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Metabolomic by ^{1}H NMR Spectroscopy Differentiates "Fiano Di Avellino" White Wines Obtained with Different Yeast Strains

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ABSTRACT: We employed ^{1}H NMR spectroscopy to examine the molecular profile of a white "Fiano di Avellino" wine obtained through fermentation by either a commercial or a selected autochthonous *Saccharomyces cerevisiae* yeast starter. The latter was isolated from the same grape variety used in the wine-making process in order to strengthen the relationship between wine molecular quality and its geographical origin. ^{1}H NMR spectra, where water and ethanol signals were suppressed by a presaturated T_1-edited NMR pulse sequence, allowed for definition of the metabolic content of the two differently treated wines. Elaboration of NMR spectral data by multivariate statistical analyses showed that the two different yeasts led to significant diversity in the wine metabolomes. Our results indicate that metabolomics by ^{1}H NMR spectroscopy combined with multivariate statistical analysis enables wine differentiation as a function of yeast species and other wine-making factors, thereby contributing to objectively relate wine quality to the terroir.

KEYWORDS: *nuclear magnetic resonance, T_1-filtered spectra, autochthonous yeast starter, "Fiano di Avellino" white wine, metabolomic, chemometric techniques*

INTRODUCTION

An ever-increasing interest is placed by large-scale wine producers on the ecology and evolution of yeasts during alcoholic fermentation of grape wine, to assess a correlation between microbial populations and wine molecular quality. The diversity of yeast communities collected from grapes was shown to depend on several factors, such as the vineyard geographical location,[1,2] grape variety, degree of grape maturation, and harvesting technique.[3,4] The use of selected yeasts in wine-making provides a number of advantages: (i) fast increase of yeast concentration into grape must, inducing a rapid start of alcoholic fermentation;[5,6] (ii) full consumption of fermentable sugars and great ethanol content in the final wine product;[7,8] (iii) inhibition of potential spoilage microorganisms with consequent assurance of wine stability;[9−11] (iv) large concentration of secondary metabolites in wine;[12,13] (v) finest control of subtlety and individuality of wine flavor.[14,15]

Within the current emphasis to relate wine properties to the *terroir* of vineyards, most wine producers began to start alcoholic fermentation by employing indigenous yeasts rather than commercial starters, which are isolated in sites away from wine-producing areas. Although the latter are reckoned to maintain constancy of wine quality, yeasts originated from microareas where wines are produced guarantee correlation between wine identity and geographical origin of grapes and even provide greater wine quality.[16] As different yeast starters mostly determine variations in wine organoleptic properties, a corresponding change in wine molecular composition is also expected.[17] In fact, although grapes provide the basic wine chemical constituents, most chemicals found in wines are produced by metabolic activities of yeasts as well as by lactic acid bacteria during the wine-making process.[18] These metabolic products should be carefully characterized by modern analytical techniques to assess the contribution of indigenous microorganisms to the wine *terroir*. Moreover, the identification and quantification of the main metabolites (metabolome) of wines becomes useful to understand the response of primary and secondary metabolism induced by environmental perturbations and wine-making practices such as fermentation.[19]

Nuclear magnetic resonance (NMR) spectroscopy is increasingly used to characterize the metabolic composition of wines.[18,20−25] Highly reproducible spectra of wine samples can be rapidly obtained by liquid-state NMR spectroscopy without sample pretreatments, thereby enabling a simultaneous determination of several low-molecular mass components in the complex wine mixtures.[26,27] Besides, these large spectral data sets can be efficiently simplified by multivariate statistical techniques[28−30] to highlight the most discriminating wine metabolites, which become the basis for the identification and classification of different wines.[20,22,23,31−33]

The aim of this study was to use ^{1}H NMR spectroscopy to evaluate the molecular profiles of two "Fiano di Avellino" wines produced with the same grape variety and oenological techniques but fermented with either a commercial (C) or an autochthonous (A) yeast starter.

