JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2008,** *56,* 8007–8016 **8007**

^{1}H Nuclear Magnetic Resonance-Based Metabolomic Characterization of Wines by Grape Varieties and Production Areas

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^{1}H NMR spectroscopy was used to investigate the metabolic differences in wines produced from different grape varieties and different regions. A significant separation among wines from Campbell Early, Cabernet Sauvignon, and Shiraz grapes was observed using principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA). The metabolites contributing to the separation were assigned to be 2,3-butanediol, lactate, acetate, proline, succinate, malate, glycerol, tartarate, glucose, and phenolic compounds by PCA and PLS-DA loading plots. Wines produced from Cabernet Sauvignon grapes harvested in the continental areas of Australia, France, and California were also separated. PLS-DA loading plots revealed that the level of proline in Californian Cabernet Sauvignon wines was higher than that in Australian and French Cabernet Sauvignon, Australian Shiraz, and Korean Campbell Early wines, showing that the chemical composition of the grape berries varies with the variety and growing area. This study highlights the applicability of NMR-based metabolomics with multivariate statistical data sets in determining wine quality and product origin.

KEYWORDS: Wine; NMR; metabolomics; metabolites; PCA; PLS-DA

INTRODUCTION

Metabolomics is a promising new approach aimed at improving our understanding of metabolic perturbations in drug toxicity (*1*−*3*), disease status (*4, 5*), and dietary intervention (*6, 7*). Metabolomics is defined as the comprehensive and quantitative analysis of all metabolites (*8*). The term metabonomics, which is defined as the quantitative measurement of the time-related multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification, is often used instead of metabolomics (*9*). Most commonly, metabolomics is supported by mass spectrometry (MS) and nuclear magnetic resonance (NMR) as parallel technologies that provide an overview of the metabolome and high-power compound elucidation. The combination of liquid chromatography (LC)-MS and NMR is a powerful methodology for identifying metabolites (*10*). The advent of powerful chemical analytical equipment and techniques with multivariate statistical modeling has led to increased adoption of large-scale metabolic analyses. NMR has the advantages of being nondestructive and intrinsically more information-rich with respect to the determination of molecular structures, especially in complex mixture analyses. NMR measurements, coupled with multivariate statistical, chemometric methods for the purpose of latent information extraction and sample classification, offer a powerful new approach for assessing metabolic function. Pattern recognition and related multivariate statistical approaches can be used to discern significant patterns in complex data sets and aim at classifying objects by identifying inherent patterns in a set of indirect measurements. In addition, pattern recognition methods can reduce the dimensionality of complex data sets by means of two- (2D) or three-dimensional (3D) mapping procedures, thereby facilitating the visualization of inherent patterns in the data. Principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA) are often used in the multivariate statistical pattern recognition methods (*11*). To provide high-quality metabolomic data, standardized work should be considered from the point of data acquisition to the validation of a statistical model (*2, 12, 13*). Most recently, the dynamic biochemical composition within living systems has proven to be fundamental systems biology, which attempts to

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10.1021/jf801424u CCC: $40.75
Published on Web 08/16/2008

Table 1. List of Red Wines

sample no.	name	grape varieties	vintage	country
17	1755 Estd	Cabernet Sauvignon	2006	France
18	in-house	Campbell Early	2006	Korea
19	in-house	Campbell Early	2004	Korea
20	in-house	Campbell Early	2007	Korea
21	Redwood Creek	Cabernet Sauvignon	2004	California, United States
22	in-house	Campbell Early	2004	Korea
23	in-house	Campbell Early	2004	Korea
24	in-house	Campbell Early	2006	Korea
25	Teuning Leaf Gallo	Cabernet Sauvignon	2005	California, United States
26	in-house	Campbell Early	2004	Korea
27	Ernest & Julio Gall	Cabernet Sauvignon	2005	California, United States
28	in-house	Campbell Early	2005	Korea
29	in-house	Campbell Early	2006	Korea
30	in-house	Campbell Early	2005	Korea
31	Calvet	Cabernet Sauvignon	2005	France
32	Blue Nun	Cabernet Sauvignon	2005	France
53	Bin 50	Shiraz	2006	Australia
54	Wombat Hill	Shiraz	2006	Australia
55	Yellow Tail	Shiraz	2006	Australia
56	Hardys	Shiraz	2005	Australia
57	Australian Reserve	Shiraz	2006	Australia
58	Wolf Blass Yellow Label	Cabernet Sauvignon	2005	Australia
59	Wombat Hill	Cabernet Sauvignon/ Merlot	2004	Australia
60	Yellow Tail	Cabernet Sauvignon	2006	Australia
61	Hardys	Cabernet Sauvignon	2007	Australia
62	Lindemans Bin 45	Cabernet Sauvignon	2006	Australia
63	in-house honey wine		2005	Korea
64	in-house	Campbell Early	2004	Korea

synergistically integrate gene expression (transcriptomics), protein translation (proteomics), and the metabolite network (metabolomics) data sets to provide a more holistic overview of living systems (*14*−*18*).

Metabolomic studies have been applied in the food sciences, as well, especially in nutrition investigations. These studies have revealed alterations of urinary metabolites after uptake of natural products (*6, 7*) and the potential to elucidate the role of bioactive foods in diseases (*19*). However, there are few studies on metabolomic approaches in fermented foods, to take account of the metabolic changes that occur during fermentation and to evaluate the quality of the foods. Wine is a microbial process product and contains many metabolites observed from grapes and from the alcoholic and malolactic (ML) fermentations. These metabolites may affect wine quality. The metabolites in wine could be affected by the "terroir", which accounts for the factors of climate, soil, and cultural practices, and by the variability of the grape composition (*20*−*23*).

In the present work, we have applied state-of-the-art metabolomic approaches to characterize the metabolites in wines vinified in different continental areas and from different grape varieties. This study was undertaken as part of the initial efforts to improve the quality of wine vinified in Korea.

MATERIALS AND METHODS

Samples. Red wines vinified with Cabernet Sauvignon and Shiraz grapes were purchased from a market in Korea. In-house red wines vinified with Campbell Early grapes were obtained from a winemaker in Korea (**Table 1**).

