic dehydrogenase activities (*32, 33*). Malic enzyme activity steadily increases between 10 and 46 °C (*26*). Thus, malate degradation is considerably accelerated during hot weather due to changes in acid breakdown, rather than to reduced malate accumulation (*34*). In the present study, there were no significant differences in the average temperatures of the three regions. However, malate contents depended on the length of sun exposure and the total amount of rainfall from April to September 2007. Therefore, we concluded that sun exposure time and rainfall more effectively explain the effects on malate content in grapes than temperature.

The average citrate content in pulp extracts of grapes cultivated in Chochiwon was higher than that in grapes from the other two regions. Many variables are related to citrate metabolism in the Krebs cycle, and little is known about the metabolic pathways involved in the catabolism during ripening

Table 3. Metabolites and Their ^1H Chemical Shifts Identified by 600 MHz ^1H NMR[a]

compound	source	^1H NMR chemical shift (S)[b]	group	^1H no.
isoleucine	grape	0.93 (t)	C5H$_3$	3
leucine	grape	0.95 (t)	C5H$_3$ + C6H$_3$	6
valine	grape	1.03 (d)	C4H$_3$ + C5H$_3$	6
2,3-butanediol	wine	1.13 (d)	C1H$_3$ + C4H$_3$	6
threonine	grape	1.32 (d)	C4H$_3$	3
ethanol	wine	1.16 (t), 3.62 (q)	C2H$_3$, C1H$_2$	5
lactate	wine	1.38 (d), 4.27 (m)	C3H$_3$	3
alanine	grape	1.47 (d)	C3H$_3$	3
arginine	grape	1.64−1.78 (m)	C3H$_2$	2
proline	grape	1.95−2.03 (m), 2.33 (m), 3.31 (m), 3.41 (m), 4.12 (m)	C4H$_2$ + C3H$_a$, C3H$_b$, C3H$_a$ C5H$_a$, C5H$_b$, C2H	8
glutamine	grape	2.38−2.47 (m)	C4H$_2$	2
GABA	grape	1.94 (m), 2.45 (t), 3.02(t)	C3H$_2$, C4H$_2$, C2H$_2$	6
acetate	wine	2.07 (s)	C2H$_3$	3
pyruvate	wine	2.37 (s)	C3H$_3$	
malate	grape	2.62−2.67 (dd), 2.79−2.85 (dd), 4.38−4.41 (dd)	C2H$_a$, C3H$_b$, C2H	3
citrate	grape	2.76 (d), 2.88 (d)	C2H$_a$ + C4H$_a$, C2H$_b$ + C4H$_b$	4
succinate	wine	2.65 (s)	C2H$_2$ + C3H$_2$	4
glycerol	wine	3.56 (m), 3.76 (m), 3.78 (tt)	C2H$_2$, C3H$_2$, C1H	5
tartarate	grape	4.43 (s)	C2H + C3H	2
fructose	grape	3.53−4.13 (m)		
β-glucose	grape	4.64 (d)	β C1H	1
α-glucose	grape	5.22 (d)	α C1H	1
gallic acid	wine	7.15 (s)	C2H + C6H	2
2-phenylethanol	wine	2.85 (t), 3.78 (m), 7.30 (m), 7.37 (m)	C2H$_2$, C1H$_2$OH, ring, ring	9
trigonelline	grape	8.01 (m), 8.82 (m), 9.12 (s)	C4H$_1$, C3H$_1$, C1H$_1$	3

