DOI:10.1021/jf900502a

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Characterization of Fermentative Behaviors of Lactic Acid Bacteria in Grape Wines through ^{1}H NMR- and GC-Based Metabolic Profiling

Jang-Eun Lee, Young-Shick Hong,* and Cherl-Ho Lee*

School of Life Science and Biotechnology, Korea University, 5-1 Anam-dong, Sungbuk-gu, Seoul 136-701, Republic of Korea

The effects of five commercial *Oenococcus oeni* strains (MCW, Enoferm α, Wyeast, Vinibacti111, and Vinibacti222) on fermentative behaviors, and variations of metabolites in Meoru (*Vitis coignetiae*) wines during malolactic fermentation (MLF) were investigated by metabolomic analysis of ^{1}H NMR and GC data sets. In the development of MLF with various *O. oeni* strains, the fastest conversions of malic acid to lactic acid occurred in wines fermented with Enoferm α and Vinibacti111 strains. Seventeen primary metabolites and 65 secondary metabolites of volatile compounds in the wines were identified by ^{1}H NMR spectroscopy and GC-MS, respectively. In pattern recognition models of principal component analysis (PCA) and orthogonal projection to latent structures discriminant analysis (OPLS-DA), significant differentiations between wines with *O. oeni* strains were identified by the secondary metabolites rather than by the primary metabolites, showing the effects of *O. oeni* strains only on the secondary metabolites. Twelve volatile compounds, 2-phenylethanol, isoamyl alcohol, 2-butanol, ethyl octanoate, ethyl hexanoate, hexadecanoic acid, diethyl succinate, butyl butyrate, octanoic acid, 9-hexadecanoic acid, isobutyric acid, and 2-ethyl-1-hexanol, contributed to the differentiation of wines according to *O. oeni* strain, including spontaneous MLF. This study demonstrates that *O. oeni* strains affect the secondary metabolites, which are easily identified through multivariate statistical analysis of GC-MS data set.

KEYWORDS: Wine; NMR; GC; metabolomics; volatile compound; metabolites

INTRODUCTION

Malolactic fermentation (MLF) is a common practice in winemaking as a second fermentation, following alcoholic fermentation. Although MLF has been known to occur naturally, inoculation technology has been developed commercially in the past two decades. Today, MLF is applied to almost all premium red wines and certain white wines. The principal effect of MLF is a reduction in acidity through the conversion of L-malic acid to L-lactic acid and carbon dioxide. This reaction is catalyzed by the enzyme malate decarboxylase, which is often referred to as the malolactic enzyme and requires the cofactors NAD^+ and Mn^{2+} (*1*). The lactic acid bacteria (LAB) mainly responsible for MLF are widely used in the winemaking process as a starter culture (*2*). In particular, *Leuconostoc oenos*, recently reclassified as *Oenococcus oeni*, is the most well-adapted species in terms of tolerances to low pH, high sulfite, and alcohol (*3*). In addition to deacidfication, MLF also improves the microbiological stability of wine and changes the flavor (*4*). It has been reported that the compositions of volatile compounds and amino acids in wines produced by *O. oeni* and *Lactobacillus plantarum* starter culture are different (*5*).

*Authors to whom correspondence should be addressed [(Y.-S.H.) e-mail chtiger@korea.ac.kr, telephone +82-2-929-2751, fax +82-2-927-5201; (C.-H.L.) e-mail chlee@korea.ac.kr, telephone +82-2-3290-3414, fax +82-2-929-5201].

Meoru (*Vitis coignetiae*), the Korean wild grape, is a fruit that contains high amounts of polyphenolic compounds such as anthocyanins and resveratrol (*6*); Meoru thus provides attractive grape juice and wine. Although Meoru produced in Korea is used for making wine or juice, its extremely high acidity and high amounts of malic acid are unfavorable for sensory aspect. Because malic acid levels do not change during alcoholic fermentation, deacidification by MLF is essential in Meoru wine.

Multivariate statistical tools such as principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) are very useful for deciphering the differentiation and characterization of wine metabolites (*7*, *8*). In addition, orthogonal projection to latent structures discriminant analysis (OPLS-DA) powerfully facilitates the interpretation of large complex data sets, separating systematic variation in data into a predictive variation and an orthogonal compound (*9*, *10*).

In the present study we reduced the acidity of Meoru wines through MLF with using five commercial *O. oeni* strains and characterized their ML-fermentative behaviors and metabolic variations through a combination of ^{1}H NMR- and GC-based metabolomic profiling coupled with multivariate statistical analysis.

MATERIALS AND METHODS

Winemaking and Experimental Design. Meoru (*V. coignetiae*), Korean wild grapes, were harvested in 2007 from the region around

 Published on Web 05/14/2009

Gamak Mountain in northern South Korea. Two hundred kilograms of grapes was destemmed and crushed. The sugar content of the must was adjusted to 22 °Brix with sucrose. The must was transferred into a 200-L stainless steel tank for alcoholic fermentation. The alcoholic fermentation was carried out with activated *Saccharomyces cerevisiae* D-47 (ICV/D-47, Lalvin) at 25 °C for 9 days. After completion of the alcoholic fermentation, the wine was pressed and distributed into 18 4-L glass carboys. For MLF, five different LAB strains of *O. oeni* were inoculated into 15 glass carboys, providing three batches for each LAB strain; the remaining 3 glass carboys were not inoculated, providing control wine. MLF was performed at 25 °C until malic acid was no longer detected. After MLF, all Meoru wines were racked and bottled at 6 months. Their total acidity and organic acid content were monitored weekly. ^{1}H NMR spectroscopic and GC-MS analyses were performed at 6 months.

LAB Strains and Culture Preparation. Five LAB strains of *O. oeni* were used in this study: MCW, Enoferm α (Vinquiry, Healdsburg, CA), Wyeast (Wyeast Lab), Vinibacti111, and Vinibacti222 (Vinobios, Copenhagen, Denmark). All LAB strains were grown in Rogosa medium (20% nonpreservation apple juice, 20 g/L tryptone, 5 g/L peptone, 5 g/L glucose, 5 g/L yeast extract, 0.005% Tween-80) to obtain the appropriate biomass of 3×10^8 cfu/mL. Each activated strain was mixed with Meoru wine (1:1) and incubated at 25 °C for 24 h. The wine base cultures were inoculated as 2% concentration into each Meoru wine for MLF.

Organic Acid Analysis. The pH and total acidity of wine were determined by pH-meter (Orion 3star, Thermo Scientific) and sodium hydroxide titration, respectively (*4*).

The Gilson HPLC series was used for organic acid analysis. Meoru wines were filtered with a 0.45-μm syringe filter and directly injected on the Prevail organic acid column (250 mm × 4.6 mm, Alltech). The injection volume of the prepared sample was 20 μL. The mobile phase, 25 mM KH_2PO_4 (pH 2.5 by phosphoric acid), was used at a flow rate of 1.0 mL/min, and UV detection was carried out at 210 nm (*11*).