MATERIALS AND METHODS

Yeast Selection and Wine Production. Eight "Fiano di Avellino" vineyards were subjected in October 2009 to sampling of grapes and berries within the production area of this grape variety in

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Campania Region (Italy), marked as *controlled and guaranteed denomination of origin* (CGDO). Sampling and storage of grapes and berries were conducted by a method already described.[34]

The wild yeasts isolated from samples underwent a genotypical screening (5.8S internal transcribed spacer-PCR, inter-δ-PCR, and random amplified polymorphic DNA-PCR) to enable differentiation at strain level.[34] Moreover, 17 yeast strains of the *S. cerevisiae* species were selected and subjected to a number of oenological tests,[34] and the strain that revealed the greatest technological potentials was used as a starter culture to produce wine A. A common commercial *S. cerevisiae* strain was employed as a yeast starter to produce wine C. Both wines A and C were obtained in a large-scale wine-making process at the "MASTROBERARDINO s.p.a." winery in Avellino (Campania, Italy) to produce the CGDO "Fiano di Avellino" wine.

Wine fermentation and aging were conducted in steel tanks. Microbiological screenings were again performed on must and wine samples collected during the most critical technological phases of the two parallel wine-making processes, to verify that the inoculated strains remained dominant during the entire fermentation process. Finally, the two experimental wines were bottled after six months.

Among the 40 bottles of "Fiano di Avellino" wine that underwent NMR spectroscopy, 20 were obtained with autochthonous (A) and 20 with commercial (C) yeast starter. The pH was measured on 100 mL of wine immediately after bottle uncorking, and the average pH of wines A and C was 3.17 and 3.28 $\pm$ 0.03, respectively.

NMR Experiments. Wine samples were prepared by mixing 0.25 mL of wine with 0.75 mL of deuterated water (99.8% D_2O/H_2O, Armar Chemicals), containing 2% (v/v) formic acid (98−100% RG, Merck Chemicals) as internal standard.[23] Each sample mixture was stirred in a vortex and transferred into a stoppered 5 mm NMR tube, whose remaining void volume was degassed gently by a N_2 flux. The content of ethanol and acetic acid was quantified by acquiring a proton spectrum with only water presaturation and comparing signal intensity to 3-(trimethylsilyl) propionic acid sodium salt (TMSPA) standard (Merck, Darmstadt, Germany) added in a 0.2 mg mL^{-1} concentration to five replicates of both A and C wines in the NMR tube.

A 400 MHz Bruker Avance spectrometer, equipped with a 5 mm BBI Bruker probe and working at the ^1H frequency of 400.13 MHz, was used to conduct all liquid-state NMR measurements at a temperature of 298 $\pm$ 1 K. Monodimensional ^1H spectra were acquired with 3.5 s of thermal equilibrium delay, a 90° pulse length ranging between 8 and 8.6 μs (−2 dB of attenuation), 96 transients, 32 768 time domain points, and 16 ppm (6410.3 Hz) as spectral width. Intense ethanol signals were suppressed by adopting a T_1-filter consisting of an inversion−recovery pulse sequence.[23,35,36] Because ethanol protons showed a longitudinal relaxation time significantly longer than wine metabolites, a 3 s delay (τ) was introduced in the inversion recovery pulse sequence (180°−τ−90°). This delay provided an almost complete recovery of metabolites signals before complete relaxation of ethanol multiplets, thereby ensuring a total annulment of ethanol resonances. In addition, the combination of such a sequence with on-resonance presaturation (4.706 $\pm$ 0.001 ppm) enabled the efficient multisuppression of both ethanol and water signals. The free induction decays (FIDs) were Fourier transformed with a function size of 65 536 points, applying a 0.2 Hz line-broadening.

