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Metabolic Influence of *Botrytis cinerea* Infection in Champagne Base Wine

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Supporting Information

ABSTRACT: *Botrytis cinerea* infection of grape berries leads to changes in the chemical composition of grape and the corresponding wine and, thus, affects wine quality. The metabolic effect of *Botrytis* infection in Champagne base wine was investigated through a ^{1}H NMR-based metabolomic approach. Isoleucine, leucine, threonine, valine, arginine, proline, glutamine, γ-aminobutyric acid (GABA), succinate, malate, citrate, tartarate, fructose, glucose, oligosaccharides, amino acid derivatives, 2,3-butanediol, acetate, glycerol, tyrosine, 2-phenylethanol, trigonelline, and phenylpropanoids in a grape must and wine were identified by ^{1}H NMR spectroscopy and contributed to metabolic differentiations between healthy and botrytized wines by using multivariate statistical analysis such as principal component analysis (PCA). Lowered levels of glycerol, 2,3-butanediol, succinate, tyrosine, valine derivative, and phenylpropanoids but higher levels of oligosaccharides in the botrytized wines were main discriminant metabolites, demonstrating that *Botrytis* infection of grape caused the fermentative retardation during alcoholic fermentation because the main metabolites responsible for the differentiation are fermentative products. Moreover, higher levels of several oligosaccharides in the botrytized wines also indicated the less fermentative behavior of yeast in the botrytized wines. This study highlights a metabolomic approach for better understanding of the comprehensive metabolic influences of *Botrytis* infection of grape berries in Champagne wines.

KEYWORDS: *Botrytis cinerea*, Champagne, wine, grape, NMR, metabolomics

INTRODUCTION

Fungal infection of the grape berry by *Botrytis cinerea* induces significant modifications in the chemical or metabolite compositions in the grape berry itself and the corresponding wine,[1,2] leading to the production of sweet white wines, such as Sauternes, made from fungus-infected overripe berries and characterized by an exceptional range of aromas and an increased sugar concentration.[3,4] Although the noble rot caused by *B. cinerea* is desired for the production of high-quality sweet white wines, gray rot leads to undesirable or negative effects on wine quality mainly through the degradation of aroma compounds and the production of off-flavors, such as "moldy" and "earthy" aromas.[4,5] In the Champagne vineyard, *B. cinerea* infection rates can reach 15−25%, depending on the vintage, and might cause considerable economic losses for winemakers. Therefore, *Botrytis* infection is considered to be undesirable in Champagne wines with respect to organoleptic defaults.[1] In particular, the loss of the foaming properties of Champagne base wines was found to be caused by *Botrytis* infection through the degradation of grape proteins by *Botrytis*-secreted proteases.[6,7]

To date, most studies have focused on the influence of *Botrytis* infection on aromas or volatile compounds.[3,8,9] Because volatile compounds in wine are directly associated with the primary compounds, such as amino acids, organic acids, and polyphenols,[10] the comprehensive investigation on the influence of *Botrytis* infection on the primary metabolites in wine could be an important feature for a better understanding of the relationship between fungal infection and wine quality. Metabolomics is a useful approach for a comprehensive investigation of metabolite variations, which are coupled with global metabolic profiling and multivariate statistical analysis. In particular, the ^{1}H NMR-based metabolomic approach provides better and global understanding of grapevine physiology[11−13] and wine fermentation.[14] For example, a strong relationship between wine metabolome and climate conditions in a vineyard and a dependence of metabolic variations on wine bacteria have been reported recently in wine through ^{1}H NMR-based metabolomics.[5,15−18]

This study aimed at characterizing and understanding the global metabolic effect of *Botrytis* infection in Champagne base

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wine. Champagne base wines were vinified from healthy and botrytized grape berries harvested in the same vineyard in 2008, 2009, and 2010. The grape must and wine metabolites were analyzed by using ^{1}H NMR analysis, and the influence of *Botrytis* infection was investigated with the global profiled metabolites coupled with multivariate statistical analysis.

MATERIALS AND METHODS

Chemicals. All chemical reagents were of analytical grade. The standard reagents, D_2O (99.0% ^{2}H) and TSP (98%), were purchased from Sigma (St. Louis, MO).

Vinification. Grape berries of the Chardonnay variety were hand-harvested in the same vineyard in the Champagne area (France) in September of 2008, 2009, and 2010. Champagne base wines were vinified five times from musts prepared with healthy (H) and *B. cinerea*-infected (B) grapes. Musts from 2008 and 2009 were stored at −20 °C prior to vinification, but the musts from 2010 were used for the vinification without storage. The rate of *Botrytis* infection was expressed as the percent of infected grape berries versus the total number of grape berries on a bunch. The rates were visually estimated at about 20% (B20) in 2008 and 2010 and 20% (B20) and 40% (B40) in 2009 (see Figure S1 of the Supporting Information). Moreover, *Botrytis*-infected and desiccated berries (BD) in 2008 were also collected for this study. The Chardonnay musts were racked and chaptalized with sucrose. The alcoholic fermentation was carried out by *Saccharomyces cerevisiae* var. *bayanus* (active dry yeast, IOC 18-2007, Institut Oenologique de Champagne, Epernay, France) at 18 °C. The base wines were centrifuged at 9500*g* for 10 min, filtered through a 0.45 μm membrane filter (Alltech), and stored at −20 °C until analysis.

Gluconic Acid Determination. pH values and other enological parameters were determined according to official methods.[19] D-Gluconic acid concentration was quantified using an enzymatic analysis kit (R-Biopharm GmbH, Darmstadt, Germany) and expressed in grams per liter.