^{1}H NMR Spectroscopic Analysis. One milliliter of must or 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 a chemical shift reference (^{1}H, δ 0.00), respectively. ^{1}H NMR spectra were acquired on a Varian INOVA-600 MHz NMR spectrometer (Varian Inc., Palo Alto, CA) operating at 599.84 MHz ^{1}H frequency and a temperature of 298 K, using a triple-resonance 5-mm HCN salt-tolerant cold probe. A 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 0.3-Hz line-broadening function was applied to all spectra prior to Fourier transformation (FT).

Volatile Compound Analysis. A headspace solid-phase microextraction (SPME) method was utilized to prepare for GC-MS analysis. The polydimethylsiloxane (PDMS) 100-μm fiber (Supelco, Bellefonte, PA) was selected for the whole range of different volatile and polar compounds according to the instructions of Setkova et al. (*12*). Glass screw-cap vials with polytetrafluoroethylene (PTFE)/silicone septa (20 mm) were obtained from Agilent Technologies. The SPME experiments were optimized using 1 mL of Meoru wine and 0.2 g of NaCl in a 20-mL vial. The vial was soaked in water in a beaker on a hot plate with a magnetic stirrer; the water was heated to 40 °C. The wine was agitated at 500 rpm using a tiny magnetic bar in the vial. The wine sample was incubated for 5 min, ensuring that the NaCl dissolved completely within 5 min. One microliter of 3-octanol solution (8.22×10^{-4} g/mL in methanol) was added to the wine in the vial as an internal standard prior to sample incubation. The extraction time was 2 min. Thermal desorption into the GC injector was carried out for 3 min at 250 °C.

Gas chromatograph 7890 (Agilent Technologies, Palo Alto, CA) coupled to mass spectrometer 5975C (Agilent Technologies) was used to analyze volatile compounds. The DB-WAXetr (Agilent 122-7322, 30 m × 250 μm × 0.25 μm) column was used for GC analysis. Splitless injection mode was applied, and helium gas was used as the carrier gas with a constant flow rate of 1.0 mL/min. The GC oven temperature was set at 40 °C for 5 min and increased at a rate of 3 °C/min to 80 °C, at 4 °C/min to 180 °C, and at 5 °C/min to 200 °C. The mass spectrometer (MS) was operated in electron impact (EI) mode (70 eV). Data acquisition was performed in full-scan mode from *m*/*z* 50 to 650 with a scan time of 2.9 s.

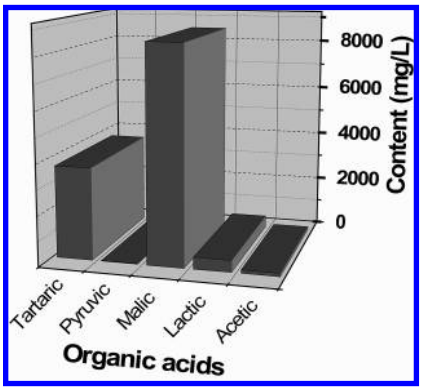

Figure 1. Organic acid composition of Meoru juice.

Multivariate Data Analysis. 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 ppm) was removed. In addition, the regions for residual ethanol (1.15−1.20 and 3.59−3.72 ppm) from incomplete removal during lyophilization and for DSS (−0.5 to 0.7 ppm) were also removed. The spectra were then normalized to the 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 (*7*, *13*). The resulting data sets were then imported into SIMCA-P version 12.0 (Umetrics, Umeå, Sweden) for multivariate statistical analysis. Signal assignment for representative samples was facilitated via acquisition of two-dimensional (2D) total correlation spectroscopy (TOCSY), heteronuclear multiple bond correlation (HMBC), heteronuclear single quantum correlation (HSQC), spiking experiments, and comparisons to the literature (*7*, *8*).

Selected GC-MS peaks were identified by comparing the mass spectra and the retention index of the peaks with those from the National Institute of Standards and Technology (NIST) mass spectral library (Wiley registry). The signal-to-noise threshold level was set at 17 for selection of major volatile compounds. All GC-selected peaks were integrated and normalized to integral peak area of the internal standard. The normalized peaks were imported into SIMCA-P software for multivariate statistical analysis.

Mean-centered scaling was applied for all multivariate analysis by SIMCA-P version 12.0 (Umetrics). PCA, an unsupervised pattern recognition method, was initially performed to examine the intrinsic variation in the data set. A supervised pattern recognition method, OPLS-DA, was used to extract maximum information on discriminant compounds from the data. OPLS-DA provides a way to remove systematic variation from an input data set *X* (compounds) not correlated to the response set *Y* (spectral intensities in NMR spectra and spectral areas in GC chromatogram) (*14*). Hotelling's *T*2 region, shown as an ellipse in the scores plots, defines the 95% confidence interval of the modeled variation (*15*). The quality of the models is described by R^2 and Q^2 values. R^2 is defined as the proportion of variance in the data explained by the models and indicates goodness of fit, and Q^2 is defined as the proportion of variance in the data predictable by the model and indicates predictability.

Statistical Analysis. The statistical analysis system (SAS package ver. 9.20) was used for data analysis. Significances of organic acids content by HPLC and peak areas of volatile compounds by GC-MS were analyzed by ANOVA and Duncan's multiple-range test.

Chemicals. All chemical reagents were of analytical grade. All organic acid standards, D_2O (99.9%), and DSS (97%) were purchased from Sigma (St. Louis, MO). 3-Octanol (99%) was obtained from Aldrich (Milwaukee, MI).

RESULTS AND DISCUSSION

Organic Acids in Meoru Juice. Figure 1 shows the organic acid contents of Meoru juice before alcoholic fermentation. High

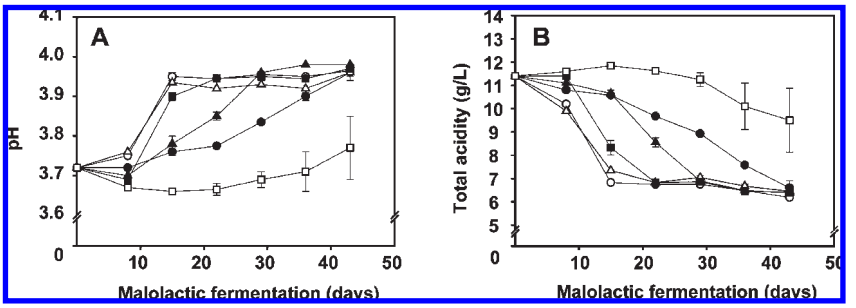

Figure 2. Changes of pH (**A**) and total acidity (**B**) in Meoru wines during MLF with various *O. oeni* strains, including spontaneous MLF: ●, MCW; ○, Enoferm α; ▲, Wyeast; △, Vinibacti111; ■, Vinibacti222; □, spontaneous MLF.