^{1}H NMR Spectroscopic Analysis of Wines. One milliliter of wine was lyophilized in a 1 mL Eppendorf tube and dissolved in 99.9% deuterium oxide (400 μL, D_2O), mixed with 400 mM oxalate buffer (140 μL, pH 4.0) and 5 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (60 μL, DSS, 97%), and then 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 (^{1}H, δ 0.00), respectively. ^{1}H NMR spectra were recorded on a Varian VnmrS 600 spectrometer, operating at 599.84 MHz ^{1}H frequency and a temperature of 298 K, using a 5 mm ^{1}H{^{13}C/^{15}N} triple resonance indirect detection probe. The noesypresat pulse sequence was applied to suppress the residual water signal. For each sample, 16 transients were collected into 76924 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 suite4.6 software, professional edition (Chenomx Inc., Canada). The NMR spectral data were reduced into 0.001 ppm spectral buckets, while the region corresponding to water (4.6−4.8 ppm) was removed. In addition, the regions of residual ethanol (1.15−1.20 and 3.59−3.72 ppm) from incomplete removal during lyophilization and of DSS (−0.5−0.5, 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 (*24*). The resultant data sets were then imported into SIMCA-P version 11.0 (Umetrics, Umeå, Sweden) for multivariate statistical analysis. Signal assignment for representative samples was facilitated via acquisition of 2D total correlation spectroscopy (TOCSY), spiking experiments, and comparison to literature. In addition, Chenomx NMR suite4.6 software was also utilized to assign the metabolites in wine.

Multivariate Data Analysis. The mean center was applied for all multivariate analysis by SIMCA-P version 11.0. PCA, and 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. The PLS-DA can be described as the regression extension of PCA, giving the maximum covariance between measured data (X) and the response variable (Y). PLS-DA models were calculated to compare continental Cabernet Sauvignon wines from France, California in the United States, and Australia. The quality of the PLS-DA models was described by the total variance of PLS1 and PLS2 at a confidence level of 95%.

Chemicals. All chemical reagents were of analytical grade. D_2O (99.9%), DSS (97%), 2-phenylethanol (2-PE), and gallic acid were purchased from Sigma (St. Louis, MO).

RESULTS

^{1}H NMR Spectroscopy of Wine. Representative one-dimensional (1D) ^{1}H NMR spectra of wines vinified from Campbell Early grapes harvested in Korea, Cabernet Sauvignon grapes in France, Australia, and California, and Shiraz grapes in Australia are shown in **Figure 1**. The assignments of the metabolites have been carried out on the basis of the analysis of 2D NMR and spiking experiments and of information published elsewhere (*20*−*22, 25*). Sixteen metabolites were identified in the ^{1}H NMR spectra of the wines (**Figure 2** and **Table 2**). As shown in **Figure 1**, malic acid was observed only in Korean Campbell Early wines because these wines were not fermented by ML bacteria, which convert malic acid to lactic acid. Significant variations in the chemical shifts of most metabolites were observed in all wines, mainly due to large pH variations. The pH values of samples ranged from 3.40 to 4.20 and were therefore adjusted to 4.00 using oxalate buffer (400 mM, pH 4.0) to minimize the chemical shift variations. However, a real pH was not fixed to 4.00 because of different compositions or levels of organic acids in the wines; thus, we still expected chemical shift variations, even in buffer. ^{1}H NMR spectra were therefore aligned by the COW method (*24*). The

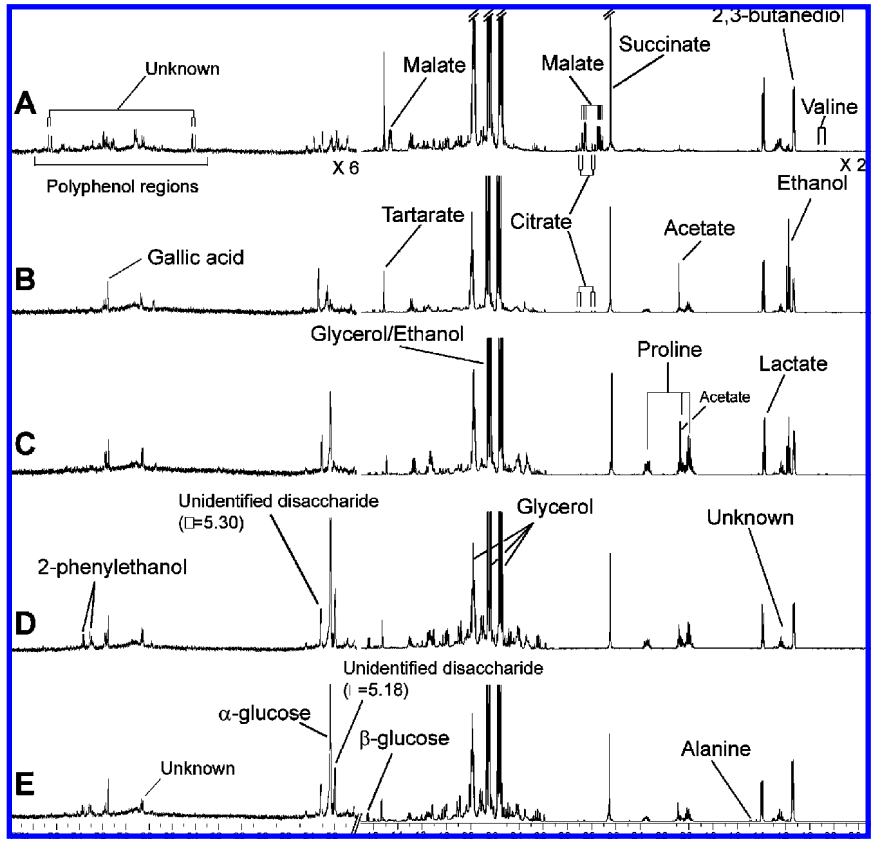

Figure 1. Representative ^{1}H NMR spectra of wines vinified with Campbell Early grapes harvested in Korea (**A**), with Cabernet Sauvignon harvested in France (**B**), California (**C**), and Australia (**D**), and with Shiraz harvested in Australia (**E**).

