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NMR Analysis of Seven Selections of Vermentino Grape Berry: Metabolites Composition and Development

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The goal of this work was to study via NMR the unaltered metabolic profile of Sardinian *Vermentino* grape berry. Seven selections of Vermentino were harvested from the same vineyard. Berries were stored and extracted following an unbiased extraction protocol. Extracts were analyzed to investigate variability in metabolites concentration as a function of the clone, the position of berries in the bunch or growing area within the vineyard. Quantitative NMR and statistical analysis (PCA, correlation analysis, Anova) of the experimental data point out that, among the investigated sources of variation, the position of the berries within the bunch mainly influences the metabolic profile of berries, while the metabolic profile does not seem to be significantly influenced by growing area and clone. Significant variability of the amino acids such as arginine, proline, and organic acids (malic and citric) characterizes the rapid rearrangements of the metabolic profile in response to environmental stimuli. Finally, an application is described on the analysis of metabolite variation throughout the physiological development of berries.

KEYWORDS: Nuclear magnetic resonance; statistical analysis; grape berry extract; metabolic profiling; *Vitis vinifera L.* (Vermentino)

INTRODUCTION

Vermentino is a wine grape variety traditionally cultivated in the west Mediterranean region and recently introduced in the new areas of viticulture diffusion such as Australia, USA, South Africa and Argentina. The most important geographical regions for the diffusion of this cultivar are Sardinia and Corsica, covering 3000 ha (*1*) and 1110 ha respectively. Vementino is also found in Piedmont and Tuscany, and DNA analysis established its genetic identity with the traditional varieties Pigato and Favorita.

The high quality attained by the monovarietal wines, which has allowed the growers to achieve a DOCG (Controlled and Guaranteed Denomination of Origin) district in Northern Sardinia, is one of the reasons of the growing market achievements of this variety. Vermentino is a relevant product of Italian enology, as it represents the fifth most sold wine in 2008 in Italian large retail stores and shows a continuous increase in the average selling price (*2*).

Clonal selection of Vermentino has been carried out in France and Italy, and several clones were selected (*3*, *4*). Each clone is characterized by having a particular size, shape and morphology of the bunch and grape, variable productivity, typical flavors and different disease resistance. In relation to the different geographic area selection, each clone evidenced dissimilar agronomic and technological performance. It should be observed that morphologic and genetic analyses are somewhat insensitive toward the current metabolic state of the plant, which changes very rapidly in response to environmental stimuli such as ground exposure to light and drought conditions during ripening. On the contrary, plants and fruits are believed to be very sensitive to these changes, so that the term *terroir* has been coined in enology to embrace all the complex effects of environmental factors and agronomical practices that lead to grape quality and, after the wine-making process, to wine uniqueness. Climate, soil, vine (rootstock and cultivar) and human practices are the four main parameters which define terroir (*5*).

Metabolites are the intermediates and end products of complex cellular regulatory processes, and their levels can be regarded as the ultimate response of biological systems to genetic or environmental changes.

Metabolite fingerprinting is a well-known technique suitable for studying variation of the metabolic profile of plants due to physiological or external perturbations (*6*). Moreover, it would be desirable to discriminate among plant samples with respect to their genotype, growth conditions and phenological phase (*7*).

Recently, the concept of metabolomic analysis was introduced in plant biochemistry to provide comprehensive insight into the metabolic state of the plant and to allow underlying subtle peculiarities and alterations (*8*, *9*).

Among all the analytical methods, proton nuclear magnetic resonance (^{1}H NMR) spectroscopy is a powerful technique for determining metabolite fingerprinting and/or metabolomic analysis of plants (*10*−*13*). ^{1}H NMR allows for the simultaneous detection and quantification of all proton-containing compounds in a complex mixture, and is used to determine the cell or tissue

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extract composition without the necessity of *a priori* knowledge of the sample composition (*14*, *15*). NMR also has the advantages of being nondestructive and intrinsically very informative. In addition, the sample does not require any particular pretreatment, separation or derivatization before analysis with the exception of extraction.

^{1}H NMR experiments are usually rapid and reproducible and can potentially provide large data sets that turn out to be suitable for statistical interpretation. If coupled with multivariate chemometric methods, with the purpose of unraveling information hidden in complex systems, NMR represents a potent new tool for assessing metabolic function (*16*) and for highlighting the variations of metabolite concentrations linked to typical phenotypes and/or pathologic conditions. Moreover, this approach allows performing sample classification.