[a] The chemical shifts were determined at pH 4.0 and expressed as relative values to those of DSS at 0 ppm. [b] Letters in parentheses indicate the peak multiplicities: s, singlet; d, doublet; t, triplet; dd, doublet of doublet; tt, triplet of triplets; q, quartet; and m, multiplet.
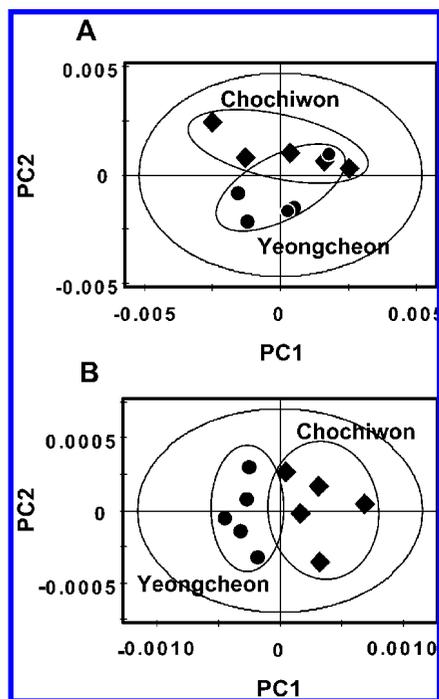
Figure 3. Principal component analysis (PCA) scores plot derived from the ^1H NMR spectra of pulp from Yeongcheon (circles) and Chochiwon (diamonds) before (**A**) and after (**B**) excluding sugar regions. Exclusion of sugar regions in PCA models improved predictability as indicated by increases of R_X^2 and Q^2 from 0.52 to 0.91 and from 0.15 to 0.84, respectively.

(*35*). Although the relationship between citrate and climatic factors is not clear, some studies have shown that phospho-enolpyruvate carboxykinase (PEPCK) might function in the catabolism of malate and citrate (*36−38*). PEPCK is the enzyme that decarboxylates part of the oxaloacetate formed in the Krebs cycle. Oxaloacetate can be transformed into glucose by gluco-neogenesis with elimination of malate and citrate. This suggests that malate and citrate are strongly connected to each other; therefore, citrate could also be a key metabolite to discriminate regional differences.

Tartarate is a secondary product of the metabolism of sugars, and tartarate concentration remains relatively constant despite the increase in berry volume during maturation (*26*). Tartarate is difficult to metabolize and thus is often more stable than malate during maturation (*19*). Different levels of tartarate were observed only in skin extracts from the three different regions. However, tartarate contents of grape were not closely related to climatic conditions compared with malate and citrate contents.

Total Phenol in Grape Extracts. Phenolic compounds are secondary products of sugar catabolism. Environmental conditions can modify certain transformation speeds, sometimes to the point of upsetting the order of physiological changes in the ripening grape (*26*). This is caused by the competition between primary metabolism (growth) and secondary metabolism (accumulation) when grape sugar alimentation is poor (*26*). Many researchers have reported the effects of environmental factors such as sunlight exposure and temperature on the accumulation of phenolic compounds (*15, 39−41*). Sun-exposed grapes contain more flavonols than grapes grown in shady regions, but contain little anthocyanin due to high temperature (*15, 41*). Mori et al. (*42, 43*) demonstrated that higher temperature can result in less accumulation of anthocyanins and variations in phenolic composition. Also, the phenolic composition of shaded and light-exposed grape clusters remained the same in Cabernet Sauvignon (*44*).

No significant differences in total phenolic compound levels were observed among grape extracts from the three regions (**Table 2**). To investigate the differences in phenolic compounds according to different climate regions, more specific analytical methods such as HPLC might be necessary to measure total phenol contents.