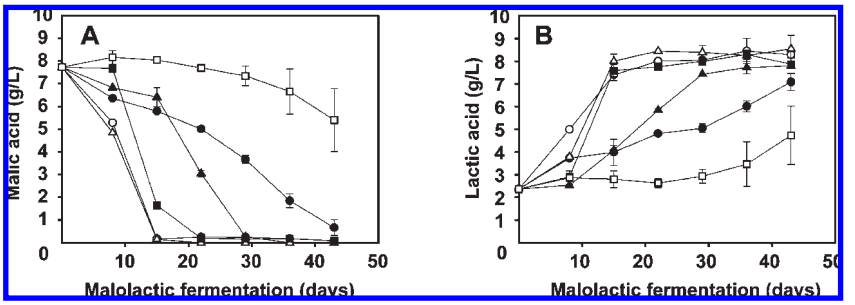

Figure 3. Changes of malic (**A**) and lactic acid contents (**B**) in Meoru wines during MLF with various *O. oeni* strains, including spontaneous MLF: ●, MCW; ○, Enoferm α; ▲, Wyeast; △, Vinibacti111; ■, Vinibacti222; □, spontaneous MLF.

contents of malic acid (8552.0 ± 252.0 mg/L) were observed in Meoru juice. In general, tartaric and malic acids are the key acids in vine fruit with concentration ranges of 2−3 and 1−2 g/L, respectively (*1*). In addition, the tartaric/malic acid ratio ranges from 1.1 to 2.0 (*16*). In the present study, the ratio was 0.43, indicating that malic acid levels were much higher than tartaric acid levels in Meoru grape. Contents of tartaric, pyruvic, lactic, and acetic acids were 3667.8 ± 114.9, 22.5 ± 0.5, 470.0 ± 9.6, and 130.0 ± 1.1 mg/L, respectively.

Malolactic Fermentation of Meoru Wines. MLF was induced with five different LAB strains in the wines, following completion of alcoholic fermentation with *S. cerevisiae* D-47. During MLF, pH increased from 3.72 to 3.95 and total acidity decreased from 11.4 to 6.4 g/L (**Figure 2**). These results are consistent with typical changes in pH and total acidity during MLF (*17*). The pH and total acidity were not further changed after day 42, except in control wine. It has been reported that total acidity decreased by about 0.6 g/L as a consequence of 1 g/L of malic acid fermentation (*1*); this is consistent with our result that total acidity reduction of 5.0 g/L at the end of MLF resulted in 8 g/L of malic acid consumption (**Figures 2** and **3**). In general, the total acidity decreases from 1 to 3 g/L and pH increases by 0.1−0.3 units during MLF (*17*). However, the reduction in the total acidity of Meoru wines was 2 times higher than that in most vine wines, demonstrating the intrinsic high content of malic acid in the Meoru berry.

Malic acid in induced-MLF wines was completely converted into lactic acid within 42 days. It was interesting to note that the LAB strains used in the present study exhibited different malic acid conversion rates. Strains Enoferm α and Vinibacti111 completed the MLF within 2 weeks; Vinibacti222 within 3 weeks; Wyeast within 4 weeks; and MCW within 6 weeks. However, malic acid levels continued to decrease in control wines until day 42, indicating spontaneous MLF was still occurring. Because *O. oeni* strains accounted for 98.5% of the predominant species in spontaneous MLF wines (*18*), *O. oeni* strains would be dominant in the spontaneous MLF of control wines. It is well-known that the time frame for MLF generally shortens as the pH increases (*19*). It has been reported that MLF by *O. oeni* (ML-34) was completed in 164 days at pH 3.15, whereas it only took 2 weeks at pH 3.83 (*20*). In addition, MLF with *O. oeni* strains (IS-18 and IS-159) at pH 3.49 were completed in 14 and 28 days, respectively (*21*). The fermentative behaviors of *O. oeni* strains in the present study were consistent with these reports; strains Enoferm α and Vinibacti111 caused the highest pH and the fastest malic acid reduction rate compared to other LAB strains, indicating the fastest MLF (**Figures 2** and **3**). In most cases, it is important that MLF be completed rapidly to save processing time and to achieve early stability of the wine. Strains Enoferm α, Vinibacti111, and Vinibacti222 could therefore be suitable for MLF in Meoru wine.

Table 1 shows organic acid contents of Meoru wines after racking at 6 months. Tartaric acid mainly originates from the grapes and usually precipitates as an insoluble salt during the winemaking. Large amounts of tartaric acid precipitate were observed during MLF and aging. Control wine contained the highest tartaric acid levels, whereas these levels was lowest in wines with MLF induced by MCW and Vinibacti111, indicating that the settling property of tartaric acid against pH resulted in significant variations in tartaric acid levels after racking according to each LAB strain. Lactic acid levels likely increased as the main product of malic acid conversion during MLF.

Acetic acid is the most common byproduct from MLF along with diacetyl, acetoin, and 2,3-butanediol. It is also produced by yeast during alcoholic fermentation. Acetic acid concentrations were not significantly different in all wines. LAB metabolizes citric acid to acetic acid toward the end of fermentation (*3*).

^{1}H NMR Spectroscopic Analysis of Meoru Wines. Representative one-dimensional (1D) ^{1}H NMR spectra of Meoru wines are shown in **Figure 4A**. The dominant primary metabolites of Merou wines were leucine, isoleucine, valine, unknown compound (U), 2,3-butanediol, lactic acid, alanine, acetic acid, proline, succinic acid, γ-aminobutyric acid (GABA), choline, glycerol, tartaric acid, α- and β-glucoses, and polyphenols.

To investigate significant differences in the primary metabolites according to LAB strain, PCA was applied to all Meoru wines. There were no significant differentiations between Meoru

Table 1. Organic Acid Concentrations (Milligrams per Liter)[a] in Meoru Wine at 6 Months

	lactic acid bacteria strains					control
	MCW	Enoferm α	Wyeast	Vinibacti111	Vinibacti222	spontaneous MLF
oxalic acid	330.0 ± 4.71	322.7 ± 33.6	329.1 ± 11.5	344.9 ± 4.88	334.2 ± 17.1	361.0 ± 26.7
tartaric acid**	1532.3 ± 122.2d	1813.4 ± 114.7abc	1725.5 ± 60.5bc	1651.9 ± 90.9cd	1740.8 ± 64.3bc	1903.8 ± 74.6ab
malic acid	nd[b]	nd	nd	nd	nd	nd
lactic acid***	6966.8 ± 11.8d	9017.5 ± 44.1a	7742.9 ± 38.3c	8430.9 ± 46.7b	9022.4 ± 72.2a	8797.9 ± 96.4ab
acetic acid	957.8 ± 17.9	1062.5 ± 15.1	1058.7 ± 57.1	943.0 ± 82.0	1042.2 ± 36.7	1022.9 ± 141.8

[a] Means ± SD. Values with different letters are significantly different by Duncan's multiple-range test at ***, $p < 0.001$; and **, $p < 0.01$. [b] nd, not detected.