Primary metabolites of plant tissue extracts (e.g., sugars and amino acids) and secondary metabolites such as phenolic compounds can be easily identified and quantified by ^{1}H NMR (*17*). Some of them are potential precursors of aromatic compounds of wine. For instance, amino acids are primarily responsible for forming active aroma compounds such as esters, alcohols and acids after their degradation by yeast metabolism in must (*18*).

Pereira and co-workers (*14*) discussed the capability of NMR and chemometric data analysis in discriminating grape skin homogenates from different vintages. In the same study, the authors demonstrated that NMR coupled to PCA analyses does not allow discrimination between different cultivars such as Merlot Noir, Cabernet franc and Cabernet Sauvignon. Other authors reported similar results obtained analyzing wines from different grape varieties (*19*).

Wine aroma is variably influenced by a number of factors (*20*−*22*). Grape berries, must and wine have an intrinsic complexity, and the aroma of the final product of enology is the result of a controlled balance of different factors such as berry metabolite composition (amino acids, organic acids and sugars), appropriate agronomical practices and a proper wine-making process. Currently, the evaluation of molecular factors which mainly influence grape and wine quality arouse great interest in the science and technology of wine (*23*).

Surprisingly, despite the great commercial interest, very little is reported about molecular composition of Vermentino grapes in the scientific literature (*24*).

The present study aimed to identify and quantify the principal metabolites of Sardinian Vermentino grape berry by means of nuclear magnetic resonance spectroscopy (NMR). The experimental approach follows a detailed protocol (*7*) for the extraction of polar metabolites from plant tissues, which was carefully adapted to grape berry. It is worth noting that a clear distinction exists between the analysis of grape berry performed in this study and other investigations on must, grape homogenates (*8*, *14*, *24*) or wines (*16*, *19*, *22*, *25*).

Our data demonstrate for the first time the suitability of NMR spectroscopy in characterizing the actual metabolic profile of different clones and biotypes of Vermentino, with particular attention to small molecular weight polar metabolites. The selected clones and biotypes appear to be genetically and morphologically different, and a careful analysis of their metabolic profiles would be highly desirable to highlight differences and similarities involved in plant growth and fruit maturation.

Statistical analysis of collected data spotlights differences and correlations between samples.

MATERIALS AND METHODS

Sample Preparation. Five Vermentino clones were selected: 640 (France), CAPVS3 and CAPVS12 (Sardinia), VCR1, VCR2 (Piedmont and Tuscany) and two biotypes, RP and SN (Alghero). Grape berries were sampled from the same vineyard in Sardinia (Alghero, SS) at four different phases of grape berry development (August 26, September 8, September 18, and September 23, 2008). As spatial heterogeneity of the vineyard can influence the experiments, vines relative to each clone were grown in an agronomic field experiment which is implemented through the randomized block design. Therefore, in the following we will refer to a block as a specific region (area) of the vineyard. The vineyard was divided into three blocks.

Bunches of each clone were randomly harvested from each block. Immediately after harvesting, intact bunches were rapidly brought on ice to the laboratory and frozen by immersion in liquid nitrogen, protected from light to prevent photoinduced reactions and then stored at −80 °C until extraction. Single frozen berries were freeze-clamped, rapidly and accurately weighted (~500 mg), and ground in a mortar filled with liquid nitrogen. The seeds were manually removed before weighing. Polar metabolites (mainly sugars, amino acids and organic acids) were extracted from a single berry per time using perchloric acid (PCA, 65%, Carlo Erba, Milan, Italy) to prevent enzymatic activity and to remove proteins and acidic macromolecules. Great care was taken to avoid partial thawing of berries before the addition of $HClO_4$, as detailed in the following. Thus, liquid nitrogen was continuously poured to the mortar containing the pulverized frozen berry, until 1 mL of ice-cold 3 M $HClO_4$ was added to the powder. Then, the extract was allowed to thaw on ice for 15 min. The next steps were achieved by accurately following the guidelines detailed in ref 7. Consequently, the extracts were subjected to several cycles of centrifugation (9000 rpm for 15 min at 4 °C; centrifuge ALC PK121R) in order to remove potassium perchlorate $KClO_4$, and to two freeze−drying steps (20 and 12 h, freeze-dryer VirTis, Warminster, PA, USA, mod. Genesis 12ES) to reduce the residual water signal in the NMR spectra. The pH of extracts was carefully monitored during several steps, until a final value of 7.5 was reached (before the last freeze-drying step) by using NaH_2PO_4/$NaHPO_4$ buffer (Merck, Darmstadt, Germany). Finally, all the lyophilized extracts were stored at −80 °C until NMR analysis was performed.