Must Analyses. The sugar contents of musts of the grapes grown in Chochiwon were lower than those of grapes grown
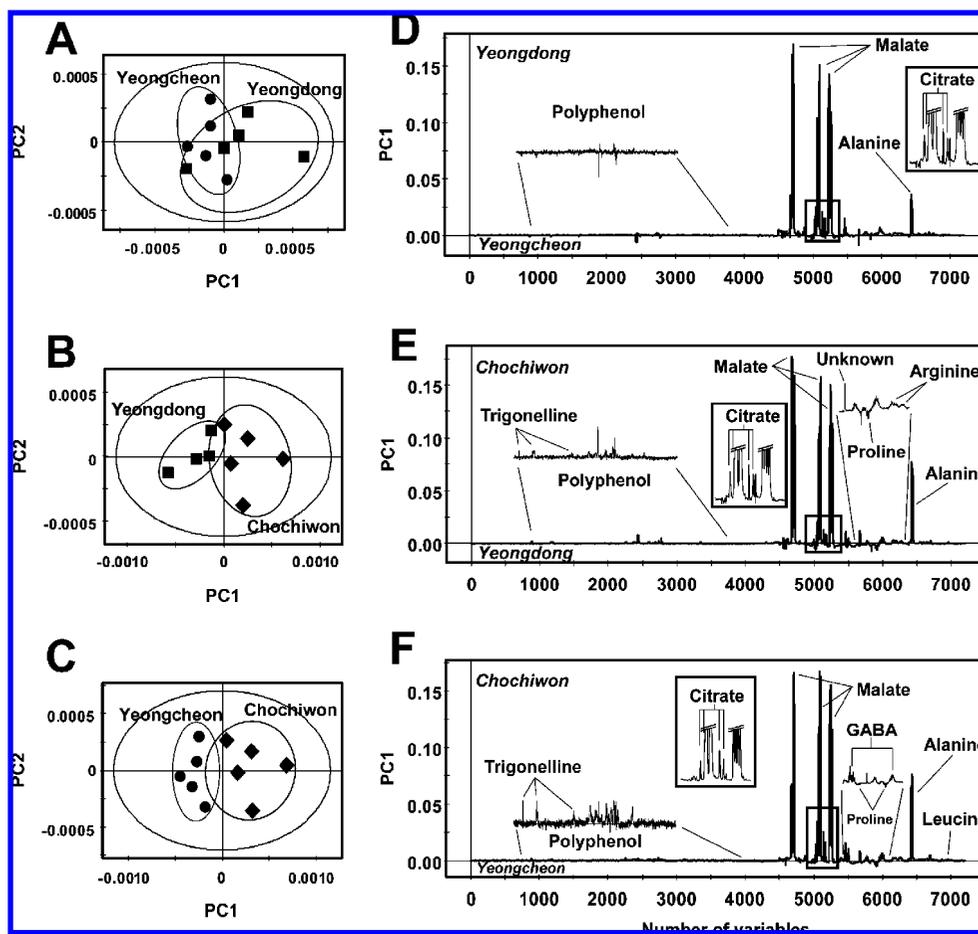
Figure 4. PCA score (**A**−**C**) and loading (**D**−**F**) plots derived from the ^{1}H NMR spectra of pulp extracts demonstrating the separation between pulps of Muscat Bailey A grapes from Yeongdong and Yeongcheon (**A**, **D**), Yeongdong and Chochiwon (**B**, **E**), and Yeongcheon and Chochiwon (**C**, **F**). Symbol: squares, Yeongdong; circles, Yeongcheon; diamonds, Chochiwon.

in the other two regions (**Table 2**). The accumulation of sugar in grapes is known to be strongly affected by climatic conditions. Our results agree with reports that grapes grown in regions with high light exposure, high temperature, and low rainfall contain more sugar than grapes grown in other environments (*26*). The relationship between sugar content in must and the climatic factors of temperature, rainfall, and length of light exposure was not further considered in this study because sugar was added to musts before yeast inoculation to produce wines with constant alcoholic content [approximately 13% (v/v)].

The titratable acidity was determined by neutralization with a sodium hydroxide solution of known normality. Musts from Chochiwon showed lower titratable acidity than those from the other two regions. This was inconsistent with the result that grape extracts from Chochiwon contain higher malate levels than grapes from the other regions. The titratable acidity of must takes into account all types of acids, that is, inorganic acids such as phosphoric acid and organic acids including malate and tartarate as well as amino acids. Furthermore, the contribution of each type of acid to the titratable acidity is determined by its strength, which is defined by its state of dissociation and the degree to which it has combined to form salts (*45*). Tartarate is converted to salt forms as ripening progresses, whereas malate exists entirely as a free acid in both pulp and skin at all stages (*46*). Thus, our results suggest that the concentrations of calcium and potassium in grapes from Yeongcheon and Chochiwon may result in increased salt formation, with a subsequent decrease in titratable acidity.

The UV spectra of red wines showed maximum values at

520 nm due to anthocyanins and their flavylium combinations and minimum values in the region of 420 nm. The color analysis of wine therefore requires optical density measurements of yellow at 420 nm and of red at 520 nm. In addition, optical density measurement at 620 nm provides the blue component in young red wines (*45*). The color intensity of grapes from Chochiwon was lower than of those of grapes from the other regions (**Table 2**). Exposure to sunlight increased the color intensity and the concentrations of phenolic compounds such as anthocyanin (*47, 48*). Low light exposure in Chochiwon could therefore be responsible for the low color intensity.