Structural identification of wine metabolites was achieved by 2D NMR experiments, consisting of homonuclear $^1H-^1H$ COSY (correlation spectroscopy) and TOCSY (total correlation spectroscopy) as well as heteronuclear $^1H-^{13}C$ HSQC (heteronuclear single-quantum correlation) and HMBC (heteronuclear multiple bond correlation). Because an excitation sculpting block was implemented in both 2D homonuclear experiments to remove the protonic ethanol signals, a specific shape pulse was built by modifying the basic shape pulse SQUA100.1000 reported by Bruker Topspin. The best shape pulse length and power calibration consisted of 8000 μs for all samples, respectively, and in a range from 25.45 to 33.9 dB, depending on the sample. All 2D experiments had a spectral width of 16 (6 410.3 Hz) and 300 (30 186.8 Hz) ppm for ^1H and ^{13}C nuclei, respectively, and a time domain of 2 048 points (F_2) and 512 experiments (F_1). All homonuclear 2D spectra consisted of 16 dummy scans and 64 total transients, while for the TOCSY experiment a mixing time of 80 ms and a trim pulse length of 2 500 ms were adopted. HSQC and HMBC heteronuclear experiments were acquired with 16 dummy scans, 80 total transients, and 0.5 μs of trim pulse length. Moreover, optimization of the latter experiments was achieved with 145 and 6.5 Hz as the optimal $^1H-^{13}C$ short and long-range J-couplings, respectively. A baseline correction was applied to all mono- and bidimensional spectra, and the proton frequency axis was calibrated by associating the formic acid signal at 8.226 ppm.[37] Spectra were processed by using both Bruker Topspin Software (v.2.1) and MestReC NMR Processing Software (v.4.9.9.9).

Multivariate Data Analyses. The multivariate analyses were applied to ^1H NMR spectra which were preliminarily divided into 115 buckets. The bucket intervals had variable widths (the largest one comprised 0.08 ppm), so that each bucket included either individual signals or distinct multiplets, when no signals overlapping occurred. Buckets were selected over the 7.8−0.4 ppm spectral region and were applied to all spectra because no significant chemical shifts drifts were observed for either A or C wines. Prior to multivariate analysis, spectra were normalized by dividing each single bucket area by the sum of all signal areas and Pareto-scaled for PCA (principal component analysis), DA (discriminant analysis), and HCA (hierarchical cluster analysis). The DA method consisted of building a validation set, achieved by dividing all samples in either a training or a test set. The former set used a discriminant model formed by 27 of the 40 samples, whereas the remaining 13 samples comprised the latter set to test the model. A similar partition, containing around 32.5% of samples in the test set, allowed a sufficient number of samples in the training set as well as a representative number of samples in the test set.[22] This validation was repeated 5 times by changing, each time randomly, the samples included in the training and test set. An HCA was conducted by executing a hierarchical ascendant clusterization. The extent of similarity among samples was measured by Euclidean distances, whereas cluster aggregation was based on the Ward method.[28,38] Statistical data elaboration was achieved by the XLStat software v.7.5.2 (Addinsoft).

RESULTS

On-resonance presaturation combined with T_1-filter[23,35] was applied to suppress ethanol (a triplet at 1.184 ppm and a quartet at 3.641 ppm) and water (a singlet at 4.706 ppm) signals, because their large intensities not only masked other meaningful peaks but also overlapped other nearby signals. Figure 1 shows a ^1H spectrum of wine C resulting from multisuppression, whereby water and ethanol as well as the acetic acid signal (2.08 ppm) were removed (the corresponding negative intensities were due to relatively longer T_1 relaxation times).

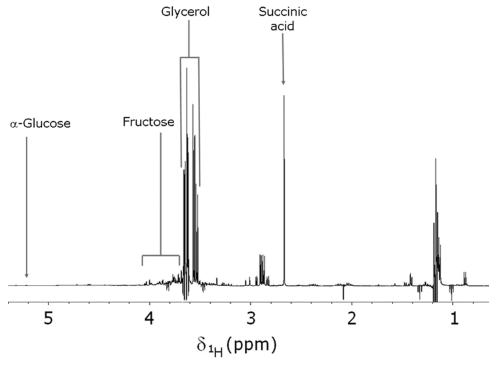

Figure 1. ^1H NMR spectrum of wine C obtained by suppressing ethanol and water signals by T_1-filter. Arrows indicate signals of molecules that significantly contributed to differentiate wines samples.