^{1}H NMR Spectroscopic Analysis of Wines. One milliliter of wine was lyophilized in a 2 mL Eppendorf tube and dissolved in deuterium oxide (400 μL, D_2O), mixed with 400 mM oxalate buffer (140 μL, pH 2.5) and 5 mM 3-(trimethylsilyl) [2,2,3,3-$^2H_4$]propionate (60 μL, TSP, 98%), and then centrifuged at 9500*g* for 10 min. Supernatants (550 μL) were transferred to 5 mm NMR tubes. D_2O and TSP provided a field frequency lock and a chemical shift reference (^{1}H, δ 0.00), respectively. ^{1}H NMR spectra were acquired on a Bruker Avance III 800 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 800.35 MHz ^{1}H frequency and a temperature of 300 K, using a standard broadband probes with *z*-gradient (Bruker Biospin). A standard Bruker water presaturation pulse sequence ("zgpr") was used over a spectral width of 12820.5 Hz with 64 scans collected into 32K data points after four dummy scans, using a 5 s relaxation delay and 5 s total acquisition time. A 0.3 Hz line-broadening function was applied to all spectra prior to Fourier transformation. Signal assignment for representative samples was facilitated by two-dimensional (2D) total correlation spectroscopy (TOCSY), correlation spectroscopy (COSY), heteronuclear multiple-bond correlation (HMBC), heteronuclear single-quantum correlation (HSQC), and comparisons to the literature.[5,11,15] Furthermore, statistical TOCSY (STOCSY)[20] was also used for the signal assignment.

NMR Data Processing and Multivariate Statistical Analysis. All NMR spectra were phased and baseline corrected manually using ACD software version 9 (Advanced Chemistry Development, Toronto, Canada) and then converted to ASCII format. The ASCII format files were imported in MATLAB (R2008a, Mathworks, Inc.). Probabilistic quotient normalization of the spectra was carried out,[21] and the spectra were aligned by the recursive segment-wise peak alignment (RSPA) method to reduce variability in the peak positions.[22] Prior to the normalization and spectral alignment, the regions corresponding to water (δ 4.6−4.8), residual ethanol peaks (δ 1.043−1.086, 1.119−1.286, and 3.590−3.625) that included the satellite ethanol peaks, and TSP (−0.5 to 0.7 ppm) were removed. The latest peaks of removed ethanol included partial peaks corresponding to glycerol in wine samples. The resultant data sets were imported into SIMCA-P version 12.0 (Umetrics, Umeå, Sweden), and a mean-centered scaling method was applied for multivariate statistical analysis. PCA, an unsupervised pattern recognition method, was first performed to examine the intrinsic variation in the data set. Orthogonal projection on latent structure discriminant analysis (O-PLS-DA), a supervised pattern recognition method, was used to extract maximum information on discriminant compounds for the data. O-PLS-DA provides a way to remove systematic variation from an input data set *X* (compounds or metabolites) not correlated to the response set *Y* (discriminant classes).[23] Hotelling's T_2 region, shown as an ellipse in the score plot, defines the 95% confidence interval of the modeled variation. The O-PLS-DA models were validated using a 7-fold cross-validation method and with a permutation test (200 permutations). To facilitate interpretation of the results, the O-PLS loadings or coefficient plots that revealed metabolites responsible for the metabolic differentiations between two classes, for example, the healthy and botrytized base wines, were generated with a color-coded correlation coefficient for each data point using MATLAB (The MathWorks, Inc.) with scripts developed in-house at Imperial College London.[24] The quality of the models is described by R^2_X and Q^2 values. R^2_X 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 by ANOVA and Duncan's multiple-range test. In particular, the paired *t* test was performed for the significance analysis of each metabolite with integral area in pairs of the healthy and botrytized base wines.

RESULTS

Gluconic Acid Concentration. pH values were slightly increased in the *B. cinerea*-infected Champagne base wines compared to the healthy base wines (Table 1). As expected, higher concentrations of D-gluconic acid in the botrytized musts were observed, compared to those in the healthy musts, and the concentration was dependent on the *Botrytis* infection rate. Moreover, it was interesting to note that the botrytized and desiccated (BD) grape musts from 2008 had the highest concentration of gluconic acid among the samples.

Metabolite Assignments with ^{1}H NMR Spectroscopy. Figure 1 shows the representative ^{1}H NMR spectra of the healthy and botrytized grape musts (A−C) and corresponding Champagne base wines (D−F). The main metabolites of isoleucine, leucine, valine, arginine, threonine, proline, glutamine, γ-aminobutyric acid (GABA), succinate, malate, citrate, tartarate, fructose, glucose, oligosaccharides (C1 and C2), and phenylpropanoids were identified in the grape musts. Moreover, unknown compounds (A1 and A2), 2,3-butanediol, acetate, tyrosine, 2-phenylethanol, and glycerol were additionally observed in the base wines. However, a number of minor metabolites remain unassigned. Of these main metabolites in the musts or base wines, C1 and C2 in anomeric regions resolved at δ 5.18 (doublet, d; coupling constant, J = 3.8 Hz) and δ 5.32 (d, J = 3.8 Hz), respectively, were correlated with aliphatic regions of sugar by 2D TOCSY experiment (data not shown) and thus tentatively assigned to oligosaccharides. In particular, according to a previous study,[13]

Table 1. Changes in pH and D-Gluconic Acid in Healthy and Botrytized Chardonnay Grape Musts and Corresponding Champagne Base Wines