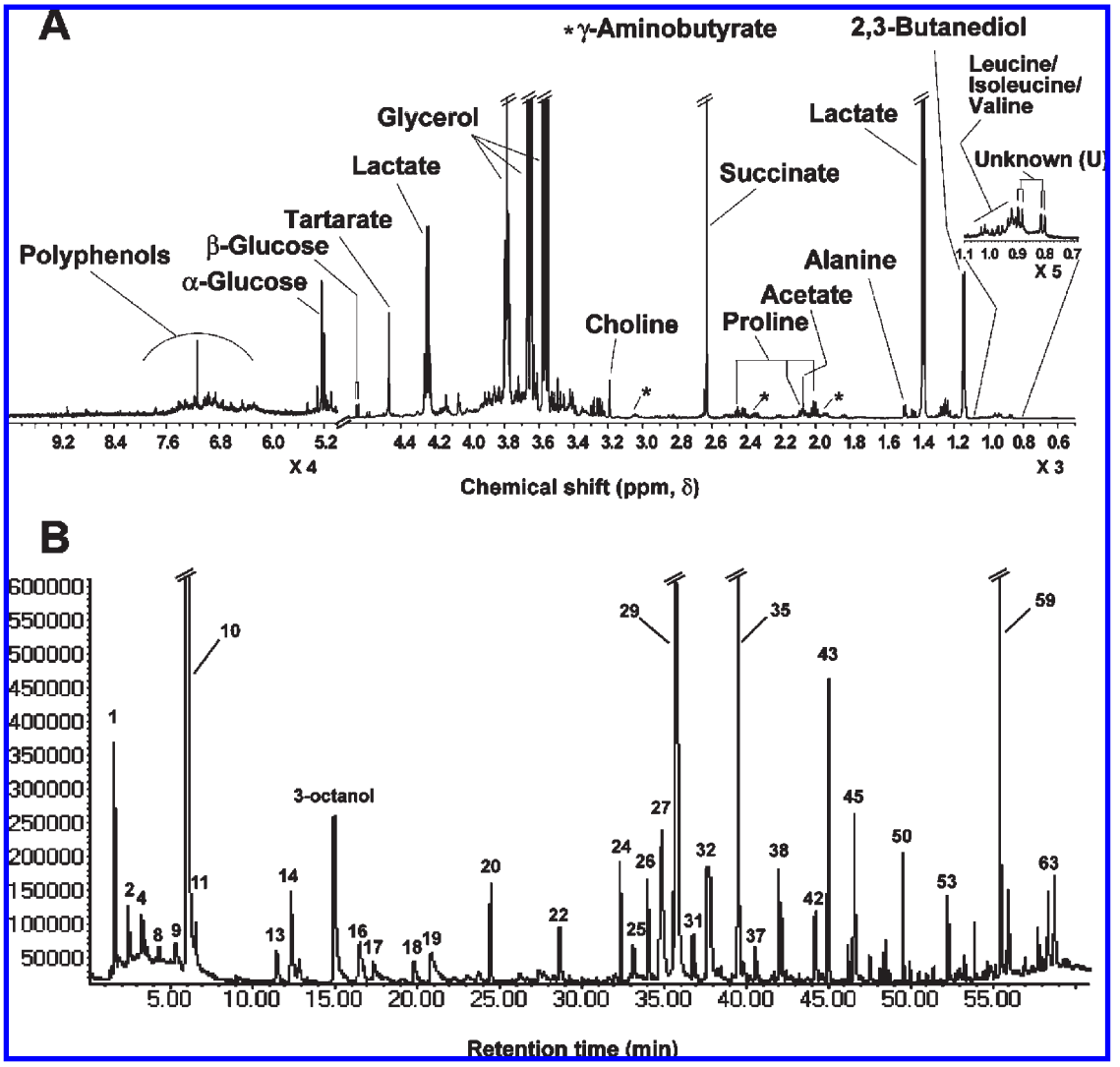

Figure 4. Representative ^{1}H NMR spectrum (**A**) and GC chromatogram (**B**) of Meoru wine fermented with *O. oeni* MCW strain, following alcoholic fermentation. In panel **B**, 3-octanol represents the internal standard, and names corresponding to the peak numbers are listed in **Table 2**.

wines in the PCA model, demonstrating no dependence of the primary metabolites on LAB strain, as shown in **Figure 5A**. In our previous metabolomic studies using ^{1}H NMR spectroscopy, we explored the metabolic differences between wines according to geographical area and grape variety, and the metabolic evolutions in time course and the fermentative behaviors of yeast strains during alcoholic fermentation (*7*, *8*). In addition, metabolic differentiations in geographical grapes and their wines were also reported (*22*). López-Rituerto et al. (*23*) showed that the simultaneous quantification of metabolites and their multivariate data analysis obtained by ^{1}H NMR spectroscopy were useful for monitoring the levels of the metabolites in wines during alcoholic and malolactic fermentations. In particular, we identified strong dependences of metabolites on wine yeast strains during alcoholic fermentation (*8*). Although no significant differentiations were observed between Meoru wines according to LAB strain in the PCA model in the present study, when we compared 1D ^{1}H NMR spectra of all Meoru wines carefully, marked differences in citric acid levels were observed, as shown in **Figure 5B**,**C**. It was clear that Vinibacti111 did not metabolize or degrade citric acid; thus, this stain resulted in higher remaining levels of citric acid in the wine compared to the other LAB strains (**Figure 5C**). In addition, citric acid was not degraded in one of the Meoru wines in which MLF occurred spontaneously, whereas citric acid in the other two spontaneous MLF Meoru wines was metabolized completely, demonstrating involvement