A second proton spectrum (data not shown) was acquired without the T_1-filter for samples added with TMSPA, with the aim to separately measure the intensities of ethanol and acetic acid. Larger amounts of ethanol were detected in wines C than wines A (3.403 ± 0.52% more abundant), while no significant differences in acetic acid content were appreciated.

NMR signals belonging to selected molecules, whose amounts were significantly different in wines treated with either autochthonous or commercial yeasts, were magnified in the four expanded ^{1}H spectra of Figure 2. Molecular attribution

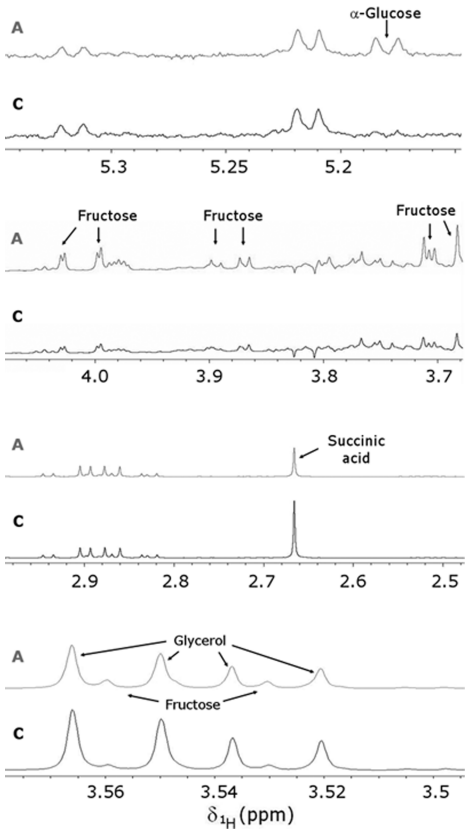

Figure 2. ^{1}H spectral regions of C (black) and A (gray) wines showing signals for α-glucose (5.35−5.15 ppm), fructose (4.08−3.78 ppm), glycerol (3.58−3.49 ppm), and succinic acid (2.98−2.48 ppm).

of these signals was achieved by combining spectral interpretation of 1D and 2D (spectra not shown) NMR spectra, which were confirmed by literature molecular assignments.[18,20,39] Signals of metabolites that most largely differentiated A from C wines corresponded to α-glucose, fructose, glycerol, and succinic acid (Figure 2, Table 1). The greatest concentrations of fructose and glucose were found in wines A, whereas wines C were richer in succinate and glycerol.

The selected buckets (115) resulting from proton NMR spectra elaboration were equally applied to all wine spectra and produced a matrix composed of 40 observations (wine samples) and 115 variables (intensities of NMR signals). An unsupervised pattern-recognition PCA was then performed to evaluate the intrinsic variation within this dense data set. PCA calculates linear combinations of a starting set of variables on the basis of their maximum variance and reduces the dimensionality of the original data matrix, while retaining the maximum amount of variability, as well as the original information contained in the data set.[28,38] Moreover, PCA

Table 1. ^{1}H Chemical Shift and Assignment of Signals That Mostly Differentiated Wine Samples

assignments	bucket number	bucket interval (ppm)	δ (ppm)
fructose	26	4.034−4.02	4.0283
	27	4.006−3.991	3.9967
	28	3.991−3.962	3.9797
	30	3.879−3.86	3.8693
	33	3.761−3.746	3.7531
	34	3.688−368	3.6831
α-glucose	5	5.191−5.167	5.1801
succinic acid	83	2.68−2.648	2.6658
glycerol	36	3.58−3.563	3.5662
	39	3.556−3.543	3.5502
	41	3.526−3.511	3.5208
leucine/isoleucine[a]	114	0.979−0.906	0.9575

[a]The bucket includes the overlapped signals of isoleucine and leucine CH_3− protons.

offers the practical advantage of exploring the response of many different samples with a large number of variables in a single output (score-plot).