		2008			2009			2010	
		H[a]	B20	BD	H	B20	B40	H	B20
pH	must	2.34	2.44	2.44	2.33	2.37	2.50	1.96	2.14
	wine	2.36 ± 0.01 c[b]	2.40 ± 0.01 b	2.44 ± 0.00 a	2.25 ± 0.02 c	2.32 ± 0.01 b	2.42 ± 0.00 a	2.08 ± 0.04 b	2.15 ± 0.02 a
D-gluconic acid (mg/L)	must	7.9	41.6	97.7	7.0	26.0	46.4	9.0	73.1
	wine	7.3 ± 0.5 c	35.1 ± 2.9 b	79.2 ± 7.7 a	5.7 ± 2.0 c	21.3 ± 1.9 b	39.0 ± 3.2 a	8.3 ± 0.8 b	65.5 ± 0.7 a

[a] H, healthy; B20 and B40, 20 and 40% botrytized; BD, botrytized and desiccated. [b] All values from the base wines were from five experimental replicates of vinification and expressed as the mean ± standard deviation. Values with different letters are significantly different by Duncan's multiple-range test at $P < 0.05$.

the unknown compound (A1) having two methyl protons (CH_3) was found only in base wines, which was consistent with the results in the present study. Therefore, these results revealed that A1 is an alcoholic fermentative product in wine. We tried to assign A1 by 2D NMR, spiking, and simulation experiments. The methyl protons of A1 were resolved at δ 0.88 (d, J = 7.0 Hz) and δ 0.94 (d, J = 6.8 Hz) of the NMR spectrum but, unfortunately, failed to be assigned completely. However, the A1 structure was similar to valine with two methyl protons resolved at δ 0.96 (d, J = 7.0 Hz) and δ 1.03 (d, J = 6.8 Hz). Furthermore, the methyl protons of A1 are correlated with CH at δ 1.95 (multiplet) linked directly to the methyl protons, as observed by 2D TOCSY experiment (data not shown), whereas those of valine have the correlation with CH at δ 2.25 (multiplet). Therefore, compound A1 was tentatively assigned to a valine derivative in the present study, which could be an intermediate produced when valine is converted to other compounds during alcoholic fermentation. Moreover, A2 (d, J = 6.8 Hz) was aliphatic and thus considered as an intermediate of the amino acid metabolism during alcoholic fermentation. In the aromatic region, olefinic protons of the phenylpropanoids that have the same coupling constants (J = 16.0 Hz) and their conjugations with malate and tartaric acid were observed in grape berry and wine, respectively, as reported by Ali et al.[11,12] Caffeic acid and its conjugations with malate and tartaric acid were clearly found by 2D TOCSY (see Figure S2 of the Supporting Information) and 1D STOCSY (Figure 2A), respectively, providing their assignments to *trans*-caffeoyl malate and *trans*-caftaric acid. In contrast to the strong correlations or correlation coefficients among the olefinic protons of the phenylpropanoids in the STOCSY analysis, the correlations between olefinic and malic/tartaric protons were found to be relatively weak, indicating that olefinic and tartaric and malic acid molecules were still undergoing conjugation in grape must or wine during winemaking, as described in the case of the terminal CH_3 group of isoleucine that showed STOCSY correlation with leucine, valine, and lysine.[25] However, the protons of malic/tartaric acids attached to the carbonyl groups of phenylpropanoids, which are known to be shifted largely downfield through the conjugation,[26] were not observed, likely due to too weak resonances or strong overlap with many unknown oligosaccharides produced or degraded during alcoholic fermentation at the regions from δ 5.3 to 5.4. Therefore, it is likely that caffeic acid and its conjugation products with malate and tartarate, that is, caffeic malate and caftaric acid, respectively, could be present simultaneously in our samples. *trans*/*cis*-Caffeoyl malate and -caftaric acid, and *trans*/*cis*-coutaric acids were also identified. Furthermore, the correlations among protons of trigonelline led to its clear assignment (Figure 2B).

Visual inspection of the NMR spectra revealed marked differences in polyphenol levels between healthy and botrytized musts. In particular, new compounds were found in the botrytized musts as highlighted by boxes in Figure 1 but were not assigned yet. To provide comparative interpretations and visualization for the metabolic effects of *Botrytis* infection in the Champagne base wines, a pattern recognition method, such as PCA or O-PLS-DA, was applied to NMR spectral data sets of the Champagne base wines vinified with the healthy and botrytized grapes harvested in the same vineyard in 2008, 2009, and 2010.

Metabolomic Changes in Botrytized Champagne Base Wine. A PCA score plot shows clear metabolic differentiations between the healthy and botrytized base wines in 2009 and also metabolic dependence on the *Botrytis* infection degree (Figure 3A). Furthermore, an O-PLS score plot exhibited more distinct differentiation among the base wines, compared to the PCA model, as shown in Figure 3B, indicating the effective removal of noncorrelated variation in X variables (metabolites in NMR spectra) to Y variables (classes or groups) in the O-PLS model. To identify the metabolites responsible for the differentiation among the base wines, pairwise O-PLS models were generated using one predictive and one orthogonal components, and all models had high R^2_X and Q^2 values, which indicated high goodness of fit and predictability, respectively: R^2_X from 0.74 to 0.98 and Q^2 from 0.88 to 0.95. Furthermore, all models were validated by a random permutated test (data not shown), and the typical permutated validations between the healthy and botrytized base wines from 2009 are provided in Figure S3 of the Supporting Information. As indicated by these results, all randomly permutated R^2_X and Q^2 values were lower than their original values and, thus, the models were validated.