At enological maturity of the berries, which will be referred to as vintage period in the following, replicates were analyzed. Hence, three berries were randomly selected from the fruit cluster of a bunch. Consequently, three NMR samples were analyzed per each clone in each block of the vineyard. To study the evolution of the metabolic profile of grape berries as a function of development and ripening, one berry was selected from the bunch.

Berries having similar size and features were always selected from the same bunch, while visibly damaged ones were discarded.

NMR Analysis. All spectra were recorded on a Bruker Avance 600 MHz NMR spectrometer equipped with a 5 mm QXI SB probe. Before analysis, dried samples were dissolved in 1 mL of D_2O (99.9%, Cambridge Isotope Laboratories Inc., Andover, MA, USA) containing 5.45 mM sodium 3-trimethylsilylpropionate (TMSP, Cambridge Isotope Laboratories Inc., Andover, MA, USA) as a concentration standard and chemical shift reference and then centrifuged at 9000 rpm for 10 min at 4 °C to remove any insoluble $KClO_4$ from the sample. Successively, 0.8 mL samples of resulting supernatants were transferred into 5 mm NMR tubes for analysis. Each spectrum was recorded with the same conditions: 512 transients were acquired with 57468 data points over a spectral width of 12 ppm. An 8 μs pulse (70°) was used with a 2 s relaxation delay and an acquisition time of 4 s. The temperature was maintained at 298 K during all measurements, and the signal of water at 4.77 ppm was suppressed by using a low-power presaturation pulse during the relaxation delay. Free induction decays (FIDs) were Fourier transformed with 1 Hz line broadening, phased and baseline corrected using XWIN NMR software. In order to facilitate signal assignment 2D ^{1}H−^{1}H COSY, 2D ^{1}H−^{1}H clean-TOCSY (clmlevphpr with 110 ms mixing time), and 2D ^{13}C−^{1}H HSQC (J = 145 Hz) spectra were acquired for some samples. Signals from all spectra were manually integrated using MestReNova software (Mestrelab Research S.L., Santiago de Compostela, Spain), taking the TMSP peak area as concentration standard reference.

Statistical Analysis. The statistical analysis was carried out using the Statistical Toolbox of the MATLAB 7.10 package (The MathWorks, Inc., https://www.mathworks.com). All collected ^{1}H NMR data were processed by means of principal component analysis (PCA), in order to examine differences and correlations between samples. In particular, PC1/PC2 scores plots were obtained by segmenting the spectra in 255 buckets of 0.04 ppm each, scaling all buckets to the total spectra intensity without residual water peak and ignoring 92 buckets corresponding to the sugar region (from 3.25 to 5.50 ppm), ethanol, acetone (an impurity residual from sample preparation in some spectra), tartaric acid and residual water.

Before obtaining the scores plot, the buckets were Pareto scaled and a minimum variance level of 10% was imposed. PCA was used to analyze, in triplicate, seven clones, grown on three different regions of the vineyard, selecting as variables the binned spectral profiles obtained via NMR. Afterward a cluster analysis was applied to characterize the molecular composition of samples by means of Bruker AMIX (Amix 3.1.6, Bruker Biospin GMBH). Anova two factors and correlation analyses were performed in order to investigate metabolite concentration differences due to the block, to the clone and to intrabunch variability. In addition mean concentration value (μ), standard deviation (STDV or σ) and relative standard deviation (RSD or σ/μ) were calculated for each metabolite identified.

RESULTS AND DISCUSSION

Molecular Characterization of Grape Berry Composition. *Sample Selection and Extraction Protocol.* When approaching a metabolomic investigation on plant tissues, aiming to detect their real metabolic profile at the time of sampling, one has to focus on suitable methods which allow the measurement of an unaltered molecular profile. Methods which imply cell disruption are to be avoided unless cell disruption is carried out in controlled conditions (*12*, *26*).

Spectroscopic results can be easily altered by sample degradation if a detailed sample preparation procedure and proper facilities are not available (*7*). In particular, it has been observed in this work that proton NMR signals (e.g., triplet at 1.18 ppm due to the methyl group of ethanol) suddenly appear upon grape berry defrost, immediately followed by perceptible sample browning. It has been previously pointed out that significant degradation of metabolites can occur in few seconds by enzyme activities in partially damaged cells (*7*). In this work, great effort has been directed to preventing oxidation and enzymatic degradation of the fruit samples during the storage time and in the extraction procedure which finally leads to the NMR sample.