The score-plot resulting from PCA achieved by combining PC1 (96.4% explained variance) and PC2 (2.46% explained variance) is shown in Figure 3. A clear differentiation among wines was observed along the PC1 where wines A were placed in negative PC1 values, whereas wines C were displayed in positive PC1 values. In addition, the fact that the observations of A and C wines were placed within distinct confidence limits (Hotelling T2 test[28]) strengthened the reliability of such separation. Because variables associated to PC2 did not contribute significantly to discriminate wines, attention was exclusively focused on PC1 and relative loading plot (Figure 4). The loading plot revealed that the variance associated to α-glucose, fructose, glycerol, and succinic acid had the largest weight in PC1, and that α-glucose and fructose were negatively correlated while glycerol and succinic acid were positively correlated. Moreover, PCA even revealed that the bucket including the overlapped leucine and isoleucine CH_3− signals (Leu/Isol) was an additional variable capable to differentiate wines. In detail, the largest, even though moderate, Leu/Isol amount was correlated to positive PC1 values (Figure 4). The analysis of variance (ANOVA) conducted for these variables showed that all of them contributed significantly to wine differentiation within a 95% confidence interval (Fisher test).[28]

Because the HCA allows recognition and distribution of data groupings in clusters of progressive dissimilarity, it was adopted as a further unsupervised method to classify wines with different yeast treatments according to their molecular affinity. Samples of different wine characteristics became organized in two distinct macroclusters (Figure 5), where the first dendrogram knot was associated to inter- and intraclass dispersions of 9.664 and 1.551, respectively.

Discriminant analysis (DA) is a supervised statistical method for sample classification. The DA discriminant functions are extracted from a data matrix composed of independent variables, with the aim to maximize interclass variance and minimize intraclass variance. This *criterium* may be supported by a validation test that evaluates the statistical confidence by which an a priori classification of part of sample observations (test set) coincides with an a posteriori DA prediction, on the basis of information provided by the remaining samples (training set). When the five DA validation tests were

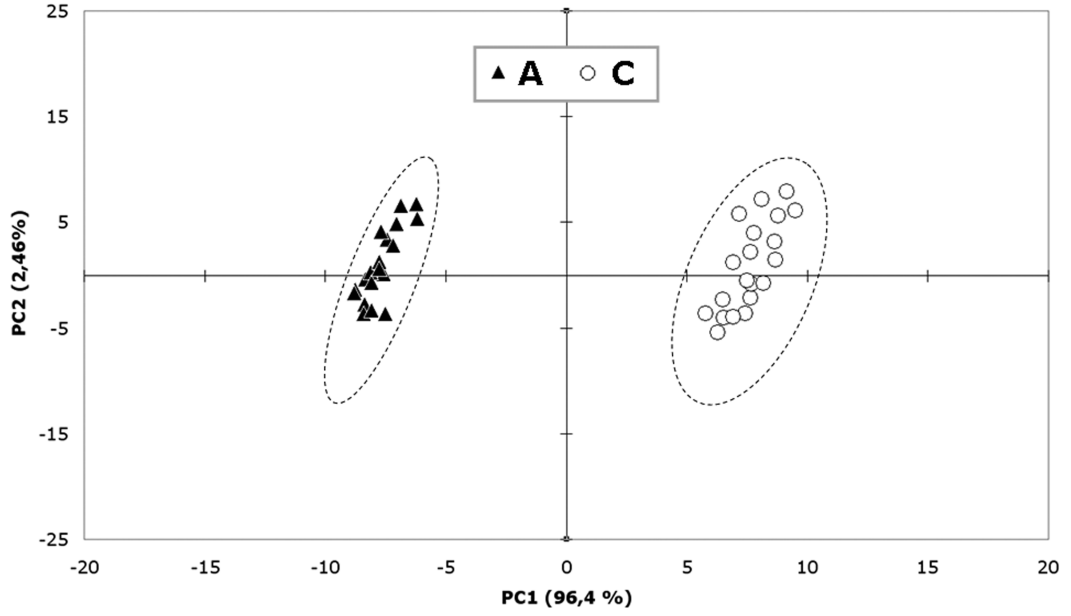

Figure 3. Principal component analysis (PCA) score-plot (PC1 vs PC2) of spectral data obtained from ^{1}H NMR spectra of wines produced with autochthonous (A; closed triangles) or commercial (C; open circles) yeast starter. The explained variance (%) is reported for each principal component, and dotted ellipses represent the 95% confidence limits for each wine type.