Individual metabolites contributing to the differentiations among the wines from 2008, 2009, and 2010 could be found in the corresponding O-PLS loading or coefficient plots (Figures 4 and 5). The direction of the ^{1}H NMR peaks in the O-PLS coefficient plot represents the relative differences in metabolites between two classes. The colors on the O-PLS coefficient plots depend on the correlation coefficient and are associated with the significance of metabolites responsible for the differentiation between the botrytized and healthy base wines. In the present study, the correlation coefficients were considered to be significant when >0.43, which corresponded to the critical value of a correlation coefficient at $P < 0.05$ verified by Student's t test with integral areas of individual metabolites. Variations in acetate and

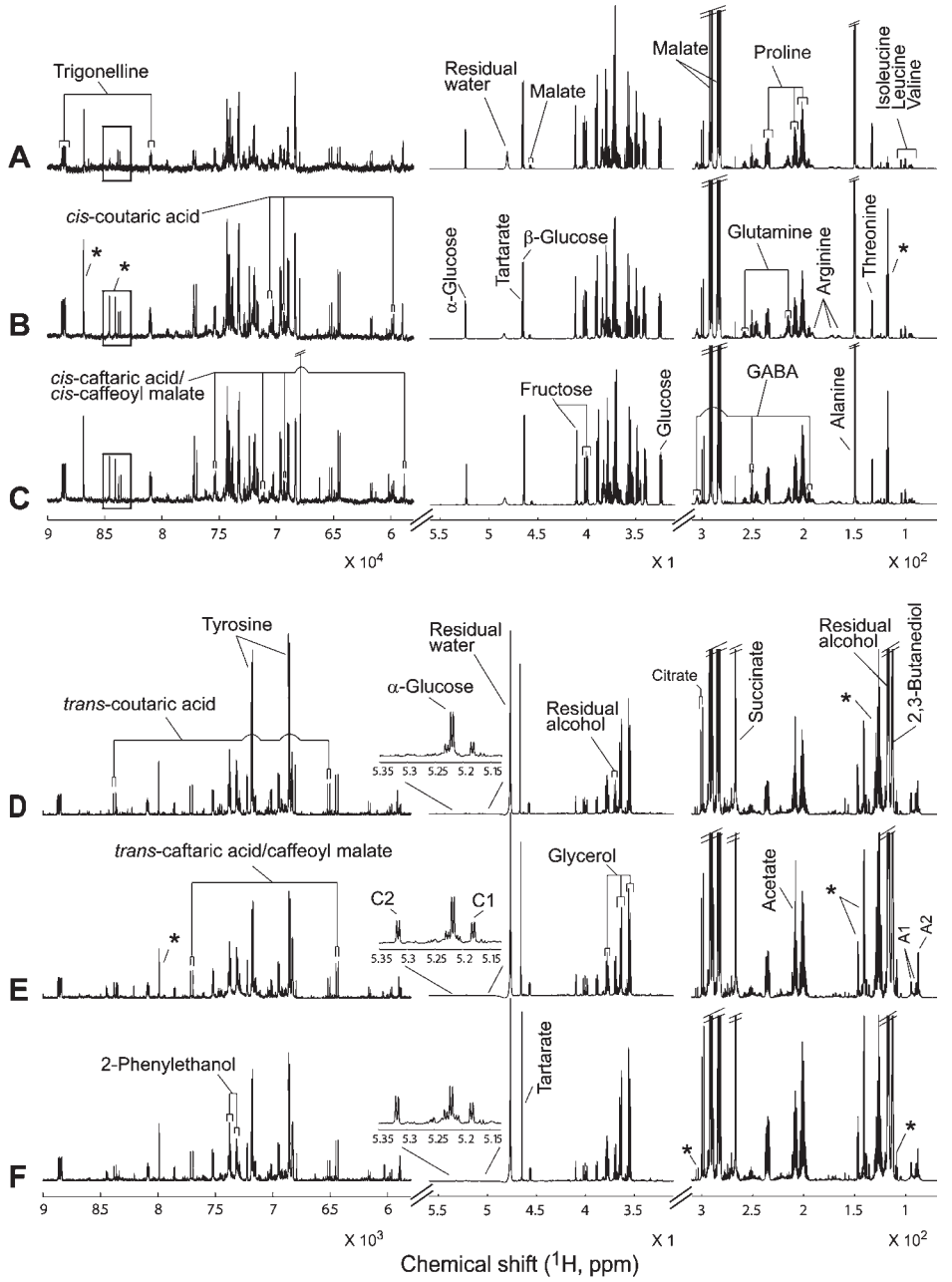

Figure 1. Typical 800 MHz ^{1}H NMR spectra of grape musts (A−C) and Champagne base wines (D−F). The grape musts and corresponding base wines were obtained from healthy (A, D), 20% botrytized (B, E), and 40% botrytized (C, F) grapes harvested in 2009. A1 and A2 were tentatively assigned to valine derivative and amino acid metabolic intermediate, respectively. C1 and C2, oligosaccharides. Asterisks denote unknown compounds.

tartarate levels were not considered in the present study because their levels were not reproducible from incomplete lyophilization and heterogeneous precipitation during freezing storage, respectively, as described in a previous study.[15]

As shown in Figure 4A, the O-PLS coefficient plot for the model differentiating the 20% *Botrytis*-infected base wine (B20) from the healthy base wine (H) from 2009 enabled the identification of the metabolites responsible for the differentiation. Lowered levels of the valine derivative (A1), succinate, 2,3-butanediol, glycerol, tyrosine, 2-phenylethanol, trigonelline, and phenylpropanoids, together with higher levels of proline, glucose, fructose, and oligosaccharides (C1 and C2), were observed in the 20% botrytized base wines (B20). The reduction and elevation of these metabolites responsible for the differentiation between the 20% botrytized (B20) and healthy (H) base wines also contributed to the differentiation between the 40% botrytized (B40) and healthy (H) base wines (Figure 4B). In the comparisons between the healthy and botrytized base wines from 2008 and 2010 (Figure 5), although only proline levels varied, the reduction and elevation of the same metabolites found in the healthy and botrytized base wines from 2009 were observed. However, lowered levels of malate, citrate, and the amino acid derivative (A2) were further responsible for the differentiations of the botrytized base wine from the healthy base wine in 2008 and 2010. In particular, marked increases in oligosaccharides (C1 and C2) and decreases in succinate levels in the botrytized base wines