In particular, when optimizing the sample preparation protocol for metabolite profiling of plants and fruits, three main problems have to be faced: the first is the number of samples or classes of samples (plants, fruits) that have to be collected or harvested; the second relates to the amount of each sample that can be processed and the time required for each extraction; finally, the need of collecting representative samples of each plant/fruit has to be carefully evaluated. In the forthcoming discussion, these critical points will be examined individually, with particular reference to the study on Vermentino berries.

The number of samples at each sampling date, as well as eventually the need of storing the samples, is a major concern in metabolomic studies. In our case, at each harvest date a large amount of grapes needed to be processed. The time needed to complete the procedure used for the preparation of NMR samples and the requirement of keeping the samples unaltered during this period ruled out the chance to work with fresh berries. The extraction was therefore carried out on frozen berries.

Thus, grape berries that arrived in the NMR lab were immediately frozen in liquid nitrogen and stored at −80 °C. Great care was directed to optimizing sample storage, defrosting and dissolution, which constitute the most critical steps of the procedure, when oxidation is believed to be significant, so as to reduce deterioration or enzymatic activity.

The second critical point is the amount of sample that can be processed in one extraction. This in turn depends on the method employed for the extraction. One of the most consistent methods for inactivating enzymes is to let frozen tissues slowly thaw in $HClO_4$ (PCA), to promote protein denaturation and precipitation. Detailed studies demonstrated over several decades the reliability of this procedure for extracting the majority of unmodified intracellular metabolites (*7*, *27*). Another potentially

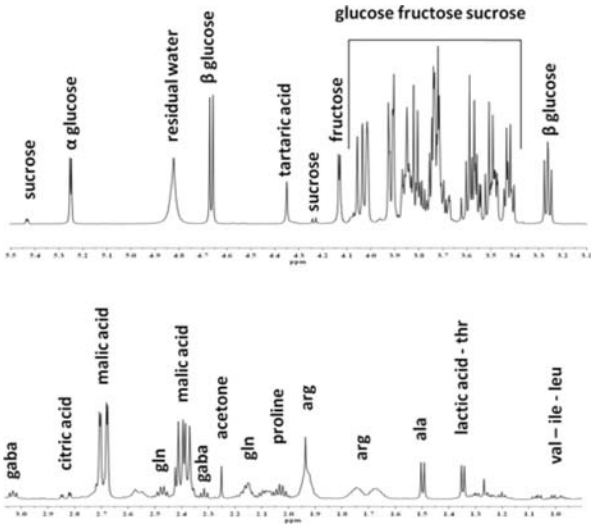

Figure 1. Typical ^{1}H NMR spectra of perchloric acid extract of Vermentino grape berry.

useful method for acid-stable, water-soluble metabolites employs a trichloroacetic acid (TCA) in diethyl ether mixture (*28*). Other extraction procedures, such as the use of biphasic chloroform: methanol:water mixtures or of other organic solvents, often turn out to be faster and more suitable for high-throughput analysis. Nevertheless, such solvents considerably preserve enzymatic activity, favoring metabolite degradation during sample preparation steps. Both TCA and PCA methods, although quite labor-intensive and time-consuming compared with the alternative procedures mentioned above, are believed to represent the methods of choice for the accurate analysis of the actual metabolite composition of plant tissues.

On the other hand, only a small quantity of tissue (0.1−0.5 g) can be harvested and extracted with perchloric acid, because subsequent pH adjustments would otherwise produce a too large volume of extract, which is difficult to handle and process during the remaining steps of the protocol (*7*).

All these concerns should also consider that, when analyzing a plant extract, a representative sample of the system should be selected. Plants and fruits may however present inhomogeneities. For instance, differences can be likely evidenced even in the composition of different berries in the same grape bunch. Additionally, in our study, it was at least desirable to know if such differences or inhomogeneities among berries of the same clone can prevail over differences among genetically distinct clones or on growing-area induced peculiarities.

Practices such as homogenizing grape material and making aliquots (*14*, *29*, *30*) would perhaps cancel the effect of intrabunch variability. As a matter of fact, such approaches would lead to a description of must, i.e. partially processed grape juice, which certainly differs from the actual metabolic profile of intact berries. In grape homogenates microbial and enzymatic activities are suddenly promoted after cell disruption, and the results would deviate from the final goal of this work.

Clones differ from each other by several means. On a macroscopic scale, morphologic features such as shape, size and compactness of the bunch, size of berries and skin thickness represent factors which usually allow their recognition. Hence, it would be interesting to survey if these differences result in dissimilar metabolisms between clones which are supposed to be different, or if other factors can significantly influence variability.