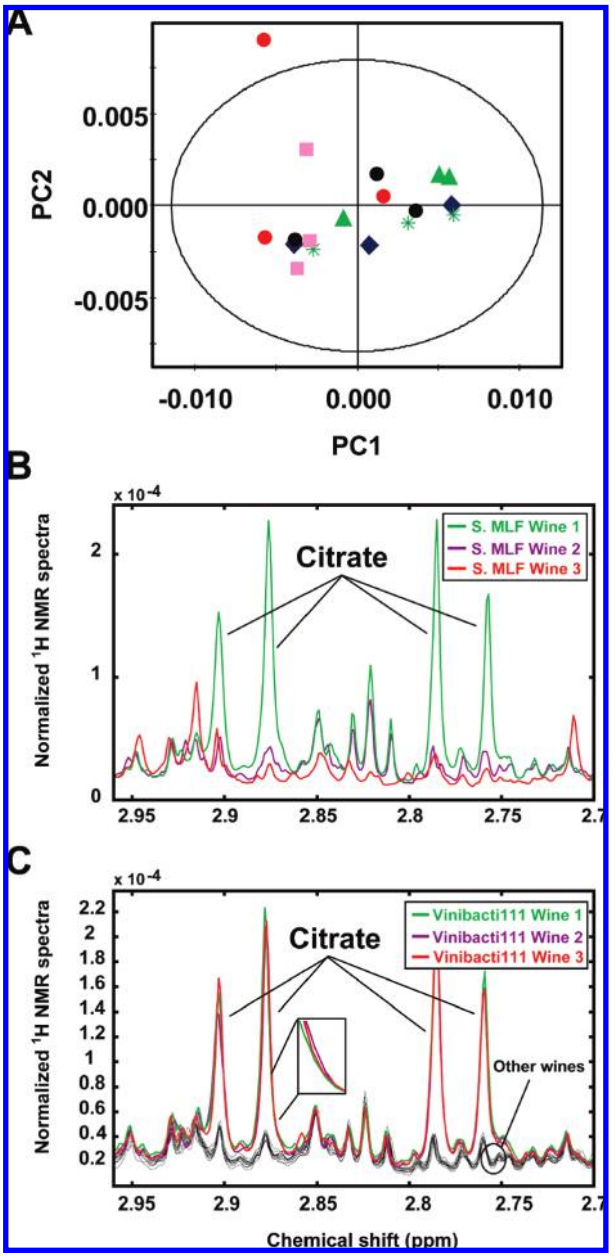

Figure 5. Principal component analysis (PCA) derived from ^{1}H NMR spectra of all Meoru wines (**A**), and normalized raw ^{1}H NMR spectra of spontaneous MLF wines (**B**) and of other wines including wines fermented with Vinibacti111 strain of LAB (**C**). **B** and **C** highlight no consumption or degradation of citric acid by LAB strains in one of three wines during spontaneous MLF and by Vinibacti111 strain during induced-MLF, respectively. Symbols in panel **A**; red, spontaneous MLF wines; blue, MLF wines with MCW strain; green, MLF wines with Enoferm α strain; orange, MLF wines with Wyeast strain; violet, MLF wines with Vinibacti111 strain; black, MLF wines with Vinibacti222 strain.

of different *O. oeni* strains in the spontaneous MLF (**Figure 5B**). Citric acid metabolism by *O. oeni* has been correlated with the synthesis of acetic acid, diacetyl, and acetoin (*23*). In addition, production of diacetyl and acetoin by *O. oeni* is reportedly simulated by increased citric acid concentration (*24*). In the present study, no significant differences in acetic acid levels were found in the HPLC analysis, due to large variations in acetic acid levels (**Table 1**). However, the different behaviors of LAB stains involved in the spontaneous MLF, in terms of citric acid metabolism, indicated the importance of controlling MLF during winemaking for consistent quality (*25*). That is, citric acid metabolism would be dependent on commercial LAB strains.

Volatile Compounds in Meoru Wines. Figure 4B shows a typical GC chromatogram obtained from Meoru wine fermented with *O. oeni* MCW strain.

Volatile compounds obtained in Meoru wines by the headspace SPME method are summarized in **Table 2**. The dominant secondary metabolites in Meoru wines were ethyl acetate, 1-propanol, isoamyl alcohol, ethyl hexanoate, 1-hexanol, ethyl octanoate, benzaldehyde, diethyl succinate, hexanoic acid, 2-phenylethanol, octanoic acid, nonanoic acid, and hexadecanoic acid, which had a large mean area in GC chromatogram and is most responsible for fruit or floral odors. In addition, 22 of 65 volatile compounds observed in the present study had significantly different mean areas according to LAB strain. These compounds are produced during both alcoholic and malolactic fermentations and enhance the complexity of the wine flavor (*26*). Alcohols, esters, and acids, which are important for the organoleptic properties and quality of wine, were increased during MLF (*5*, *27*). In addition, significant decreases of ethyl esters and acetate were observed in Tannat wine during MLF (*28*).

OPLS-DA was applied to the differentiation of Meoru wines and the identifications of metabolites contributing to the differentiations. The OPLA-DA score plot showed clear differentiations between Meoru wines according to LAB strain, reflected by a higher goodness of fit and predictability as indicated by an R_x^2 value of 0.98 and an R_y^2 of 0.89 and by a Q^2 value of 0.49, respectively (**Figure 6A**), compared to their complementary PCA model (data not shown). The OPLS-DA scatter loading plot showed that isoamyl alcohol (IAA) and 2-phenylethanol (2-PE) were most responsible for the differentiation (**Figure 6B**). 2-PE and IAA levels were highest in Meoru wine fermented with Enoferm α and in spontaneous ML fermented Meoru wine, respectively. IAA is formed in the alcoholic fermentation from leucine through the Ehrlich pathway (*1*, *26*). It constitutes quantitatively the greater fraction of higher alcohols in most wines, being considered a predictor of the sensory character of wine. 2-PE is a higher aromatic alcohol with a "rose-like" or sweet odor. It can be synthesized from L-phenylalanine by transamination of the amino acid to phenylpyruvate, decarboxylation to phenylacetaldehyde, and reduction to 2-PE, which comprise the Ehrlich pathway (*29*). Although IAA and 2-PE are metabolites produced by yeast, significant differences with LAB strains were observed in the present study. This indicates that LAB caused a side metabolic pathway and yeast−bacteria interaction. Because these two compounds (IAA and 2-PE) had the largest peak areas in GC data, they contributed to the high proportion of variance in the OPLS-DA model. To investigate the contribution of other metabolites, we regenerated the OPLS-DA after excluding IAA and 2-PE, and the resulting OPLS-DA score plot still showed clear differentiations between Meoru wines according to LAB strain with high values of R_x^2 (0.97), R_y^2 (0.99), and Q^2 (0.80), as shown in **Figure 7A**. The differentiations were caused by variations in the levels of 2-butanol, ethyl octanoate, ethyl hexanoate, hexadecanoic acid, diethyl succinate, butyl butyrate, octanoic acid, isobutyric acid, 2-ethyl-1-hexanol, and 9-hexadecanoic (**Figure 7B**).

Ethyl hexanoate levels were higher in spontaneous MLF Meoru wines, and ethyl octanoate levels were lowest in Meoru wines fermented with Vinibacti222, as shown in the OPLS-DA scatter loading plot.