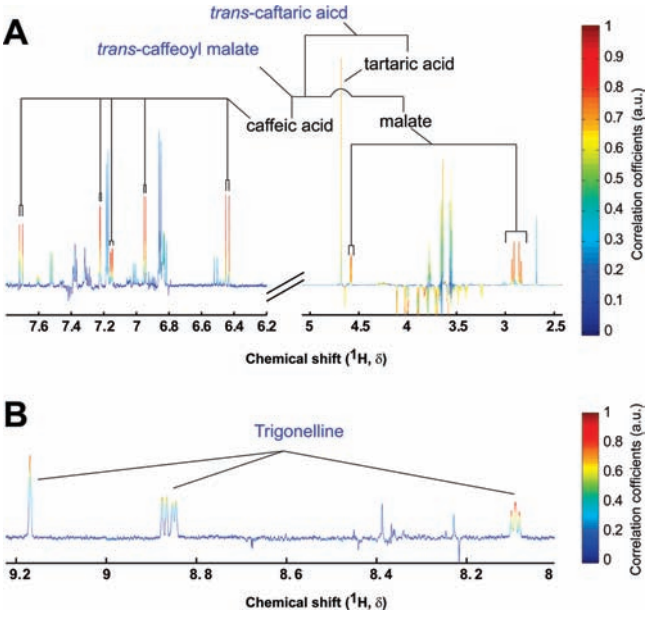

Figure 2. One-dimensional (1D) statistical total correlation spectroscopy (STOCSY) analysis for the *trans*-caffeoyl malate and caftaric acid (A) and trigonelline (B), of which caffeic acid was directly correlated with malate and tartaric acid.

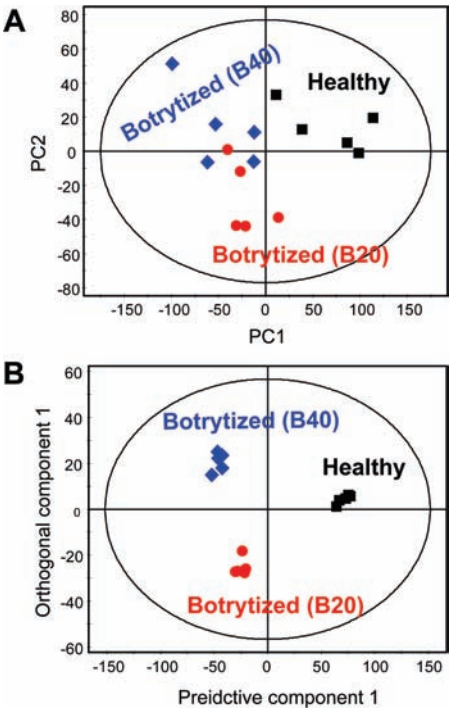

Figure 3. PCA (A) and O-PLS-DA (B) score plots derived from ^{1}H NMR spectra of healthy and botrytized (B20 and B40) base wines from 2009, indicating metabolic differences among the wines. B20 and B40 represent 20 and 40% *B. cinerea* infection, respectively.

were typically proven by normalized raw NMR spectra and their integral areas, as shown in Figures 6 and 7, respectively.

DISCUSSION

Metabolic differences in the Champagne base wines vinified with healthy and botrytized grapes harvested in the same vineyard in 2008, 2009, and 2010 were compared by using ^{1}H NMR-based global metabolic profiling. Clear differences in the metabolites were found between the healthy and botrytized base wines, and their relationships on the metabolic pathways are summarized in Figure 8. The variations in metabolites highlighted in the metabolic map were associated with fermentative behavior or activity during alcoholic fermentation because they are fermentative products. Elevated gluconic acid concentrations in the botrytized grapes and base wines exhibited a good agreement with the variations in the concentrations reported in a previous study,[6] demonstrating that gluconic acid levels can be used as an indicator of the fungal attack. The observations of the increased pH values in the botrytized musts and base wines were also consistent with the results obtained in the previous study,[6] which indicated degradation of the main organic acids by *B. cinerea*. Moreover, after completion of alcoholic fermentation, a slight consumption of gluconic acid by yeast was found, as reported in sherry wines.[27]

Metabolites Originating from Grape. Malate, citrate, and proline are known as nondegradable metabolites by yeast during alcoholic fermentation. However, malate and citrate can be converted to other compounds by lactic acid bacteria during malolactic fermentation in wine.[5,28] Therefore, variations in malate, citrate, and proline levels in the base wine were caused by intrinsically different levels in healthy and botrytized grape berries, because malolactic fermentation did not occur in the present study. Low levels of malate and citrate observed in the botrytized wines resulted from their degradations by *B. cinerea* in grapes,[29] rather than from the influence of the fungus during wine fermentation.