NMR Assignments. In total, 25 polar metabolites were identified but only 16 could be quantified due to the very low

Table 1. ^{1}H and ^{13}C NMR Assignments of Polar Metabolites Found in Extract of Vermentino Grape Berries

compound	atom	functional group	δ (ppm)	multiplicity	δ ^{13}C ppm
isoleucine	C5	δCH_3	0.94	t	
	γoC6	γCH_3	1.01	d	
	C4	γCH	1.25	m	
	^{1}C4	$\gamma^{1}CH$	1.45	m	
	C3	βCH	1.96	m	
	C2	αCH	3.66	m	
leucine	C5 + C6	$\delta CH_3 + \delta^{1}CH_3$	0.96	t	
valine	C4	γCH_3	0.99	d	
	C5	$\gamma^{1}CH_3$	1.04	d	
	C3	βCH	2.28	m	
threonine	C4	CH_3	1.33	d	
	C2	αCH	3.58	d	
	C3	βCH	4.25	m	
lactic acid	C3	βCH_3	1.33	d	
	C2	αCH	4.11	m	
alanine	C3	βCH_3	1.48	d	
	C2	αCH	3.79	q	
arginine	C4	γCH_2	1.68	m	26.6
	C3	βCH_2	1.92	m	30.0
	C5	δCH_2	3.25	t	43.0
	C2	αCH	3.76	t	57.0
gaba	C3	βCH_2	1.91	m	
	C2	αCH_2	2.30	t	
	C4	γCH_2	3.01	t	
proline	C3+C4	$\beta^{1}CH + \gamma CH_2$	2.01	m	
	C3	βCH	2.34	–	
	C5	δCH	3.33	–	
	^{1}C5	$\delta^{1}CH$	3.42	–	
	C2	αCH	4.14	–	
glutamine	C3	βCH_2	2.14	m	28.9
	C4	γCH_2	2.45	m	33.6
	C2	αCH	3.77	–	
malic acid	C3	βCH	2.37	dd	45.3
	C3	$\beta^{1}CH$	2.67	dd	45.3
	C2	αCH_2	4.30	–	
citric acid	C2+C4	$\alpha CH + \gamma CH$	2.80	d	47.3
	C2 + C4	$\alpha^{1}CH + \gamma^{1}CH$	2.83	d	47.3
β-glucose	C2	CH	3.24	t	77
	C4	CH	3.41	–	
	C3	CH	3.48	–	
	C6	CH	3.89	–	
	C1	CH	4.65	d	98.8
α-glucose	C4	CH	3.41	–	
	C2	CH	3.54	–	
	C3	CH	3.71	–	
	C5	CH	3.83	–	
	C1	CH	5.24	d	95.0
α-fructose	C3	CH	4.11	d	84.7
β-fructose	C3	CH	4.11	d	77.7
sucrose F	C3	CH	4.22	d	79
	C6	CH_2	3.82	–	
	C5	CH	3.89	–	
	C4	CH	4.07	–	
sucrose G	C1	CH	5.42	d	95.0
	C4	CH	3.48	–	
	C2	CH	3.56	–	
	C3	CH	3.76	–	
tartaric acid	C2 + C3	CH	4.33	s	76.7
fumaric acid	C2 + C3	CH	6.52	s	
cis-coumaric acid	C8^{1}	CH	5.99	d	
	C7^{1}	CH	7.08	d	
trans-coumaric acid	C8^{1}	CH	6.48	d	
	C7^{1}	CH	7.75	d	
	C3/5 ring	CH	6.90	m	
	C2/6 ring	CH	7.55	d	
cis-caffeic acid	C8^{1}	CH	5.98	d	
	C7^{1}	CH	7.00	–	
trans-caffeic acid	C8^{1}	CH	6.44	d	
	C7^{1}	CH	7.68	d	
	C5 ring	CH	6.95	d	
	C2 ring	CH	7.24	m	
formic acid	C1	CH	8.46	s	

concentration and signal overlapping of many compounds in the spectra. In particular our attention was focused on some enologically relevant sugars, organic acids and amino acids, while some phenolic compounds have been characterized as well. A typical NMR spectrum of Vermentino grape berry extract is reported in **Figure 1**. Assignments made using standard two-dimensional experiments such as $^{1}H-^{1}H$ COSY, $^{1}H-^{1}H$ TOCSY, and $^{1}H-^{13}C$ HSQC and based on the literature (*16*, *29*, *31*−*33*) are collected in **Table 1**.