Table 2. Metabolites and Their ^{1}H Chemical Shifts Identified by 600 MHz ^{1}H NMR[a]

compound	^{1}H NMR chemical shift (s)[b]	group	^{1}H no.
valine	0.87(d), 0.93(d)	$C4H_3 + C5H_3$	6
2,3-butanediol	1.13(d)	$C1H_3 + C4H_3$	6
ethanol	1.18(t), 3.64(q)	$C2H_3 + C1H_2$	5
lactic acid	1.38 (dd) 4.29 (m)	$C3H_3 + C2H$	4
alanine	1.48 (d), 3.80 (q)	$C3H_3$	3
proline	2.00 (m), 2.07 (m), 2.35(m), 3.33 (m), 3.41 (m), 4.12 (m)	$C4H_2 + C3H_a$, $C3H_b$, $C3H_a$, $C5H_a$, $C5H_b$, C2H	8
acetic acid	2.08(s)	$C3H_3$	3
malic acid	2.73 (dd), 2.86 (dd) 4.46 (dd)	$C2H_a + C3H_b + C2H$	3
citric acid	2.79(d), 2.91(d)	$C2H_a + C4H_a$, $C2H_b + C4H_b$	4
succinic acid	2.64(s)	$C2H_2 + C3H_2$	4
glycerol	3.56(m), 3.76(m), 3.78(tt)	$C2H_2$, $C3H_2$, C1H	5
tartaric acid	4.51(s)	C2H + C3H	2
α-glucose	4.64(d)	αC1H	1
β-glucose	5.23(d)	βC1H	1
gallic acid	7.15(s)	C2H, C6H	2
2-PE	2.85(t), 3.78(m), 7.30(m), 7.37(m)	$C2H_2$, $C1H_2OH$, ring, ring	9

[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 doublets; tt, triplet of triplets; q, quartet; and m, multiplet.

alignments were performed after exclusion of water and residual ethanol regions, spectral reduction into 0.001 ppm, and normalization. **Figures 3A**,**B** show the raw and aligned ^{1}H NMR spectra of 28 wines, respectively, showing no further chemical shift variations in the resonances of 2,3-butanediol, lactic acid, and alanine. No chemical shift variations of other metabolites were observed after alignment by the COW algorithm (data not shown).

In addition to residual ethanol during lyophilization, acetic acid levels were not constant among all samples due to incomplete lyophilization, a leading problem of reproducibility. The inconstant content of acetic acid was observed by inspection of 1D NMR spectra given in **Figure 1** and confirmed by comparing the ratio of acetic acid to proline concentrations in lyophilized and nonlyophilized wine samples, obtained using Chenomx software (*26*). The ratio of acetic acid to proline in lyophilized wine was reduced to half-level of that in nonlyophilized wine, whereas the ratio of lactic acid was not changed after lyophilization (data not shown). It was of interest to note that no changes in 2,3-butanediol concentrations after lyophilization were observed even in the volatile compounds such as acetic acid. These results could be from more strong binding of 2,3-butanediol, which have two hydroxyl groups, to other compounds as compared to acetic acid, which have one carboxyl group. The problems in reproducibility of acetic acid according to preconcentration methods have also been reported (*27*). A change in acetic acid levels was therefore not considered in this study.

Metabolic Characterization of Korean Campbell Early Wine. PCA identified three outlier wine samples, as shown in **Figure 4**. Of these outlying spectra, sample number 63 was an in-house honey wine, showing a quite different wine to be separated by PCA. Furthermore, sample numbers 23 and 64, of which 64 is a repeated sample preparation and NMR measurement of 23, were also outlier due to their intrinsic properties. These outliers of 23 and 64 show the reproducibility of ^{1}H NMR measurement and multivariate analysis. As outliers, these three samples were excluded from subsequent analysis. There was one wine, sample number 20, of 11 Korea Campbell Early wines that was found to have very different spectral properties with others in the 1D NMR spectrum. This wine has