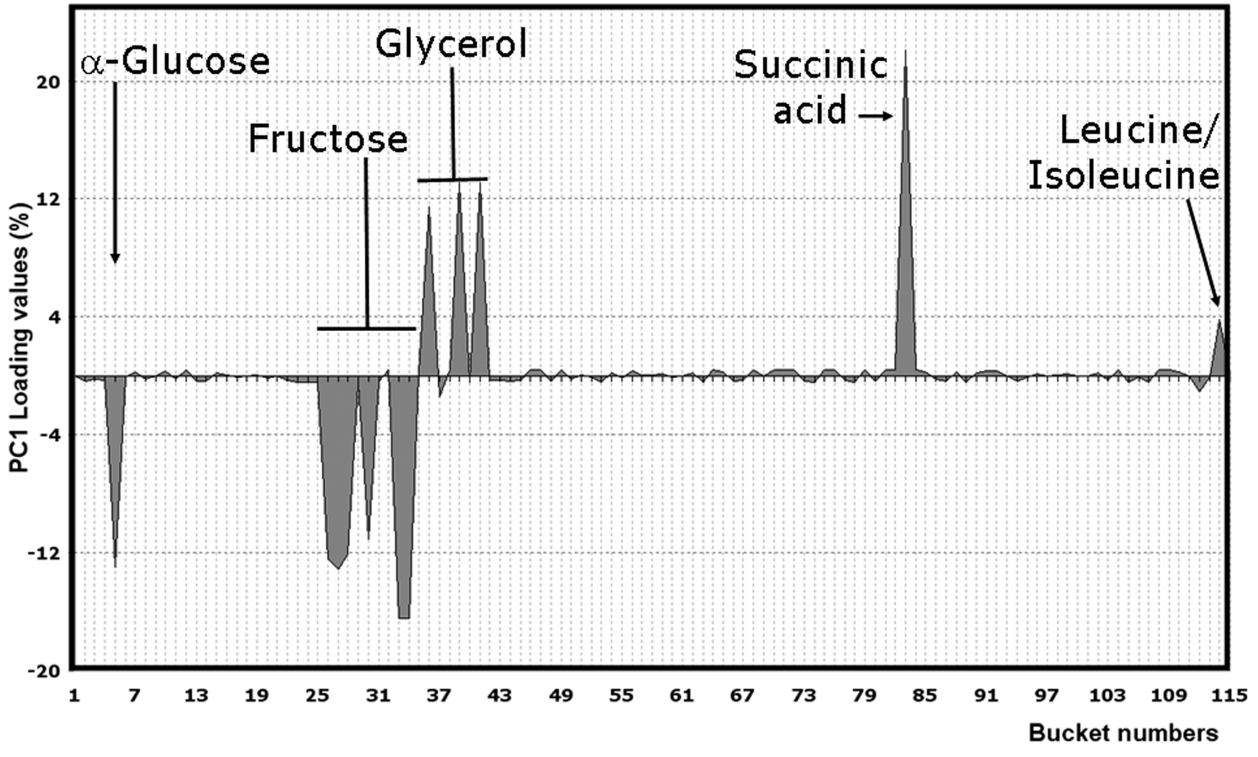

Figure 4. Loading-plot of variables associated with PC1 (96.4% explained variance). Reported assignment is for signals resonating in buckets showing the largest loading values.

performed on spectra of the differently treated wines, each sample was correctly associated to the respective group with 100% success and a significance of 0.05 for all developed discriminant models.

DISCUSSION

We found the correct conditions in the NMR pulse sequence (3 s delay between first 180° and second 90° hard pulse) to allow ethanol signals to be efficiently suppressed while retaining all other metabolic signals. Hence, we recovered all meaningful signals in spectra without any significant loss in intensity (Figure 1), except for acetic acid, whose singlet at 2.08 ppm was also canceled by the T_1-filter.