Phenolic Compounds. Some phenolic acid isomers such as *trans*- and *cis*-coumaric acids, together with caffeic acid, were identified via 2D $^{1}H-^{1}H$ clean-TOCSY (**Figure 2**). *trans*- and *cis*-caffeic vinyl proton resonances appeared at 7.68, 6.44 ppm and 7.00 ppm, 5.98 ppm, respectively, while *trans*-caffeic aromatic protons are observed at 7.24 and 6.95 ppm. Peaks at 6.90 and 7.55 ppm were assigned to *trans*-coumaric aromatic proton signals, while vinyl protons of the same species are at 6.48 and 7.75 ppm. Finally, *cis*-coumaric vinyl proton chemical shifts were identified at 5.99 and 7.08 ppm. Usually, caffeic and coumaric acids can be found in grapes in the esterified form with tartaric acid as caftaric and coutaric acids respectively. Acidification causes ester bond breakage, leading to the unesterified forms of these two cinnamates. In our spectra only unesterified forms of caffeic and coumaric acids have been found due to the acid extraction procedure.

Metabolomic Analysis. In the following, the presentation of results will be split into four principal parts. In the first, results will be presented on analyses performed in triplicate for each clone (biotype) harvested from three different zones of the vineyard, at one particular developmental phase (vintage period); the second describes the intrabunch variability. The third is focused on the evaluation of metabolic profiling variability as a function of phenological phases of grapes. The fourth describes the sources of variation of metabolite concentrations. Each part is presented with the relative statistical analysis of data, suitable to help the interpretation of metabolic profiles obtained by NMR.

Vintage Period. The most commonly applied unsupervised statistical method still remains principal component analysis (PCA). PCA provides a first indicative picture of the system under study, as it allows a relatively fast screening of experimental data relative to the presence of similarities or differences between samples. At the same time, it identifies spectral regions, i.e. molecules, that principally characterize the intrinsic variability. Once the spectra have been acquired and preprocessed (i.e., baseline corrected, scaled or normalized and binned), the obtained spectral profiles are compared to examine the overall metabolic composition of the NMR samples. This approach is usually referred to as metabolite fingerprinting.

To this end, extractions were performed in triplicate (see definition of replicates in Materials and Methods) from berries belonging to each clone from each of the three blocks of the vineyard, choosing the vintage period (September 18) as the sampling date. Sixty-three NMR spectra were acquired and submitted as input data for statistical analysis by means of PCA.