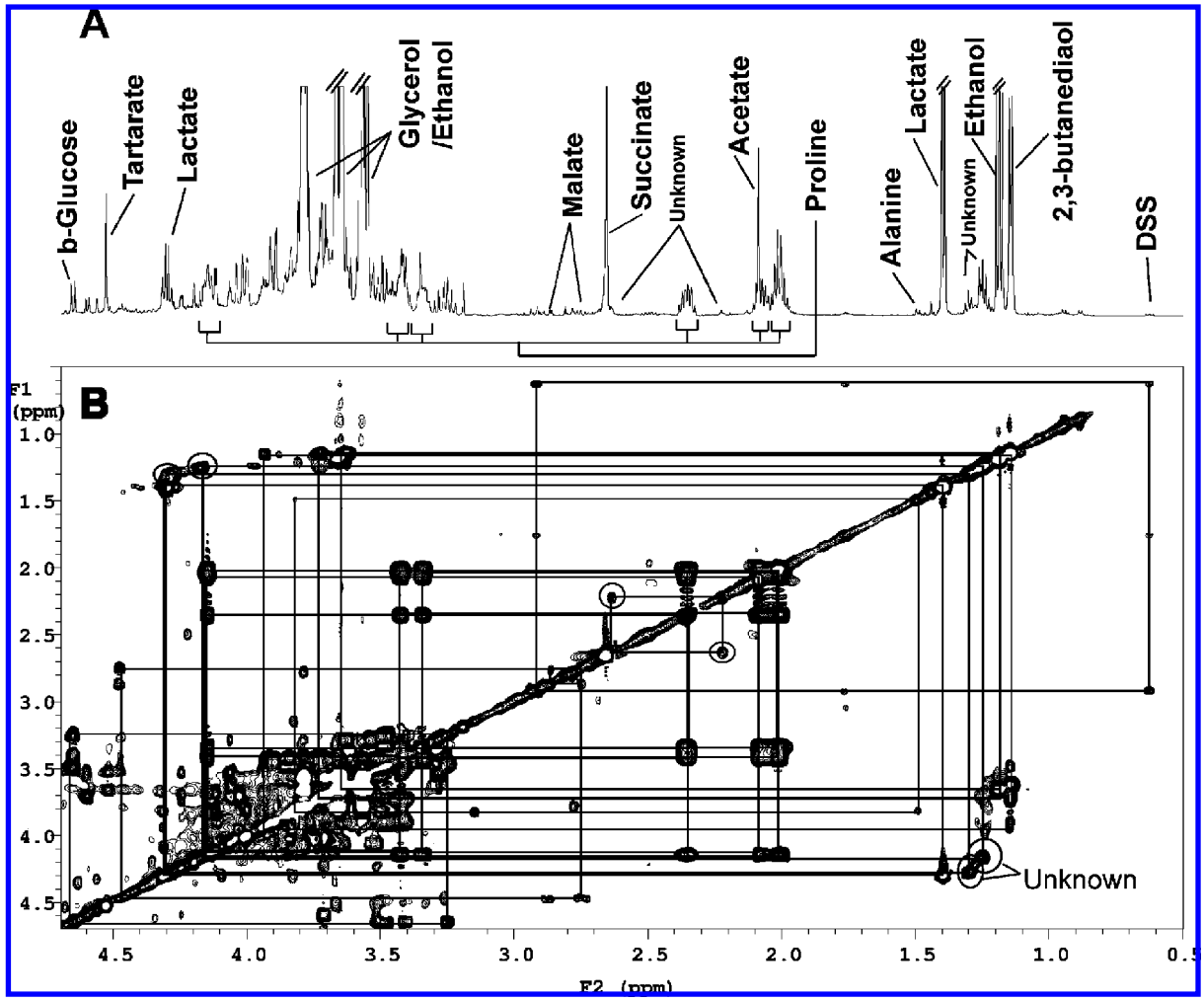

Figure 2. Two-dimensional (2D) $^1H-^1H$ TOCSY NMR spectrum of French Cabernet Sauvignon wine showing the assignment of major peaks. The 2D contour plot (**B**) corresponds to 1D spectrum acquired using PRESAT pulse sequence (**A**). Solid rectangles in panel **B** trace the ^1H-covalent network of protons as pairs of symmetric cross-peaks with respect to the diagonal axis. Solid rectangles represent metabolites having a normal number of cross-peaks, and proline reveals many cross-peaks.

been produced by wine maker's own method, especially during alcoholic fermentation, rather than that of a normal wine. This wine was removed prior to multivariate analysis.

Figure 5A shows the PCA score plot (PC1/PC2) that accounted for 74.8% of the total variance of the data set and shows the clear separation between Korean Campbell Early and Australian Cabernet Sauvignon wines by the first axis (PC1). The PCA loading plot revealed the metabolites that contributed to the separation (**Figure 5B**). The upper section of the loading plot represents metabolites that were higher in Korean Campbell Early wines, whereas the lower part represents metabolites that were lower. The separation was caused by an increase in metabolite resonances from 2,3-butanediol, alanine, succinic acid, malic acid, citric acid, glycerol, tartaric acid, and unknown polyphenols in Korean Campbell Early wines. Furthermore, increases in lactic acid, proline, α- and β-glucoses, gallic acid, and 2-PE in Australian Cabernet Sauvignon wines also contributed to the separation. Because 1D ^1H NMR spectra in **Figure 1** showed dramatic increases in malic and tartaric acids in Korean Campbell Early wines as compared to Australian Cabernet Sauvignon wines, the regions of malic acid (2.69−2.93 and 4.44−4.49 ppm), tartaric acid (4.50−4.54 ppm), and citric acid (2.77−2.79 and 2.90−2.92 ppm) were excluded from data matrix, and the PCA score (**Figure 5C**) and loading (**Figure 5D**) plots were then regenerated. After excluding these regions, the separation between these wines was still observed, with an increase in the total variance from 74.8 to 86.0%. These results indicated that other metabolites also contributed to the separation in addition to malic, tartaric, and citric acids. Korean Campbell Early wines could also be separated from French, Australian, and Californian Cabernet Sauvignon and Australian Shiraz wines.

Changes in Metabolites in Continental Cabernet Sauvignon Wines. To improve the separation among wines, based on maximizing covariance between the measured data (X) and the response variable (Y), a PLS-DA model was constructed, excluding the Korean Campbell Early wines. **Figure 6** shows the clear separations among French, Californian, and Australian Cabernet Sauvignon and Australian Shiraz wines, accounting for 44.3% of total variance. **Figure 7** shows the pairwise comparison of Australian, French, and Californian Cabernet Sauvignon wines. All three PLS-DA score plots displayed a continental discrimination of Cabernet Sauvignon wines, with total variances of 83.2, 70.6, and 82.3% for the French vs Californian wines, French vs Australian wines, and Californian vs Australian wines, respectively (**Figures 7A**,**C**,**E**). The PLS-DA loading plot showed relatively high levels of succinic and tartaric acids in French Cabernet Sauvignon wines, as well as unidentified disaccharides at $\delta = 5.18$ (doublet) and 5.30 (doublet), as compared to Californian Cabernet Sauvignon

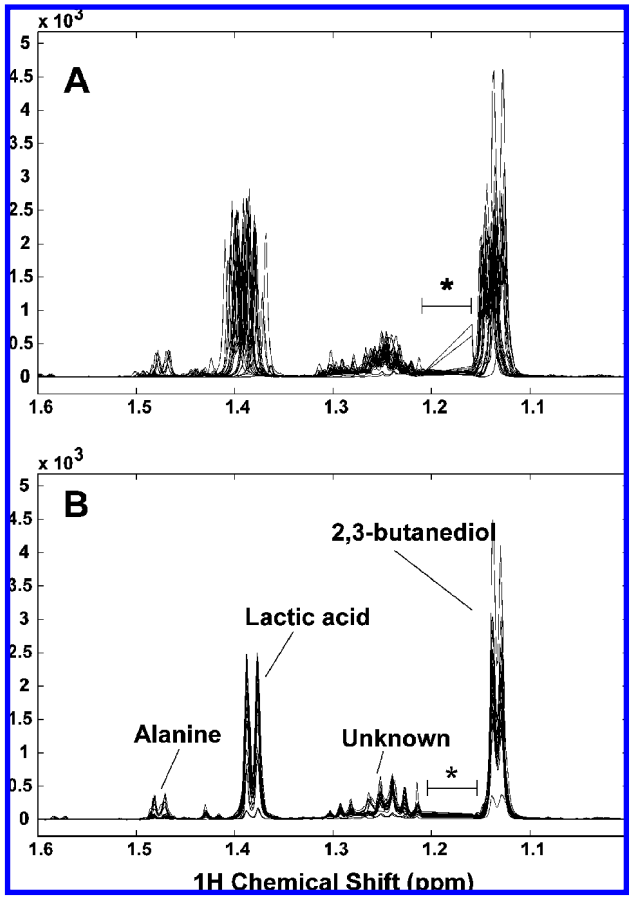

Figure 3. Raw (**A**) and aligned (**B**) ^{1}H NMR spectra of 2,3-butanediol, lactic acid, and alanine in 28 wines. Whole spectra were aligned using the COW method. The chemical shift variations of other metabolites were found and then corrected. The regions from 1.155 to 1.210 ppm (marked) and from 3.593 to 3.723 ppm (spectra not shown) were residual ethanol peaks due to incomplete removal during lyophilization and were thus cut to ensure that there was no effect of ethanol on the multivariate statistical analysis.

wines. Furthermore, higher levels of proline were observed in Californian Cabernet wines (**Figure 7B**). Other metabolites and phenolic compounds in French and Californian Cabernet wines were not significantly different. Comparing French and Australian Cabernet Sauvignon wines, higher levels of α- and β-glucose were observed in French wines. However, levels of proline, tartaric acid, gallic acid, and 2-PE were higher in Australian Cabernet Sauvignon wines than in French Cabernet Sauvignon wines (**Figure 7D**). Proline and α- and β-glucose were higher, but 2,3-butanediol, glycerol, tartaric acid, succinic acid, unidentified disaccharide ($\delta = 5.18$), gallic acid, and 2-PE were lower in Californian Cabernet Sauvignon wines than in Australian Cabernet Sauvignon wines (**Figure 7F**).