Metabolite Profiling in Grape Extracts by ^{1}H NMR Spectroscopy. Representative one-dimensional (1D) ^{1}H NMR spectra acquired from pulp and skin extracts and wines are shown in **Figure 2**. The metabolites were assigned on the basis of analysis of 2D NMR and spiking experiments and of information published elsewhere (*4, 5, 15, 49−51*). Twenty-five metabolites were identified in the ^{1}H NMR spectra of the grapes and wines (**Table 3**). Significant variations in the chemical shifts of most metabolites were not observed in the extracts, but rather in the wines, mainly due to large pH variations. The pH values of wine samples were therefore adjusted to 4.00 using oxalate buffer (400 mM, pH 4.0) to minimize the chemical shift variations. The ^{1}H NMR spectra were then aligned by the correlation optimized warping (COW) method (*17, 51*). The alignments were performed after exclusion of water regions, spectral reduction into 0.001 ppm, and normalization. Sugar resonances in ^{1}H NMR spectra of pulp and skin extracts were dominant as shown in **Figure 2A,B**. The
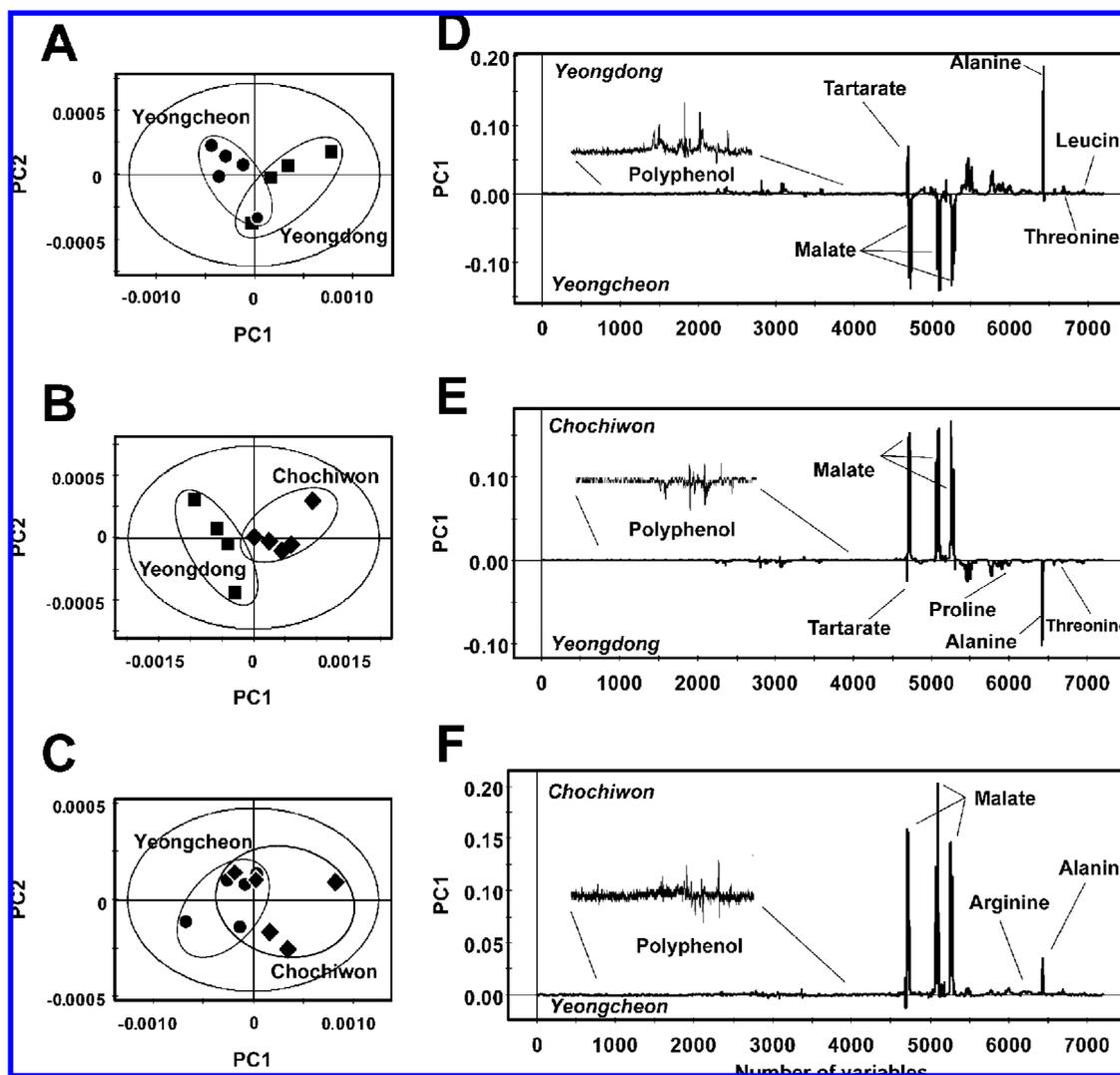
Figure 5. PCA score (**A**−**C**) and loading (**D**−**F**) plots derived from the ^{1}H NMR spectra of skin extracts demonstrating the separation between skins of Muscat Bailey A grapes from Yeongdong and Yeongcheon (**A**, **D**), Yeongdong and Chochiwon (**B**, **E**), and Yeongcheon and Chochiwon (**C**, **F**). Symbols: squares, Yeongdong; circles, Yeongcheon; diamonds, Chochiwon.