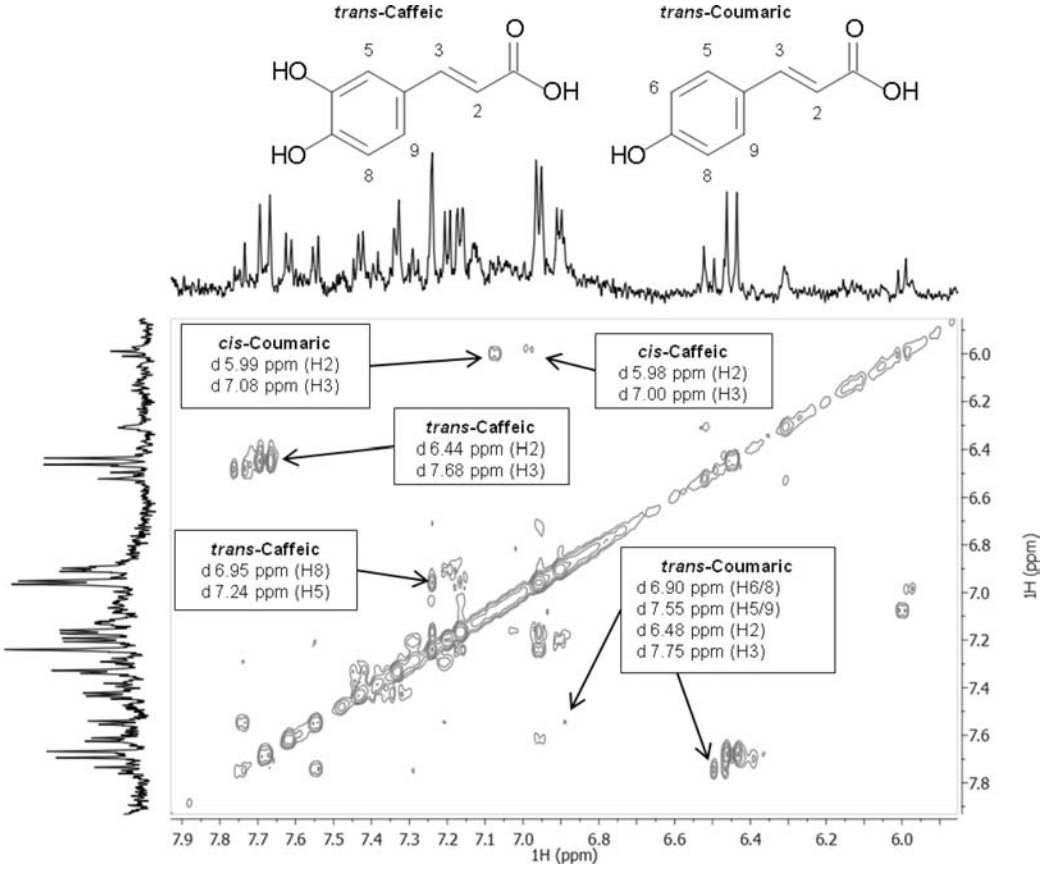

Figure 2. Aromatic region of 2D $^{1}H-^{1}H$ clean-TOCSY NMR spectrum of Vermentino grape berry extract showing assignments of *trans*- and *cis*-caffeic acid and *trans*- and *cis*-coumaric acid.

Figure 3a represents the PC1/PC2 scores plot of the principal component analysis and clearly shows the presence of two well-separated clusters. Splitting up can be associated neither to variability among the seven clones nor to distinction between the three blocks (zones) of the vineyard. It is important to underline that even berries from the same bunch can be found in two different PCA clusters of the scores plot. Variance was explained principally by minor compounds present in the grape berry, especially amino acids. In particular from PC1/PC2 loadings plot, reported in **Figure 3b**, it can be seen that gaba, arginine, glutamine and proline are the most discriminant molecules. Among them, glutamine is considered to be an important ammonium transporter, and arginine represents a varietal characteristic of grape berries (*34*).

Particularly interesting is the observed behavior of proline and gaba. As already pointed out by Pereira and co-workers (*14*), high concentrations of these two amino acids can be considered as peculiar of berries grown in a warm climate, and were found to have an effect on the wine production processes, by providing nitrogen to yeasts and contributing to the aroma profile of the wines. Millery et al. (*35*) found a linear correlation between proline concentration and the ratio of sugars/acids, expressed as ripeness index, suggesting that proline could be considered as an indicator of grape berry ripeness.

We assume that the splitting up of Vermentino samples into two clusters in PCA scores plot, as a consequence of mainly peculiar amino acid concentrations in fruit berries, can be reasonably ascribed to different berry position within the bunch. The same result can be further confirmed by the observed variability of organic acids malic and citric, which is clearly emphasized by the loading plot of the PCA analysis. These preliminary results

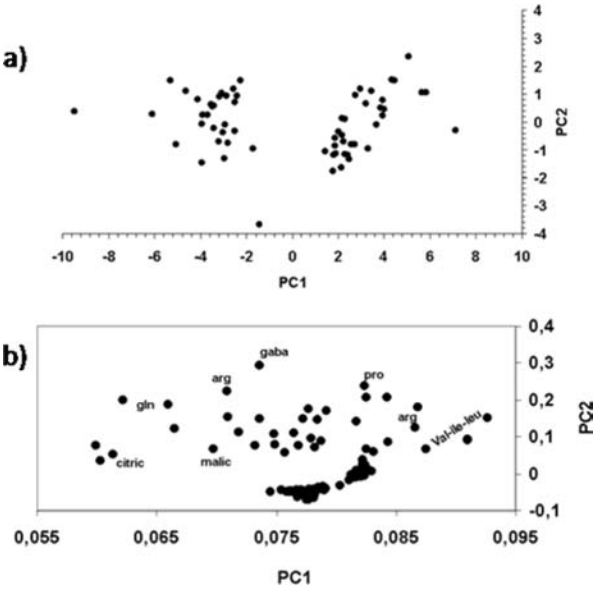

Figure 3. PCA scores (**a**) and loadings (**b**) plots of the 63 grape extracts at the vintage (September 18).

suggest that intrabunch variability represents a major discriminating effect over the difference in clone generation and block of the vineyard. A similar behavior could be reasonably derived from analyzing intrabunch variability. Consequently intrabunch variability was studied more in detail, in order to assess how metabolite concentration can be affected by the position of the berry within the same bunch.