Australian Cabernet Sauvignon and Shiraz Wines. To see the metabolic differences in wines from different grape varieties, the metabolites of Australian Shiraz and Cabernet Sauvignon wines were analyzed. The PLS-DA score plot accounted for 37.7% of total variance showing the clear separation (**Figure 8A**) between the wines. The loading plot (**Figure 8B**) showed that the separation was dominated by increases in 2,3-butanediol, tartaric acid, and α- and β-glucose in Australian Shiraz wines. However, the levels of proline and succinic acid were higher in Australian Cabernet Sauvignon wines than in Australian Shiraz wines. No significant difference in phenolic compounds between the wines was observed except for gallic acid.

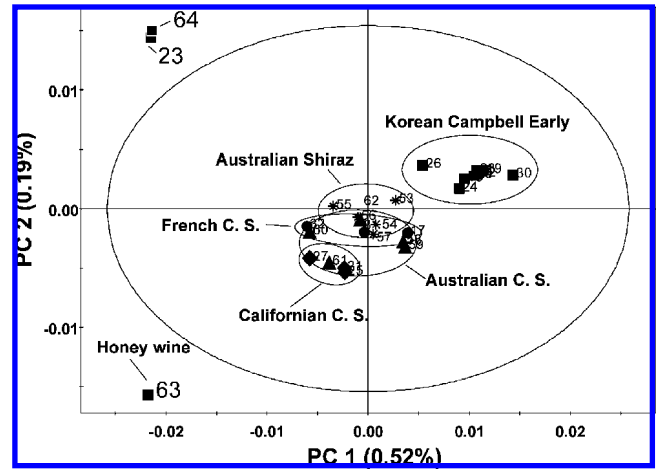

Figure 4. PCA scores plot derived from the ^{1}H NMR wine spectra. The plot shows the clear separation of Korean Campbell Early (square) wines from Australian Shiraz (star) and Australian (triangle), French (circle), and Californian Cabernet Sauvignon (diamond) wines. The plot shows the ability of ^{1}H NMR-based multivariate analysis in differentiating the quite different honey wine (number 63) from grape wines and its reproducibility giving the same outlying position of numbers 23 and 64, where number 64 was a repeated sample preparation and measurement of 23. C.S. indicates Cabernet Sauvignon.

Furthermore, 2-PE of high alcohols was higher in Cabernet Sauvignon wines. This indicated that wines from grape varieties grown in southeastern Australia could also be discriminated in the multivariate analysis according to their metabolites.

DISCUSSION

Recently, the effects of "terroir", that is, the factors of climate, soil, and cultural practices on grape and wine quality and on the variability of the grape composition, have been investigated using ^{1}H NMR and chemometrics (*20, 21*). The effects of the terroir on the metabolites of pulps and skins of grape berries have been reported and caused separation of the grape varieties according to multivariate statistical analysis like PCA and PLS-DA. Light and temperature significantly affect the contents of phenolic compounds, organic acid, and amino acids of grape berries. Pereira et al. (*22*) have reported that the most significant effect on discrimination of wines inside a vintage is from soil, and the vintage and grape variety also affect the wines. The present study was designed to show the effects of grape varieties and continental origin on metabolites in wines.

2,3-Butanediol (or 2,3-butylene glycol) is the major dialcohol found in wine and is a byproduct of fermentation, probably from pyruvic acid or reduction of acetoin. In other words, a lower level of 2,3-butanediol indicates accumulation of acetoin, which has a negative sensorial impact on wine with low taste and odor thresholds (*28*). Wine yeast strains that overproduce glycerol at moderate levels (12−18 g/L) almost completely reduced acetoin to 2,3-butanediol (*29*). Acetoin and 2,3-butanediol are therefore influenced both by the level of glycerol produced and by yeast strains, and their inverse correlation has been found in single spore cultures of a hybrid of *Saccharomyces cerevisiae* wine yeast (*30, 31*). 2,3-Butanediol is generally not expected to affect the sensory qualities of alcoholic beverages, due to its very high threshold value of about 150 mg/L. However, its concentration in wine can range from about 0.2 to 3 g/L, with a mean value of about 0.57 g/L (*32*). Therefore, 2,3-butanediol in wine may have some effect on the wine, giving a slightly bitter taste and viscous body. Glycerol is the most abundant