The majority of wine esters, such as ethyl hexanoate and ethyl octanoate in the present study, are produced by yeast during alcoholic fermentation. However, esters can also be derived from the grape and from chemical esterification of alcohol−acid

Table 2. Volatile Compounds in Meoru Wines at 6 Months[a]

				lactic acid bacteria stains					control
peak	compound	odor	t_R(s)	MCW	Enoferm α	Wyeast	Vinibacti111	Vinibacti222	spontaneous
1	ethyl acetate**	fruity	1.568	18.2ab	18.6ab	11.6c	15.9b	18.1ab	21.1a
2	hexamethylcyclotrisiloxane		1.88	2.2b	1.7b	2.7b	1.1b	6.3a	1.4b
3	1-propanol	fruit alcoholic	2.464	9.6	10.3	10.9	7.8	12.2	11.3
4	isobutyl alcohol*	fusel spiritous	3.176	8.2b	9.1b	9.2b	11.0b	12.5b	19.5a
5	2-butanol***	fusel	3.231	10.6a		5.7b	0.8c		4.4b
6	isoamyl acetate***	banana pear	3.455	1.4b	1.9b	5.6a	1.5b	2.4b	5.4a
7	unknown*		3.686	3.6ab	2.7b	2.4b	3.6ab	2.5b	5.4a
8	unknown		4.14	7.3	1.7	1.6	2.9	1.0	1.7
9	1,8-cineole		5.62	3.1		1.8	3.9		3.0
10	isoamyl alcohol***	nail polish	6.048	305.7b	245.9c	257.9bc	215.8c	264.7bc	367.3a
11	ethyl hexanoate*	green apple	6.55	10.3b	9.9b	7.1b	9.6b	5.8b	22.3a
12	unknown		9.062	4.3	2.5	4.2	4.2	3.5	2.6
13	glycine*		11.492	2.7c	4.6a	2.9bc	3.8ab	4.1a	3.8ab
14	1-hexanol	green grass	12.484	19.1abc	15.6c	18.4bc	23.5a	17.0ab	21.2ab
15	decamethylcyclopentasiloxane		12.846	2.3	3.0	4.9	5.0	4.1	4.5
16	ethyl octanoate*	sweet soap	16.672	18.0a	17.0a	12.7a	6.2ab	0.8b	16.2a
17	butyric acid	buttery	17.344	0.7	4.9	4.3	1.2		4.1
18	2-ethyl-1-hexanol*		20.005	3.1b	2.6b	2.0b	4.7ab	11.8a	4.6ab
19	benzaldehyde	almond	20.99	10.4	10.0	10.1	12.3	10.1	10.8
20	dodecamethyl cyclohexasiloxane		24.452	7.9	4.5	5.3	5.9	5.9	8.5
21	methyl benzoate	fruit	26.346	1.5		2.2	2.2	0.5	1.1
22	diethyl succinate***	faint pleasant	28.804	4.9b	11.2a	2.7b	4.4b	3.7b	4.8b
23	unknown		29.252	1.8	2.7	0.8	4.5	1.2	1.8
24	tetradecamethylcycloheptasiloxane		32.374	19.5	8.5	8.6	8.6	7.6	7.7
25	ethyl pentanoate**	apple	33.243	3.2a	4.1a	1.1b	2.8a	2.8a	2.7a
26	hexanoic acid*	pineapple	34.105	5.9ab	8.0a	4.3b	6.8a	7.0a	6.8a
27	butyl butyrate**	sweet fruity	34.995	6.0b	19.0a	4.6b	16.3a	7.3b	14.7a
28	isobutyric acid***		35.687	2.9ba	4.0b	1.5c	1.4c	9.5a	8.3a
29	2-phenylethanol*	floral rose	35.83	48.2ba	81.4a	32.2c	61.8ab	64.5ab	70.0ab
30	2-ethylhexanoate		36.821	2.6	2.3	1.6	2.5	1.9	2.6
31	heptanoic acid***	floral	37.018	1.3	1.2	0.7	1.4	1.2	1.6
32	unknown		37.717	3.6b	3.3b	1.4c	3.0ab	2.5b	2.1a
33	phenol		38.274	1.1	1.1	1.5	1.4	0.9	1.8
34	cyclodecane		38.688	2.3	2.1	3.9	0.6	1.3	3.1
35	octanoic acid*	currant-like	39.563	17.1b	28.3a	14.3b	17.7b	19.7ab	16.3b
36	isopropyl myristate		39.842		0.3		1.1	1.1	1.0
37	4-methoxyphenol		40.595		1.1		1.8	1.2	1.9
38	nonanoic acid	rancid	42.028	8.5	8.9	6.1	6.7	6.8	7.6
39	octadecamethylcyclononasiloxane		42.259	15.3	2.9	1.7	3.3	3.1	2.5
40	1-hexadecanol		42.449			1.8	0.3	0.6	0.4
41	isopropyl palmitate		44.139	1.5	1.0		1.4	1.0	
42	decanoic acid	orange-like	44.336	2.7	5.9	4.6	3.4	3.5	2.9
43	2,4-bisphenol		45.028	8.0	9.9	5.6	12.3	11.1	14.2
44	unknown		46.243	13.6	2.4	0.8	1.4	1.5	2.6
45	2-hexadecene		46.732	1.7		1.4	2.8	5.9	2.1
46	benzoic acid		47.588	2.2	2.3	1.4	1.6	1.6	4.6
47	butyl palmitate		47.805	1.1				0.4	
48	1,2,4,5-tetramethylbenzene		48.205	1.6	7.9	0.6	0.9	0.6	0.6
49	dodecanoic acid***	soap	48.511	2.1a	2.1a	0.8b	1.0b	1.0b	0.9b
50	isobutyl phthalate		49.604	1.8	2.7	1.9	3.5	1.8	4.4
51	unknown		49.957	13.8	1.9	1.0	1.4	1.5	1.3
52	hexadecanol		50.509	1.3			0.7	0.4	
53	tetradecanoic acid		52.258	9.2	3.6	1.8	2.7	3.3	
54	dibutyl phthalate		52.279		3.3	3.6	5.0	5.0	5.9
55	benzamide*		53.052	1.6a	1.2ab	0.6b	0.9ab	0.7b	0.6b
56	unknown		53.243	15.3	1.4	1.2	1.2	1.5	1.4
57	dodecanoic acid		53.392	0.8		0.7	0.4	0.3	0.3
58	pentadecanoic acid***		53.915	3.3a	1.6b	0.6c	1.8b	1.4ba	1.1ba
59	hexadecanoic acid***		55.483	20.0ab	23.2a	5.9d	13.3bc	4.6d	10.5cd
60	9-hexadecanoic acid*		55.958	2.7b	5.6b	1.0b	3.7b	17.0a	2.1b
61	1-ethanone**		57.784	4.1a	1.4c	2.4ba	2.2ba	3.0ab	1.3c
62	4-hydroxy acetophenone		58.056		3.1	1.4	1.2	1.8	1.9
63	octadecanoic acid*		58.375	6.1ab	6.8a	3.2c	4.3bc	3.9bc	3.4c
64	unknown		58.64	2.2	2.8	1.8	2.0	2.6	3.7
65	9-octadecanoic acid		58.7852	6.8	7.6	2.5	5.6	5.6	3.9

[a] Compounds are reported in order of retention time. Data expressed as peak area means (TIC $\times$ 10^6) with respect to the area of internal standard (3-octanol). Values with different letters are significantly different by Duncan's multiple-range test at ***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$.