Differences between wines treated with autochthonous and commercial yeasts were noticed in the corresponding NMR spectra. For example, NMR spectra of wines obtained by autochthonous yeasts revealed the greatest concentrations of fructose and glucose and the smallest amounts of succinate and

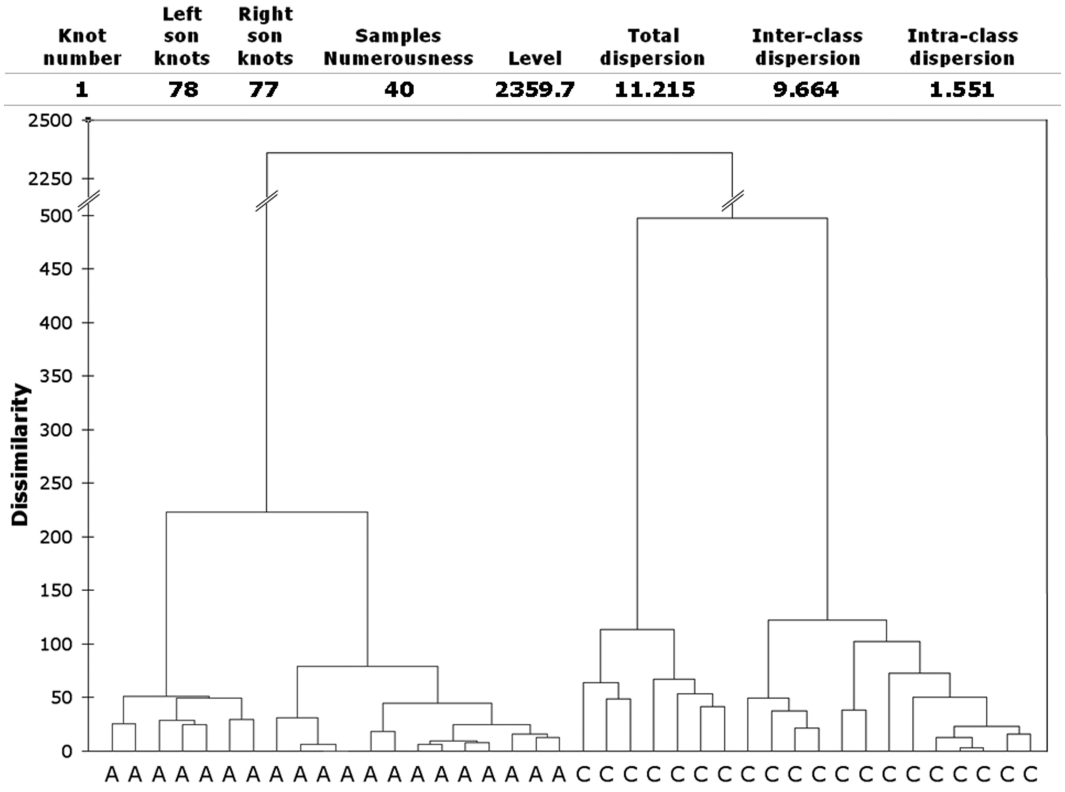

Figure 5. Dendrogram of wines C and A resulting from hierarchical cluster analysis (HCA) and main descriptive values. The dissimilarity among samples was measured by Euclidean distance, whereas cluster aggregation was achieved by the Ward method.

glycerol. The score-plot resulting from the PCA (PC1 vs PC2) elaboration of NMR spectra of the two differently treated wines is reported in Figure 3. The most informative component was the PC1 because the variance for the total data set was almost completely explained (96.4%) by this factorial axis. The PCA score-plot reveals that the best differentiation between the two wine samples occurred prevalently along the PC1 axis. The multivariate PCA projects the original observations in a new variance-controlled reference system and provides a final bidimensional score-plot, which may be interpreted on the basis of loading-plot information.[28] Consequently, the closer the objects are in a space defined by variables, the more similar are their properties. Because the loading-plot describes the correlation existing between each factorial axis and the variables, the distribution of wine samples obtained by autochthonous yeasts toward the negative PC1 values is represented in the loading-plot by a larger amount of fructose and α-glucose, and by a lower amount of glycerol and succinic acid, than for wines produced with commercial yeast (Figure 4). Similarly, PCA helped to highlight the significant contribution of the Leu/Isol signal variable, whose abundance was systematically larger in the latter than in the former wine samples (Figure 4).