Reduced Carbohydrate Metabolism. The glucose levels calculated by the integral area of glucose normalized were decreased 260, 145, and 156 times in the healthy, 20% (B20), and 40% (B40) botrytized base wines from 2009 after alcoholic fermentation, respectively (data not shown). Moreover, in the base wines vinified with healthy (H), 20% botrytized (B20), and botrytized and desiccated (BD) grapes harvested in 2008, glucose levels were decreased by 98, 99.5, and 99.3%, respectively, after alcoholic fermentation. In addition, yeast reduced the glucose levels by 99.5 and 99.9% in base wines elaborated with healthy (H) and botrytized grapes (B20), respectively, harvested in 2010. These variations in glucose levels indicated that glucose consumption by yeast during alcoholic fermentation is not dependent on *Botrytis* infection of the grape berries. However, unique low consumption of several oligosaccharides, indicated by marked high levels of oligosaccharides (C1 and C2), in the botrytized base wines from 2008, 2009, and 2010 was found (Figure 6). These reduced consumption or degradation of several oligosaccharides in the botrytized base wines may indicate retardation of the yeast metabolic activity, consistent with the report that the reduced fermentability of botrytized must have resulted from the decrease in fermented sugar contents in botrytized samples during alcoholic fermentation.[30]

Reduced Fermentative Products. Unique lower levels of succinate, valine derivative (A1), tyrosine, glycerol, and 2,3-butanediol in all botrytized base wines from 2008, 2009, and 2010 were found, as compared to those in healthy base wines (Figures 4 and 5). For example, although succinate levels were higher in botrytized grape musts than in healthy grape musts, the levels in the base wines vinified from healthy grapes were found to be increased

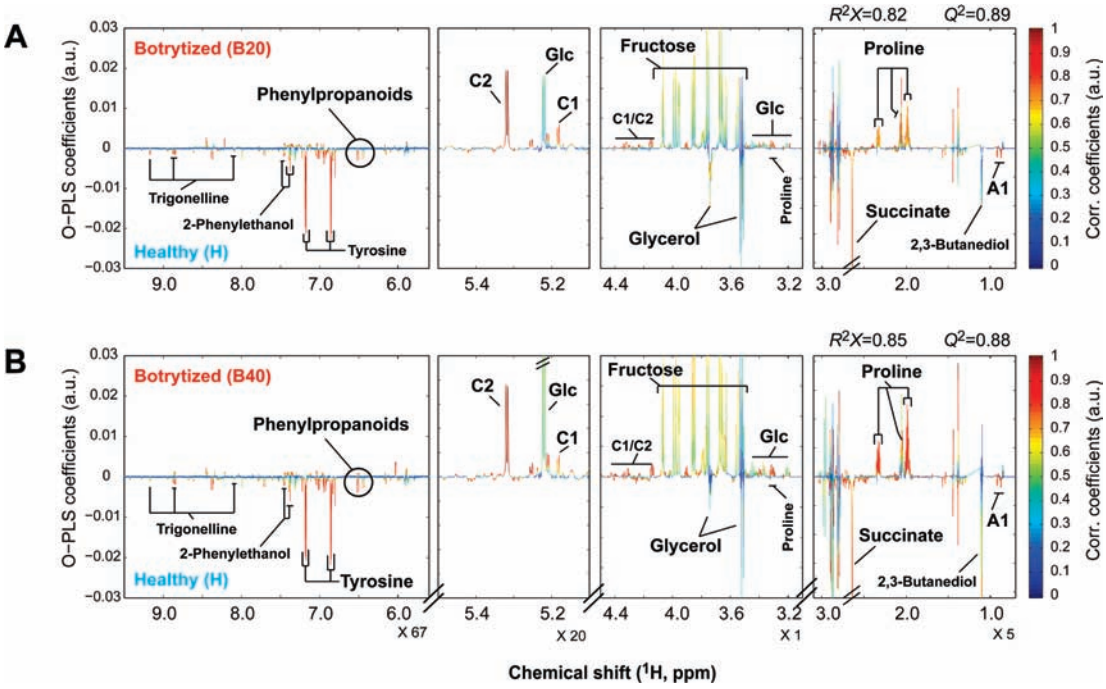

Figure 4. O-PLS coefficient plots derived from ^{1}H NMR spectra of base wines vinified with healthy and *B. cinerea*-infected grapes harvested in 2009, revealing the identifications of the metabolites responsible for the metabolic differentiation between the botrytized and healthy base wines. Glc, α-glucose; C1 and C2, oligosaccharides; A1, valine derivative. Phenylpropanoid compounds contain *cis*/*trans*-caftaric acid and caffeoyl malate, and *trans*/*cis*-coutaric acid. B20 and B40 represent 20 and 40% *Botrytis* infection, respectively.