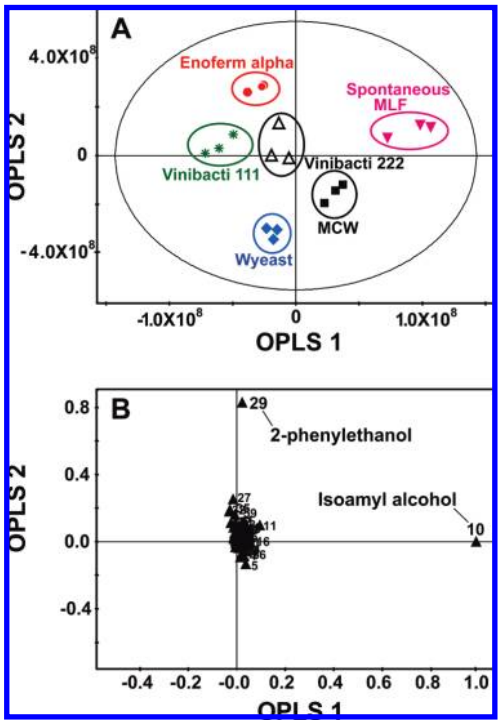

Figure 6. OPLS-DA score (**A**) and scatter loading (**B**) plots derived from volatile compounds in Meoru wines fermented with *O. oeni* strains by GC-MS, including Meoru wines with spontaneous MLF.

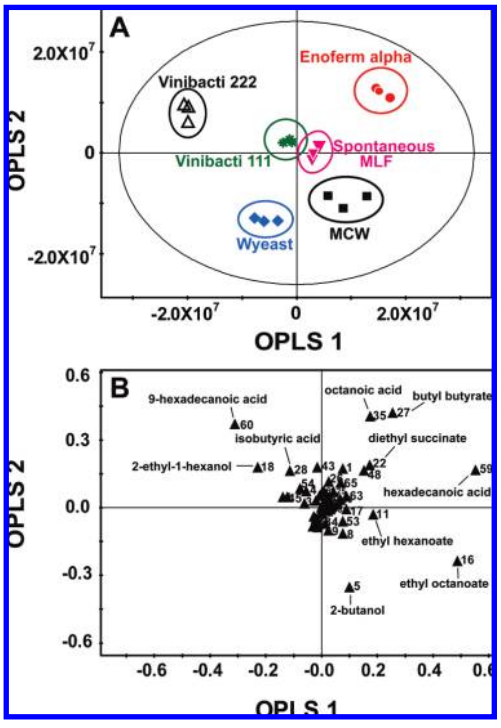

Figure 7. OPLS-DA score (**A**) and scatter loading (**B**) plots derived from volatile compounds on GC-MS of Meoru wines fermented with *O. oeni* strains, including Meoru wines with spontaneous MLF; isoamyl alcohol and 2-phenylethanol were excluded from that shown in **Figure 6**.

rearrangements during wine aging (*26*). The esterase activity of wine-associated bacterial species has not been understood clearly. Some researchers report increases in ethyl esters in wine, including ethyl acetate, ethyl hexanoate, ethyl lactate, and ethyl octanoate, as well as decreases in some esters, following MLF (*28*, *30*, *31*). Diethyl succinate is another ester produced from esterification of succinic acid in α-ketoglutarate metabolism (*32*); large amounts of diethyl succinate are found in grape wines (*33*, *34*). Diethyl succinate levels were highest in wines fermented with Enoferm α in the present study. The dependences of these esters on *O. oeni* strain indicated different behaviors by esterases produced by the LAB strain. In the present study, hexadecanoic acid levels were increased by Enoferm α and MCW, whereas octanoic and 9-hexadecanoic acid levels were enhanced by Enoferm α and Vinibacti222, respectively, revealing the dependence of volatile acids on LAB strains; these findings are consistent with the report that levels of hexanoic, octanoic, and decanoic acids were increased by the EQ 54 strain of commercial LAB (*34*). Although a number of alcohols were detected in the volatile fraction of the Meoru wines, 2-ethyl-1-hexanol and 2-butanol levels were dependent on the *O. oeni* strain, as were isoamyl alcohol and 2-phenylethanol (**Figure 6**); the highest levels of 2-ethyl-1-hexanol and 2-butanol were found in Meoru wines with Vinibacti222 and MCW, respectively.

In conclusion, our results revealed the different malolactic behaviors of five commercial *O. oeni* strains and LAB strains involved in spontaneous MLF, contributing to variations in the secondary metabolites rather than the primary metabolites. In addition, this study highlights the differentiation between wines produced with various *O. oeni* strains and the visualization of the metabolites contributing to the differentiation through the use of multivariate statistical analysis of a GC data set.

ACKNOWLEDGMENT

We thank Dr. G. S. Hwang and E. Y. Kim from Korea Basic Science Institute for assistance with NMR experiments.

LITERATURE CITED

(1) Ribereau-Gayon, P.; Glories, Y.; Maujean, A.; Dubourdieu, D. *Handbook of Enology*, 2nd ed.; Wiley: Chichester, U.K., 2006; Vol. *1*.

(2) Maicas, S.; Natividad, A.; Ferrer, S.; Pardo, I. Malolactic fermentation in wine with high densities of non-proliferating *Oenococcus oeni*. *World J. Microbiol. Biotechnol.* **2000**, *16*, 805–810.

(3) Versari, A.; Parpinello, G. P.; Cattaneo, M. *Leuconostoc oenos* and malolactic fermentation in wine: a review. *J. Ind. Microbiol. Biotechnol.* **1999**, *23*, 447–455.

(4) Zoecklein, B. W.; Fugelsang, K. C.; Gump, B. H.; Nury, F. S. *Wine Analysis and Production*; Kluwer Academic/Plenum Publishers: New York, 1999.

(5) Pozo-Bayon, M. A.; G-Alegria, E.; Polo, M. C.; Tenorio, C.; Martin-Alvarez, P. J.; Calvo de la Banda, M. T.; Ruiz-Larrea, F.; Moreno-Arribas, M. V. Wine volatile and amino acid composition after malolactic fermentation: effect of *Oenococcus oeni* and *Lactobacillus plantarum* starter cultures. *J. Agric. Food Chem.* **2005**, *53*, 8729–8735.

(6) Oh, Y. S.; Lee, J. H.; Yoon, S. H.; Oh, C. H.; Choi, D. S.; Choe, E.; Jung, M. Y. Characterization and quantification of anthocyanins in grape juices obtained from the grapes cultivated in Korea by HPLC/DAD, HPLC/MS, and HPLC/MS/MS. *J. Food Sci.* **2008**, *73*, 378–389.

(7) Son, H. S.; Kim, K. M.; Van den Berg, F.; Hwang, G. S.; Park, W. M.; Lee, C. H.; Hong, Y. S. H-1 nuclear magnetic resonance-based metabolomic characterization of wines by grape varieties and production areas. *J. Agric. Food Chem.* **2008**, *56*, 8007–8016.

(8) Son, H. S.; Hwang, G. S.; Kim, K.; Kim, E. Y.; van den Berg, F.; Park, W. M.; Lee, C. H.; Hong, Y. S. ^{1}H NMR-based metabolomic approach for understanding the fermentation behaviors of wine yeast strains. *Anal. Chem.* **2009**, *81*, 1137–1145.