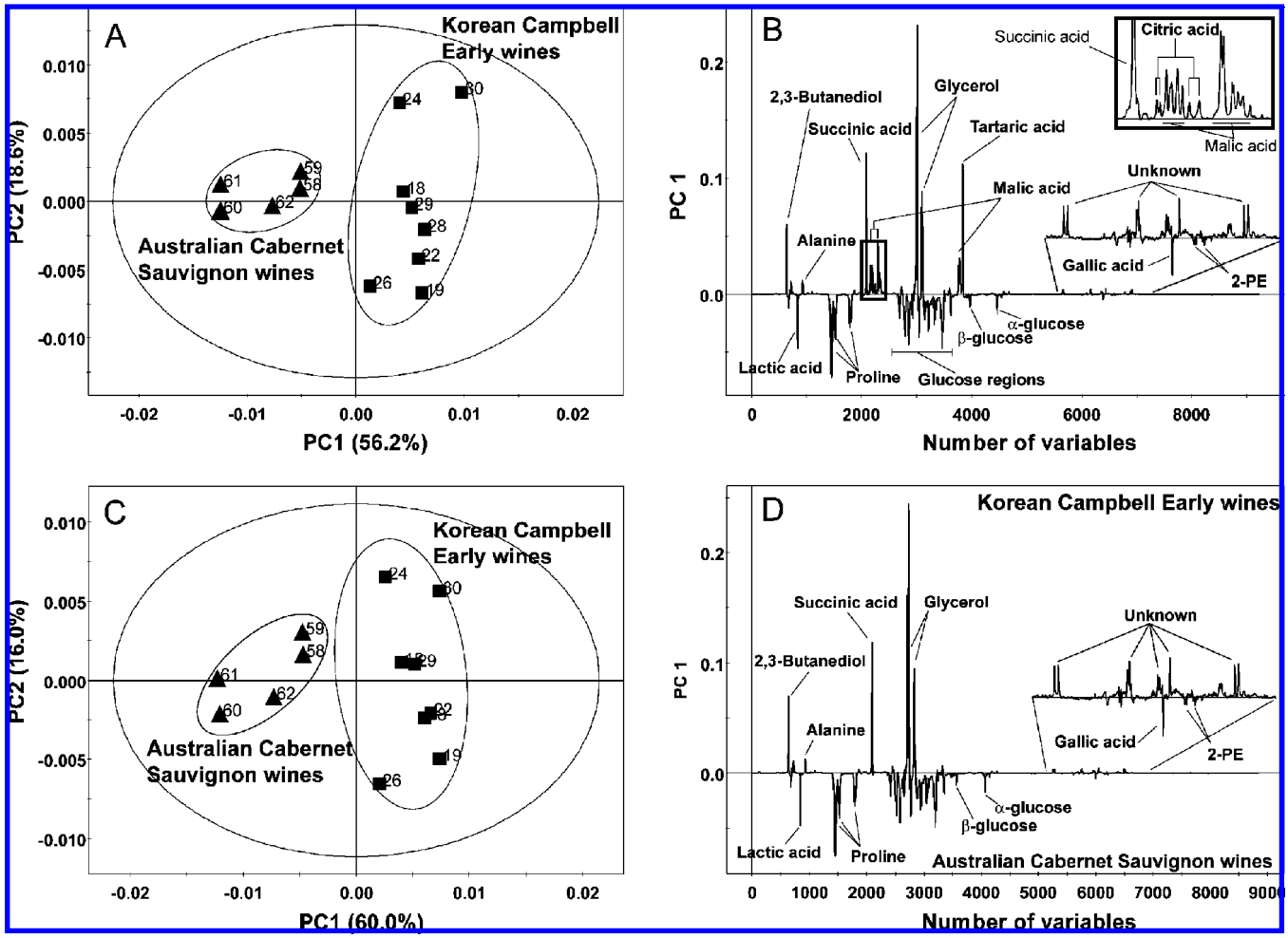

Figure 5. PCA score (**A** and **C**) and loading (**B** and **D**) plots derived from the ^{1}H NMR wine spectra demonstrating the separation between wines from Campbell Early grapes harvested in Korea (square) and from Cabernet Sauvignon grapes harvested in Australia (triangle). Panels **C** and **D** were regenerated from panels **A** and **B**, respectively, after excluding the regions of malic acid (2.69−2.93 and 4.44−4.49 ppm) and tartaric acid (4.50−4.54 ppm). The gray box inside panel **B** highlights the increase in citric acid in Korean Campbell Early wines. 2-PE indicates 2-phenylethanol.

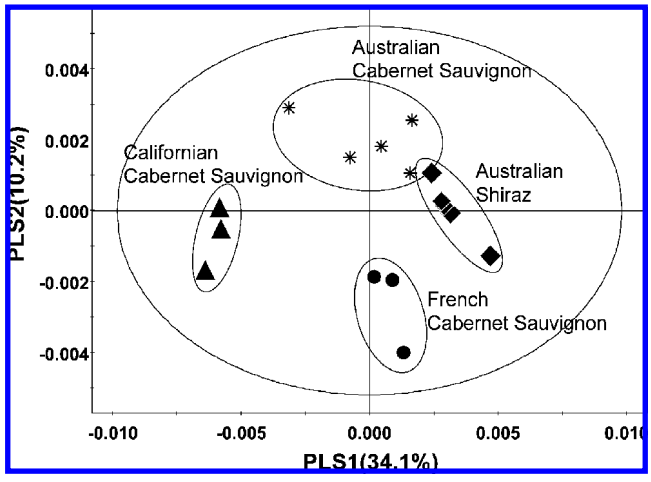

Figure 6. PLS-DA score plot derived from the ^{1}H NMR spectra of wines excluding Korean Campbell Early wines. The plot shows the clear discrimination of Australian Shiraz (square) and French (circle), Californian (triangle), and Australian (star) Cabernet Sauvignon wines.

byproduct of yeast fermentation. Many growth and environmental parameters have been reported to influence the final glycerol levels in wine. These include the grape variety and ripeness, the microbial flora on grape berries, and the sulfite concentration, pH, fermentation temperature, nitrogen source, and yeast strain (*33*−*35*). It is frequently suggested that glycerol contributes positively to wine quality. The perceived contribution has been defined in terms of mouth-feel and texture properties (*20*). Furthermore, at the concentration at which glycerol is normally found in wine, the impact that it could have on the viscosity of wine would probably not be perceived by even the most experienced tasters. At levels at which glycerol is normally found in wine, from 1.0 to 9.0 g/L, its primary contribution to the sensory properties of wine is sweetness. Furthermore, below a concentration of 25.8 g/L, glycerol does not produce a detectable increase in perceived viscosity (*36*). Glycerol overproduction is accompanied by acetic acid accumulation. Excessive production of acetic acid (above 1 g/L) is a major side effect, since the maximum amount desirable in wine is around 0.6 g/L. Abnormally high volatile acidity levels, however, are due to the breakdown of residual sugars, tartaric acid, and glycerol by anaerobic lactic bacteria.

Higher levels of 2,3-butanediol and glycerol were observed in Korean Campbell Early wines than in other wines under study (**Figure 5**). This result could indicate that yeast strains used in the fermentation of Korean Campbell Early wines produced more glycerol than those used in other wines. Furthermore, the Korean Campbell Early wines were chaptalized to increase the alcohol content of the final wine. Chaptalization, in which sugar is added to the grape must or juice before fermentation, is believed to enhance the synthesis of higher alcohols and is