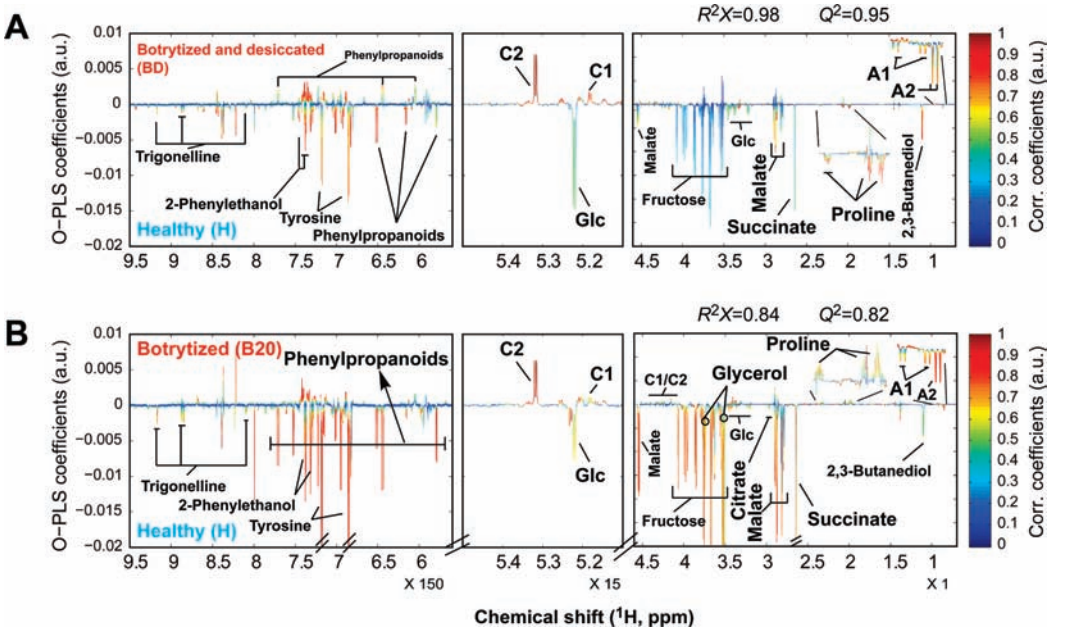

Figure 5. O-PLS coefficient plots derived from ^{1}H NMR spectra of base wines vinified with healthy and *B. cinerea*-infected grapes harvested in 2008 (A) and 2010 (B). Glc, α-glucose; C1 and C2, oligosaccharides; A1, valine derivative; A2, amino acid metabolic intermediate. Phenylpropanoid compounds contain *cis*/*trans*-caftaric acid and caffeoyl malate and *trans*/*cis*-coutaric acid.

from 1.3 to 2.5 times, which were calculated by integral area of the NMR peaks and significant below the probability of 0.05 (Figure 7). When we consider the report that *Botrytis*-infected grape berries revealed a 2−7-fold reduction in total amino acid concentration, as compared to healthy berries,[31] the alterations in amino acid levels in the botrytized grape could influence, consequently, the succinate levels in the corresponding wine. Indeed, amino acids, such as glutamine, GABA, and threonine, contribute to the production of succinate during alcoholic fermentation.[4] However, although levels of these amino acids, typically glutamine and GABA, varied between the healthy and botrytized grape musts harvested in 2008, 2009, and 2010 (see

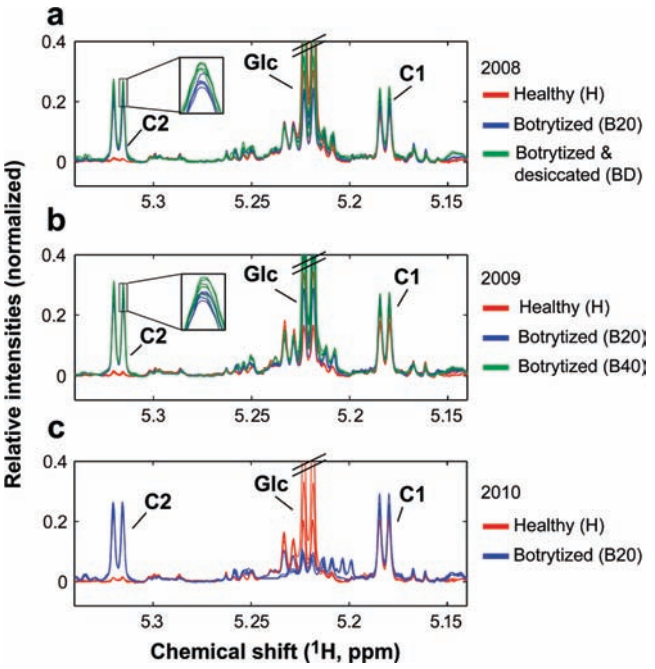

Figure 6. Changes in glucose (Glc) and oligosaccharides (C1 and C2) between the base wines vinified with healthy and botrytized grapes harvested in 2008 (a), 2009 (b), and 2010 (c), highlighting the reduced consumption of oligosaccharides (C1 and C2) in the botrytized base wines after alcoholic fermentation. B20 and B40 represent 20 and 40% *Botrytis* infection, respectively.

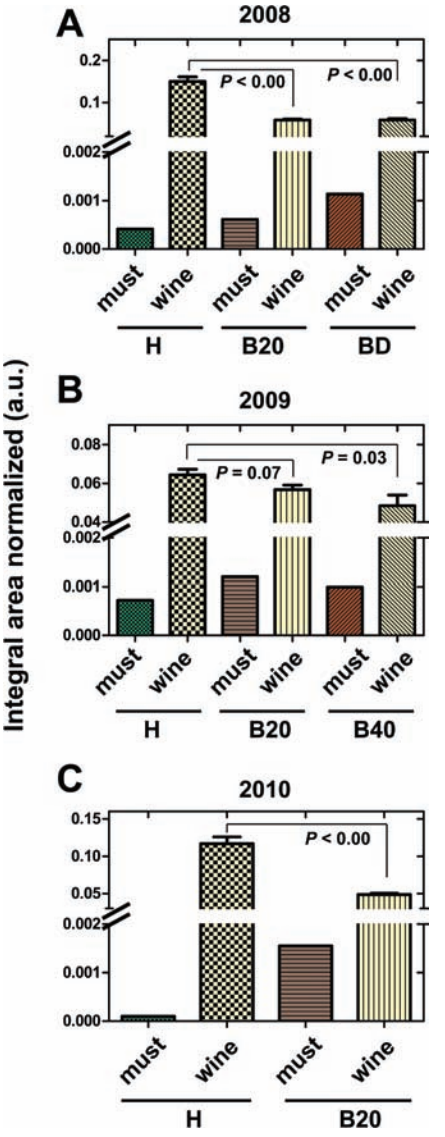

Figure 7. Comparisons of succinate levels in grape musts and base wines obtained and vinified from healthy and botrytized grape berries harvested in 2008 (A), 2009 (B), and 2010 (C). H, healthy; B20 and B40, 20 and 40% botrytized; BD, botrytized and desiccated.