(9) Cloarec, O.; Dumas, M. E.; Trygg, J.; Craig, A.; Barton, R. H.; Lindon, J. C.; Nicholson, J. K.; Holmes, E. Evaluation of the orthogonal projection on latent structure model limitations caused by chemical shift variability and improved visualization of biomarker changes in ^{1}H NMR spectroscopic metabonomic studies. *Anal. Chem.* **2005**, *77*, 517–526.

(10) Bylesjo, M.; Rantalainen, M.; Cloarec, O.; Nicholson, J. K.; Holmes, E.; Trygg, J. OPLS discriminant analysis: combining the strengths of PLS-DA and SIMCA classification. *J. Chemom*. **2006**, *20*, 341–351.
(11) Mato, I.; Suarez-Luque, S.; Huidobro, J. F. A review of the analytical methods to determine organic acids in grape juices and wines. *Food Res. Int*. **2005**, *38*, 1175–1188.
(12) Setkova, L.; Risticevic, S.; Pawliszyn, J. Rapid headspace solid-phase microextraction−gas chromatographic−time-of-flight mass spectrometric method for qualitative profiling of ice wine volatile fraction I. Method development and optimization. *J. Chromatogr*. **2007**, *1147*, 213–223.
(13) Larsen, F. H.; van den Berg, F.; Engelsen, S. B. An exploratory chemometric study of H-1 NMR spectra of table wines. *J. Chemom*. **2005**, *20*, 198–208.
(14) Trygg, J.; Wold, S. Orthogonal projections to latent structures (O-PLS). *J. Chemom*. **2002**, *16*, 119–128.
(15) Hotelling, H. The generalization of Student's ratio. *Ann. Math. Stat*. **1931**, *2*, 360–378.
(16) Margalit, Y. *Concepts in Wine Chemistry*; Wine Appreciation Guild: San Francisco, CA, 2004.
(17) Davis, C. R.; Wibowo, D.; Eschenbruch, R.; Lee, T. H.; Fleet, G., H. Practical implication of malolactic fermentation: a review. *Am. J. Enol. Vitic*. **1985**, *36*, 290–301.
(18) López, I.; Tenorio, C.; Zarazaga, M.; Dizy, M.; Torres, C.; Ruiz-Larrea, F. Evidence of mixed wild populations of *Oenococcus oeni* strains during wine spontaneous malolactic fermentations. *Eur. Food Res. Technol*. **2007**, *226*, 215–223.
(19) Wibowo, D.; Eschenbruch, R.; Davis, C. R.; Fleet, G.; Lee, T. H. Occurrence and growth of lactic acid bacteria in wine. A review. *Am. J. Enol. Vitic*. **1985**, *36*, 302–313.
(20) Fugelsang, K. C. *Wine Microbiology*; Chapman and Hall: New York, 1997.
(21) Coucheney, F.; Desroche, N.; Bou, M.; Tourdot-Maréchal, R.; Dulau, L.; Guzzo, J. A new approach for selection of *Oenococcus oeni* strains in order to produce malolactic starters. *Int. J. Food Microbiol*. **2005**, *105*, 463–470.
(22) Son, H. S.; Hwang, G. S.; Kim, K. M.; Ahn, H. J.; Park, W. M.; van den Berg, F.; Hong, Y. S.; Lee, C. H. Metabolomic studies on geographical grapes and their wines using ^{1}H NMR analysis coupled with multivariate statistics. *J. Agric. Food Chem*. **2009**, *57*, 1481–1490.
(23) López-Rituerto, E.; Cabredo, S.; López, M.; Avenoza, A.; Busto, J. H.; Peregrina, J. M. A thorough study on the use of quantitative ^{1}H NMR in rioja red wine fermentation processes. *J. Agric. Food Chem*. **2009**, *57*, 2112–2118.
(24) Shimazu, Y.; Uehara, M.; Watanabe, M. Transformation of citric acid to acetic acid, acetoin and diacetyl by wine making lactic acid bacteria. *Agric. Biol. Chem*. **1985**, *49*, 2147–2157.
(25) Nielsen, J. C.; Richelieu, M. Control of flavour development in wine during and after malolactic fermentation by *Oenococcus oeni*. *Appl. Environ. Microbiol*. **1999**, *65*, 740–745.
(26) Nielsen, J. C.; Prahl, C; Lonvaud-Funel, A. Malolactic fermentation in wine by direct inoculation with freeze-dried *Leuconostoc oenos* cultures. *Am. J. Enol. Vitic*. **1996**, *47*, 42–48.
(27) Swiegers, J. H.; Bartowsky, E. J.; Henschke, P. A.; Pretorius, I. S. Yeast and bacterial modulation of wine aroma and flavour. *Aust. J. Grape Wine Res*. **2005**, *11*, 139–173.
(28) Maicas, S.; Gil, J. V.; Pardo, I.; Ferrer, S. Improvement of volatile composition of wines by controlled addition of malolactic bacteria. *Food Res. Int*. **1999**, *32*, 491–496.
(29) Gámbaro, A.; Boido, E.; Zlotejablko, A.; Medina, K.; Lloret, A.; Dellacassa, E.; Carrau, F. Effect of malolactic fermentation on the aroma properties of Tannat wine. *Aust. J. Grape Wine Res*. **2001**, *7*, 27–32.
(30) Etschmann, M. M. W.; Bluemke, W.; Sell, D.; Schrader, J. Biotechnological production of 2-phenylethanol. *Appl. Microbiol. Biotechnol*. **2002**, *59*, 1–8.
(31) Delaquis, P.; Cliff, M.; King, M.; Girard, B.; Hall, J.; Reynolds, A. Effect of two commercial malolactic cultures on the chemicals and sensory properties of chancellor wines vinified with different yeast and fermentation temperatures. *Am. J. Enol. Vitic*. **2000**, *51*, 42–48.
(32) de Revel, G.; Martin, N.; Pripis-Nicolau, L.; Lonvaud-Funel, A.; Bertrand, A. Contribution to the knowledge of malolactic fermentation influence on wine aroma. *J. Agric. Food Chem*. **1999**, *47*, 4003–4008.
(33) Radler, F. Microbial biochemistry. *Experientia* **1986**, *42*, 884–893.
(34) Izquierdo Canas, P. M.; Garcia Romero, E.; Gomez Alonso, S.; Palop Herreros, M. L. L. Changes in the aromatic composition of Tempranillo wines during spontaneous malolactic fermentation. *J. Food Compos. Anal*. **2008**, *21*, 724–730.
(35) Ugliano, M.; Moio, L. Changes in the concentration of yeast-derived volatile compounds of red wine during malolactic fermentation with four commercial starter cultures of *Oenococcus oeni*. *J. Agric. Food Chem*. **2005**, *53*, 10134–10139.

Received February 12, 2009. Revised Manuscript Received April 27, 2009. We acknowledge the Korea University Grant for the research professorship of Y.-S.H.