huge resonances of sugars in the NMR spectra of pulp and skin extracts prevented development of PCA models with high predictability. **Figure 3A** shows the PCA score plot of pulp spectra between Yeongcheon and Chochiwon, showing poor predictability as indicated by the Q^2 value of 0.15. Therefore, sugar regions from 3.10 to 4.30 ppm and from 4.60 to 5.50 ppm were excluded to improve the predictability of the PCA model, and the PCA model was then regenerated. After exclusion of the sugar regions, the statistical values of R_X^2 and Q^2 in the PCA model increased from 0.52 to 0.91 and from 0.15 to 0.84, respectively, resulting in clearer separations between Yeoncheon and Chochiwon pulp extracts (**Figure 3B**). R_X^2 represents the goodness of fit of the PCA model, and Q^2 reveals the predictability of the PCA model.

Panels **A**−**C** of **Figure 4** show the PCA score plots (PC1/PC2) derived from the ^{1}H NMR spectra of pulp extracts from the three regions for visualizing separations among these extracts. PCA modeling between Yeongdong and Yeongcheon, between Yeongdong and Chochiwon, and between Yeongcheon and Chochiwon revealed R_X^2 and Q^2 values of 0.77 and 0.37, 0.80 and 0.65, and 0.91 and 0.84, respectively. The PCA score plot between pulp extracts from Yeongdong and Yeongcheon showed separation by the first principal component (PC1), with overlapping of some samples, resulting in low predictability as indicated by Q^2 of 0.37 (**Figure 4A**). The complementary PCA

loading plot indicated the metabolites that contributed to the separation (**Figure 4D**). The upper section of the loading plot represents metabolites that were higher in Yeongdong grape extracts, whereas the lower section represents metabolites that were lower. Malate, citrate, and alanine levels were increased in pulp extract from Yeongdong compared to that from Yeongcheon. Clearer separation between pulp extracts from Yeongdong and Chochiwon was observed (**Figure 4B**). The loading plot showed relatively high levels of alanine, arginine, malate, citrate, trigonelline, and unidentified compounds at δ 2.23 (singlet) and 7.57 (doublet), together with lower levels of proline and an unknown compound at δ 2.06 (singlet) in pulp extract from Chochiwon compared to those from Yeongdong (**Figure 4E**). The PCA score plot between pulp extracts from Yeongcheon and Chochiwon also showed a clear separation (**Figure 4C**). The separation was caused by higher levels of malate, citrate, leucine, threonine, alanine, γ-aminobutyrate, trigonelline, and unidentified compounds at δ 1.92 (singlet), 2.13 (doublet), and 2.43 (triplet), together with lower levels of proline in the pulp extract from Chochiwon compared to that from Yeongcheon (**Figure 4F**).