Figure S4 of the Supporting Information), succinate levels were clearly lowered in all botrytized base wines. Therefore, lowered succinate levels in the botrytized base wines may be due to less effective regulation of the tricarboxylic acid (TCA) cycle than in the healthy base wine, indicating a reduced alcoholic fermentative activity in the botrytized wines because succinate is one of the major intermediates of the TCA cycle. This reduced fermentability in the botrytized wines could also be explained by reductions in glycerol, 2,3-butanediol, tyrosine, and valine derivative (A1) levels observed in the present study, because these metabolites are the main products of the alcoholic fermentation.[14]

Marked accumulations of glycerol and gluconic acid in botrytized grape berry are well-known phenomena.[32] It is therefore likely that glycerol concentration is substantially increased in wines vinified from botrytized grape berries.[33] However, glycerol levels in the botrytized wines were significantly lowered compared to those in healthy wines in the present study. It may also be a consequence of the reduced fermentability of the botrytized base wines because glycerol is one of the main products from the alcoholic fermentation. These results indicated that the different glycerol levels between the healthy and botrytized wines were dominated by the differences of glycerol produced during alcoholic fermentation rather than by intrinsic concentration in the grapes. However, high differences in glycerol concentrations between the healthy and botrytized grapes could affect the concentrations of glycerol in corresponding wines, but no changes in glycerol levels were found between healthy (H) and botrytized and desiccated (BD) wines in 2008 (Figure 5A). Although glycerol was not detected in ^{1}H NMR spectra of the grape musts due to overlap with huge NMR peaks of glucose and fructose in the present study, relative glycerol concentration in grape musts could be comparable using gluconic acid concentrations as provided in Table 1. Indeed, more accumulation of gluconic acid usually leads to higher levels of glycerol in grapes.[32] Therefore, high glycerol levels in the corresponding base wines following large amounts of gluconic acid in botrytized and desiccated (BD) must from 2008 may lead to a similar level of glycerol with healthy (H) base wines.

To date, few studies have reported the metabolic or fermentative influences of *Botrytis* infection of grape berry in the corresponding wine. Ribereau-Gayon[30] suggested that the reduced fermentability in botrytized samples could be caused by accumulation of antiyeast substances, such as acetic acid or mannoproteins. However, the identification of the metabolites responsible for the antiyeast activity still require further investigation. Moreover, the disappearance of numerous grape proteins and the finding of two pectinolytic enzymes secreted by *B. cinerea* in the botrytized Champagne base wine were reported.[7] Therefore, it is likely that the lesser fermentability in the botrytized base wine

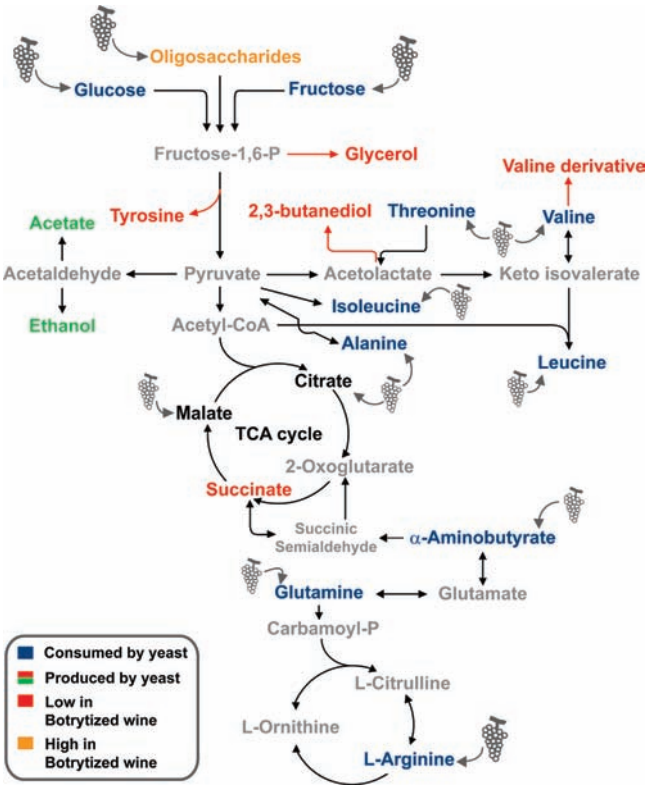

Figure 8. Schematic summary of metabolic differences between the healthy and botrytized base wines. Blue denotes the decreased metabolites during alcoholic fermentation, whereas red and green represent the increased metabolites. In particular, the metabolites in red increased during alcoholic fermentation and highlight the lowered metabolites in the botrytized base wine compared to those in the healthy base wine. Oligosaccharides marked in orange were less consumed by yeast in the botrytized base wine.

would be a consequence of compositional differences, including grape metabolites and proteins of *Botrytis*-infected grape berries. Furthermore, with respect to the degradation of numerous proteins in botrytized grape berries, the lack or loss of enzymes able to degrade polyphenolic compounds from their conjugates may cause the reduced degradations or extractions of phenylpropanoids in the botrytized base wines during alcoholic fermentation as observed here, but this needs further study. Moreover, reduced levels of 2-phenylethanol in the botrytized base wine could be evidence of perturbations of protein or amino acid metabolism by *Botrytis* infection (Figures 4 and 5). Indeed, 2-phenylethanol is synthesized by yeast through the Ehrlich pathway via transamination of L-phenylalanine to phenylpyruvate,[34] which indicates a lower amount of L-phenylalanine in the botrytized grape berries. Therefore, *B. cinerea* infection in grape berries could influence wine quality through metabolic modifications in both grape berries and corresponding wines, as, for example, the loss of foaming properties and protein in botrytized Champagne wines.[6,35]

This research marks the first investigation on the comprehensive metabolic effects of *B. cinerea* in Champagne base wine through NMR-based metabolomic approach, demonstrating that the global analysis of metabolites could provide better understanding of metabolic perturbations in the botrytized Champagne base wine and useful information on their associations with Champagne wine quality.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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