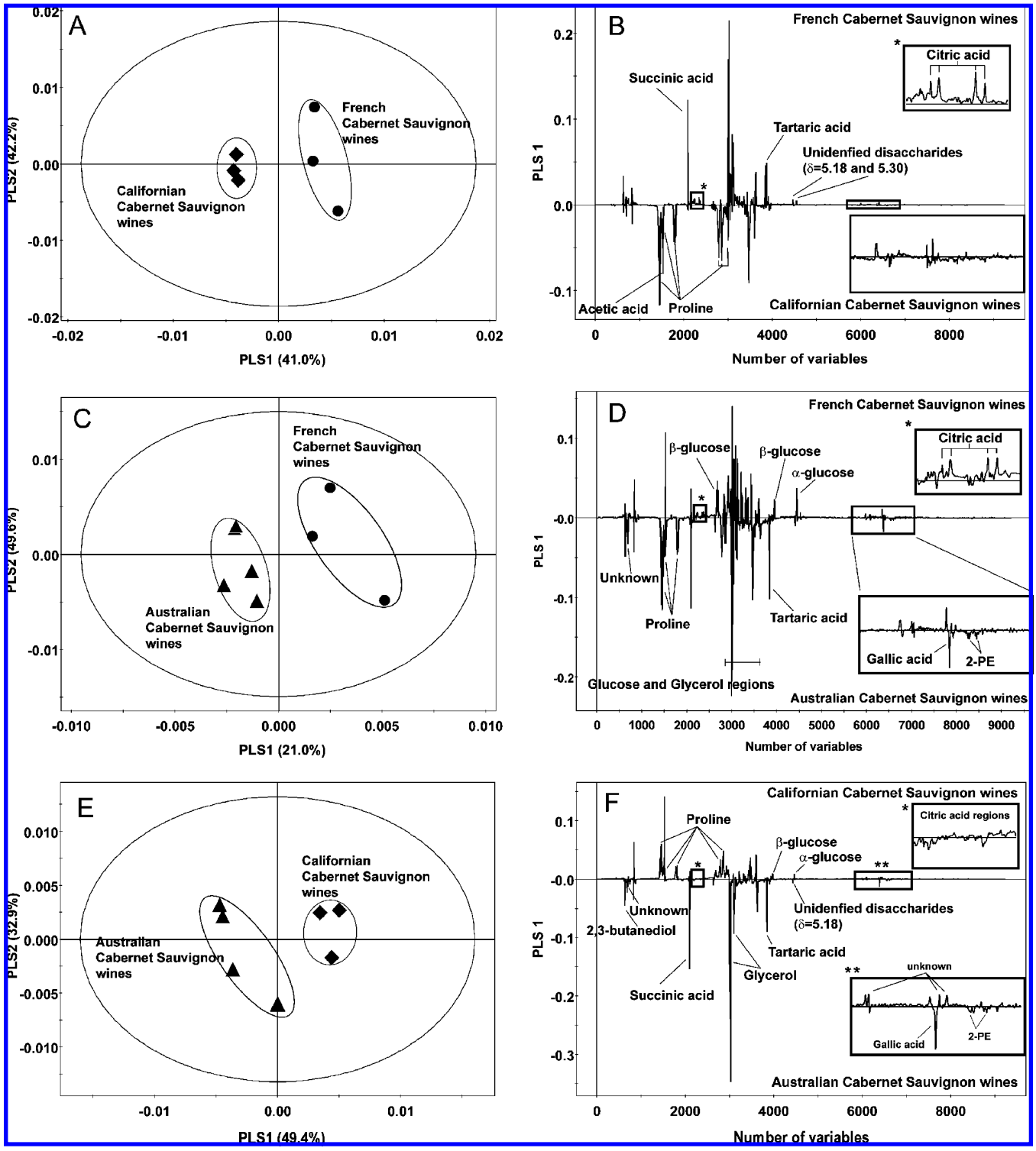

Figure 7. PLS-DA score plot derived from the ^{1}H NMR spectra of wines as pairwise comparison of geographical Cabernet Sauvignon wines: (**A**) PLS-DA score plot discriminating between French (square) and Californian wines (diamond), (**B**) complementary loading plot of the first component (PLS 1), (**C**) PLS-DA score plot discriminating between French (circle) and Australian (triangle) wines, (**D**) loading plot of the first component, (**E**) PLS-DA score plot discriminating between Californian (diamond) and Australian (triangle) wines, and (**F**) loading plot of the first component. 2-PE indicates 2-phenylethanol. The expanded gray boxes correspond to each small box and highlight the changes in citric acid and in polyphenol levels, respectively.

thought to augment the production of glycerol, succinic acid, and 2,3-butanediol and even the synthesis of some aromatic esters (*37*). Although there is no apparent scientific evidence for its effect, the increases of glycerol, succinic acid, and 2,3-butanediol in Korean wines might be due mainly to the chaptalization rather than to the various yeast strains or other parameters.

Acetoin was not observed in all wines used in this study, indicating that it had already been reduced to 2,3-butanediol during fermentation or that its levels were undetectable by NMR. In addition to an increase in glycerol by yeast strains, grape berries infected by *Botrytis cinerea* may explain the high glycerol level as a result of the metabolism of the fungus (*38*). Among Cabernet Sauvignon wines produced in different

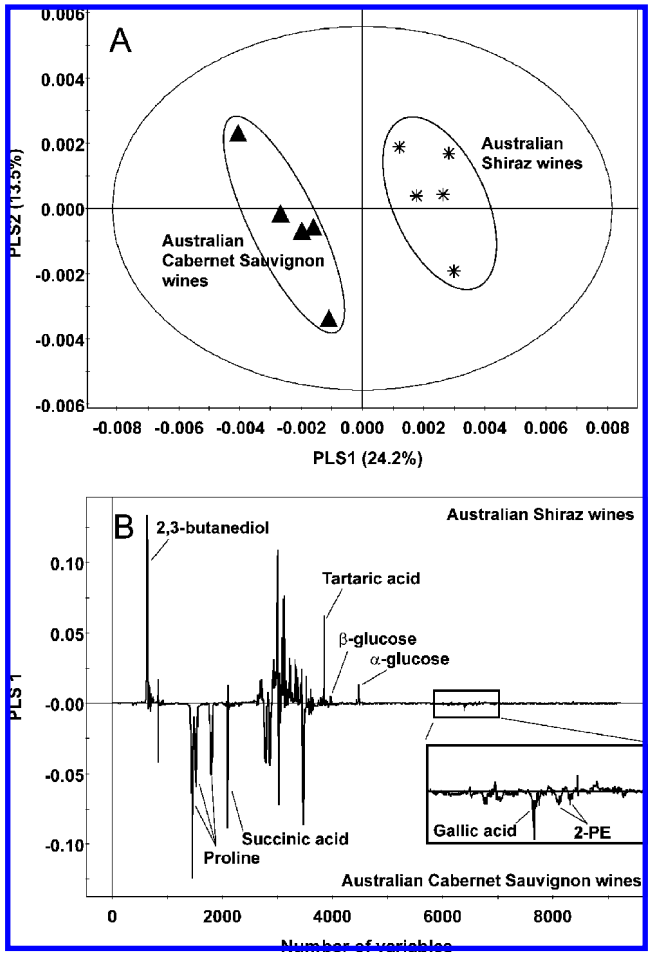

Figure 8. PLS-DA score (**A**) and loading (**B**) plots derived from the ^{1}H NMR spectra of Australian Shiraz (star) and Cabernet Sauvignon (triangle) wines. The gray box inside panel **B** highlights the increase in gallic acid and 2-PE in Australian Cabernet Sauvignon wines.

continental areas, the highest levels of 2,3-butanediol and glycerol were observed in Australian Cabernet Sauvignon wines, probably indicating the effect of various yeast strains used in the fermentation (**Figure 7E**).