Panels **A**−**C** of **Figure 5** show the PCA score plots (PC1/PC2) derived from the ^{1}H NMR spectra of skin extracts from the three regions, with high values of R_X^2 from 0.78 to 0.87 and Q^2 from 0.43 to 0.62. The PCA score plots revealed clear
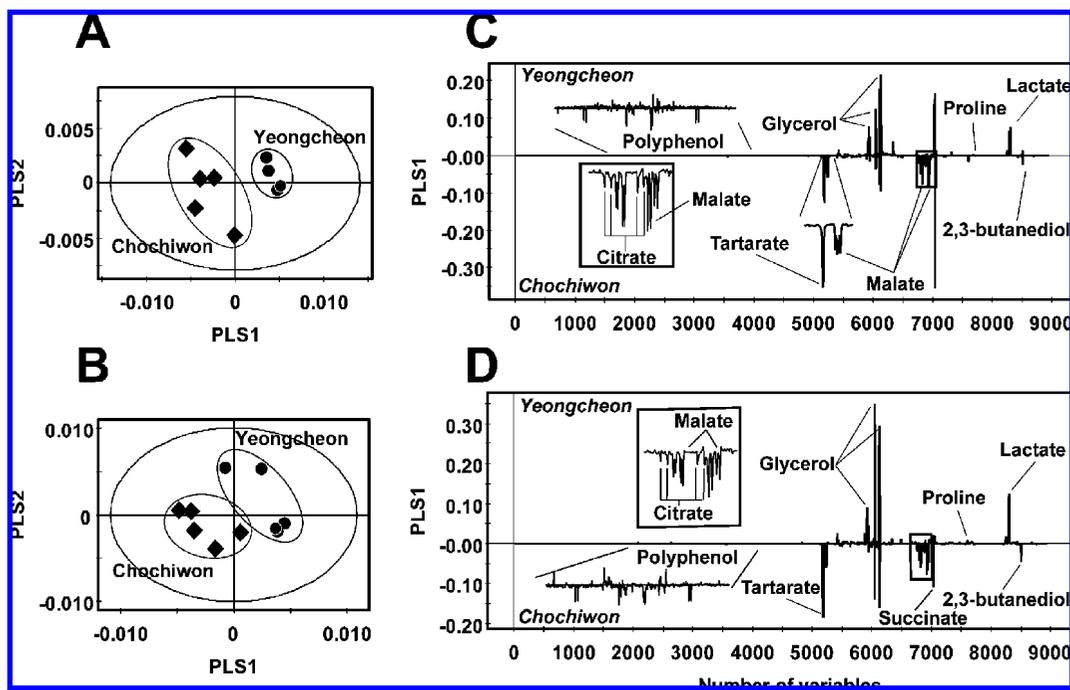
Figure 6. PLS-DA score (**A**, **B**) and loading (**C**, **D**) plots derived from the ^{1}H NMR spectra of wines from Muscat Bailey A grapes harvested in Yeongcheon (circles) and Chochiwon (diamonds) and aged for 3 months (**A**, **C**) and 6 months (**B**, **D**).

separations between skin extracts. The loading plot of skin extracts from Yeongdong and Yeongcheon showed that the skin extract of Yeongdong contained more leucine, threonine, alanine, and polyphenol compounds but less malate compared to that from Yeongcheon (**Figure 5D**). In the comparisons of skin extracts from Yeongdong and Chochiwon, higher levels of threonine, alanine, proline, and tartarate and lower levels of malate were observed in Yeongdong (**Figure 5E**). The loading plot of skin extracts from Yeongcheon and Chochiwon showed that the separation was dominated by increases in alanine, arginine, and malate levels in Chochiwon (**Figure 5F**). No significant differences in metabolite levels were observed in seed extracts from the three regions (data not shown). Berries in shaded and sun-exposed conditions that depend on the side of the row (east or west), the local leaf density, and the position of the berries outside or inside the canopy in the same vineyard have shown differences in chemical composition or metabolite, demonstrating the effects of microclimates (*15*). In the present study, we investigated metabolic differences in grapes from geographical regions considerably far from each other, demonstrating effects of geographical climate on metabolites of grape. Unfortunately, we did not investigate whether microclimates could affect metabolites of grapes in local vineyards of three geographical regions or not, even though the largest heterogeneous localization of vineyards within Yeongcheon as compared with other vineyards may be due to the small number of local grapes used, as shown in **Figure 1**.