The diversity detected in the two wine metabolomes should be mainly explained by a different response in yeasts metabolism, because other parameters in the wine-making technological processes were kept strictly similar for both wine samples. The abundant content of glucose and fructose in wines produced with autochthonous *S. cerevisiae* suggests a slower glycolytic activity than that by the commercial yeast during alcoholic fermentation and, thus, a less efficient conversion of carbohydrates in ethanol.[5] Such a lower activity for autoctonous starters also may be inferred by the greater amount of glycerol in wines produced with commercial yeasts, because glycerol is the final product of the glycerol pyruvate fermentation. In addition, the NMR spectra recorded without the T_1-filter revealed a greater ethanol content in the C wine, because glycerol formation accompanies ethanol production during the anaerobic sugar fermentation in wines.[40] These findings are in line with new oenological approaches aiming to produce wines with low ethanol content through yeasts that enhance glycerol pyruvate fermentation.[41] Furthermore, the large content of succinic acid in C wines is additional evidence of the larger fermentation properties of commercial yeasts, with succinic acid being one of the main fermentation byproducts.[17] Thus, our results suggest that the progressive consumption of glucose, accompanied by glycerol, succinate, and ethanol production, reflected the yeast activities during wine-making. The relationship between yeast and wine composition was already indicated by Son et al.,[18] who followed the fermentation behavior of yeasts at different stages of wine-making by applying ^{1}H NMR spectroscopy to musts and wines. Finally, the different contents of Leu/Isol in the two wine samples may be explained by the different capacity of the employed yeasts to synthesize these amino acids.[10,42]

The efficacy of NMR spectroscopy to reveal a metabolic composition that discriminates wine samples was further indicated by the HCA elaboration, which showed clear-cut separation between the two different wines investigated here (Figure 5). In fact, the HCA showed that the interclass dispersion was around 6.2 times greater than the intraclass dispersion and confirmed the results from PCA score-plot. Moreover, the discriminating capacity of the statistical method was also proved by a DA exercise in which 100% of success (0.05 significance) was achieved at each performed validation.

This study not only confirmed that ^{1}H NMR spectroscopy, combined with multivariate statistical analyses, can rapidly assess the molecular profile of wines but also enabled us to efficiently distinguish wines whose fermentation was achieved by different yeast starters. In particular, NMR spectra obtained here provided sufficient metabolomic data to successfully differentiate between two "Fiano di Avellino" wines produced with the same grape must and oenological techniques, except for fermentation that was started by either autochthonous or commercial yeast strains. The introduction of a calibrated T_1-filter in the NMR pulse sequence allowed acquisition of greatly reproducible and resolved proton spectra without the interference of ethanol and water signals. Even though other advanced NMR pulse sequences are available to efficiently suppress undesired signals,[31,43] the T_1-filter sequence adopted here was effective in removing both ethanol and water signals, while remaining simple and rapid to apply.[23] The statistical PCA elaboration of NMR spectral data enabled the identification of differences in wine metabolomes, such as the diverse content of fructose, glucose, glycerol, fructose, isoleucine, and leucine. The discrimination capacity of the combined use of NMR spectroscopy and PCA multivariate statistics was further proved by hierarchical cluster analysis. The results of the metabolomic investigation reported here can be correlated to the diverse fermentation efficiency exerted by either autochthonous or commercial yeasts, thus indicating the potential of the NMR metabolomic approach in evaluating wine quality and wine-making processes.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

A, wines produced by autochthonous yeast; C, wines produced by commercial yeast; PCA, principal component analysis; DA, discriminant analysis; HCA, hierarchical cluster analysis

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