Tartaric acid is one of the major acids in grapes and is carried into the wine, along with malic and citric acids. The high level of tartaric acid found in Korean Campbell Early wines as compared to the other wines might be due to the lack of cold precipitation to reduce it in the Korean wines. Furthermore, Korean Campbell Early wines showed the highest level of malic acid because there was no ML fermentation in these wines. In other words, the other wines were produced by ML fermentation, showing complete elimination of malic acid, as shown in 1D NMR spectra (**Figure 1**) and PLS-DA loading plots (**Figures 7B**,**D**,**F**). Except for Korean Campbell Early wines, tartaric acid was highest in Australian Cabernet Sauvignon wines.

It was of interest to note that there were no significant differences of lactic acid in all Cabernet Sauvignon wines or Shiraz wines, suggesting unique controlled ML fermentation since lactic acid is the main product of ML fermentation. Moreover, these Cabernet Sauvignon wines had significantly higher lactic acid levels than Korean Campbell Early wines, confirming that there was no ML fermentation in the Korean wines. This difference of lactic acid levels might result in a milkier taste in Cabernet Sauvignon and Shiraz wines than in Korean wines.

Succinic acid is the predominant nonvolatile organic acid formed during alcoholic fermentation, contributing to the total acidity; succinic acid is very stable and does not change during aging (*39*). The increase in succinic acid during alcoholic and ML fermentation processes has been demonstrated by quantitative ^{1}H NMR (qHNMR) spectroscopy (*40*).

The level of citric acid was highest in French wines among Cabernet Sauvignon-based wines, as shown in PLS-DA loading plots (**Figure 7**). Significantly higher levels of citric acid were found in Korean Campbell Early wines as compared to other wines, as shown in **Figure 5**. This difference of citric acid strongly indicated the involvement of ML fermentation by bacteria in wine. That is, in the presence of ML fermentation, a decrease in citric acid was observed.

Of the prominent amino acids in must, proline is not used by yeast as a nitrogen nutrient, while arginine, glutamic acid, alanine, and aspartic acid are used during yeast growth (*41, 42*). Cabernet Sauvignon has the highest concentrations of proline among the grapes. The second abundant amino acid in grapes is arginine. The ratio of proline/arginine differs significantly among grape varieties and ranges from 0.5 in Pinot Noir to 11 in Cabernet Sauvignon, thereby serving as a marker of varietal differences. Arginine was not observed in all wines used in this study due to its reduction during fermentation. The proline level was higher in Australian Cabernet Sauvignon wines than in Australian Shiraz wines, indicating a high content of proline in Cabernet Sauvignon grapes as compared to Shiraz grapes (**Figure 8**). This difference in proline level between Cabernet Sauvignon and Shiraz showed good agreement with the concentrations of proline in Cabernet Sauvignon and Shiraz grapes harvested in south Australia in 2001, which were 42.8 and 14.3 (μm/g fresh wt), respectively (*43*). In the present study, the highest proline level was found in Californian Cabernet Sauvignon wines, indicating the relatively high content of proline in Cabernet Sauvignon grapes grown in California as compared to those grown in continental areas (**Figure 7**). Higher proline levels have been found in sun-exposed grape berries than in shaded berries (*21*). The high level of proline in Californian Cabernet Sauvignon wines therefore indicated longer or stronger light exposure of these grapes than those in other areas. This proline content may be important to give wine the perceived "mouth-feel" or "body", because proline is a component of salivary proline-rich proteins, which have a strong affinity for polyphenols and contribute to mouth-feel (*44*−*46*).

Gallic acid, a phenolic compound, is present as free gallic acid and as an ester attached to procyanidin polymers in grape seeds (*41*). Gallic acid is also present in grape stems and may be increased by whole cluster fermentation. Thus, gallic acid found in wine is largely derived from grape seeds and stems, as well as from contact with oak during the fermentation process. To increase the amount of gallic acid in a particular wine, a winemaker can use whole cluster fermentation to expose the product to more stems. However, prolonged exposure to new oak is the main source of the acid. There was no gallic acid in Korean Campbell Early wines because fermentation or ripening occurred in stainless steel tanks with no addition of grape stems. However, a higher level of unknown polyphenol was found in Korea Campbell Early wines than in other wines (**Figure 1**). Gallic acid was found in Australian, Californian, and French wines, indicating fermentation or ripening in oak barrels.

2-PE is a high aromatic alcohol with a roselike odor and is an important compound contributing to the overall flavor quality of wine (*47*). Bacteria, fungi, and yeasts are known for their ability to synthesize 2-PE using L-phenylalanine as a substrate. Biochemically, 2-PE is synthesized through the Ehrlich pathway via transamination of L-phenylalanine to phenylpyruvate, which

is decarboxylated to phenylacetaldehyde and finally reduced to 2-PE (*48, 49*). In the present study, 2-PE was found only in Australian Cabernet Sauvignon- and Shiraz-based wines (**Figure 1**), indicating a higher level of L-phenylalanine in grapes grown in Australia than in grapes grown in other countries. Significantly higher 2-PE levels were found in Australian Cabernet Sauvignon wines as compared to Australian Shiraz wines (**Figure 8**). These results indicate that the level of L-phenylalanine in grapes could be affected by both the grape variety and the growing area.

This research marks the first characterization of wines from continental areas of France, California, Australia, and Korea using NMR spectroscopic analysis coupled with multivariate analysis. The global analysis of metabolites in wine could provide useful information on each wine related to alcoholic and lactic fermentations and intrinsic properties of grape varieties. This study further demonstrates the possibility of NMR-based metabolomic research to characterize wine quality and applied fermentation methods and product origin.

ACKNOWLEDGMENT

We thank Eun-Young Kim from the Korea Basic Science Institute for assistance with the Varian NMR measurements.

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Received for review May 7, 2008. Revised manuscript received July 11, 2008. Accepted July 11, 2008. This work was partially supported by a Korea University Grant.

JF801424U