Each amino acid profile is characterized by a climatic condition (*52*). Amino acid contents in pulp and skin showed similar patterns in response to regions. Grapes with low sun exposure in Chochiwon contained more alanine, arginine, leucine, and GABA but less proline in the pulp. However, grapes with more sun exposure in Yeongcheon contained less leucine, threonine, alanine, and arginine but more proline in the skin. These results are consistent with reports on metabolic differences according to environmental conditions (*15, 53*).

At the mature stage, arginine is often the predominant amino acid, representing 6−44% of the total nitrogen in grape juice

(*26*). Arginine plays a very important role as the main storage amino acid in grape berry nitrogen metabolism (*54*). The proline concentration can increase during maturation through arginine transformation. In sun-exposed berries, less arginine and more proline were found, indicating greater accumulation of proline in sun-exposed berries than in shaded berries (*15*). Net accumulation of free proline in the developing grape berry may result from changes in the balance of metabolite transport into the berry, biosynthesis and degradation of proline, and its incorporation into alternative nitrogen sinks such as cellular protein (*53*). The enzymes involved in proline synthesis and degradation are controlled by plant responses to the environment (*55*). Thus, the activity of the enzymes is affected by sun exposure. Of the prominent amino acids in must, proline is not used by yeast as a nitrogen nutrient. Therefore, the proline content in the grape may be important to give wine the perceived "mouthfeel" or "body", because proline is a component of salivary proline-rich proteins, which have a strong affinity for polyphenols and contribute to mouthfeel (*56, 57*). The high level of proline in wines indicated longer or stronger light exposure for these grapes. Alanine and threonine were also very significant biomarkers for distinguishing among the regions and were lower in regions of high sun exposure in this study. However, we did not explore the relationships between alanine and threonine and the environmental factors.

Metabolite Profiling in Wines by ^{1}H NMR Spectroscopy. Wines vinified with grapes from three different regions were analyzed to determine whether there were significant differences in metabolite concentrations. PLS-DA models were applied to ^{1}H NMR spectra data sets of wines aged for 3 and 6 months. The PLS-DA model was used to maximize covariance between measured data (X) and the response variable (Y). Panels **A** and **B** of **Figure 6** show the PLS-DA score plots separating wines from Yeongcheon and Chochiwon that were aged for 3 and 6 months; the values of R_X^2, R_Y^2, and Q^2 were 0.43, 0.95, and 0.49 at 3 months and 0.46, 0.87, and 0.24 at 6 months. Panels **C** and **D** of **Figure 6** show the pairwise comparison of Yeongcheon and Chochiwon wines aged for 3 and 6 months,

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respectively. The PLS-DA loading plot showed relatively high levels of lactate, proline, and glycerol but low levels of 2,3-butanediol, malate, tartarate, citrate, and succinate in Yeong-cheon wines compared with Chochiwon wines aged for 3 and 6 months. Metabolites such as sugars and amino acids are consumed by yeast as nutrients, whereas glycerol, 2,3-butanediol, lactate, succinate, and other volatile acids are produced during fermentation and aging (*58*). Although some metabolites changed during fermentation and aging, PLS-DA models revealed regional discrimination of wines aged for 3 and 6 months. These results indicate that the metabolites or chemical compositions of grapes grown in different climates affect the metabolites of their wines.

Conclusions. In the present study, conventional targeted analysis and global NMR spectroscopic analysis coupled with multivariate statistical data sets revealed that the chemical compositions or metabolites in the pulps, skins, seeds, musts, and wines harvested from different regions strongly depend on the duration of sun exposure and the precipitation in the environmental conditions. This approach will be useful for understanding the metabolic profile of grapes grown in specific environments and can be used to determine appropriate harvest times for grapes according to individual circumstances.

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