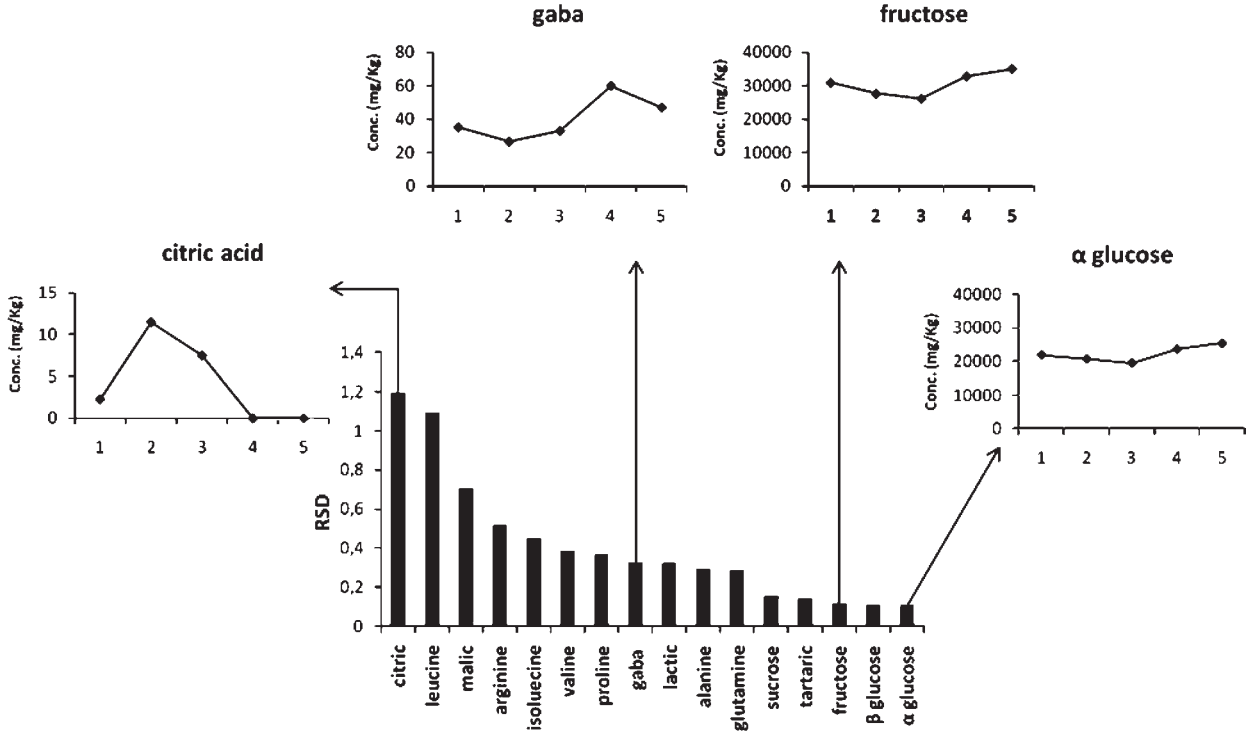

Figure 4. Graphical representation of intrabunch variability, for the five berries analyzed of VCR2 clone. Variability is expressed as the relative standard deviation (RSD), calculated for each metabolite in the five VCR2 berries (vintage period) analyzed. Concentration (mg/kg) trends are highlighted for citric acid, which shows the highest RSD value, gaba, fructose and α-glucose with the lowest one.

Intrabunch Variability. Intrabunch variability was investigated by analyzing different berries from clone VCR2 at the vintage period. Proton NMR spectra of the extracts from five berries were analyzed and quantitative analysis carried out.

The metabolite concentration variability, calculated as RSD of the concentration values expressed in mg/kg, is reported in **Figure 4**.

Sugar concentration values are rather unvarying (RSD = 0.155, 0.118, 0.106, 0.106 for sucrose, fructose, β-glucose and α-glucose, respectively). Organic acids citric and malic show higher RSD values (RSD = 1.19, 0.704 respectively). This is particularly true for citric acid, because it is rarely present in Vermentino grapes or it can be found very diluted, and therefore under the NMR detection threshold in some of the berries analyzed. Variability is significant also for some amino acids. It can be seen that concentration of arginine (RSD = 0.519), leucine (RSD = 1.09), proline (RSD = 0.366) and glutamine (RSD = 0.287) are considerably affected by the position of the berry in the bunch. It is worth noting that the proton NMR signals of these compounds are well resolved and not overlapped with other resonances; therefore, the variability in the quantitative data reported are reliable and entirely ascribable to variations in plant metabolism. The analysis of intrabunch variability is in very good agreement with the aforementioned PCA plots, pointing out the relevance of some enologically important amino acids and organic acids in characterizing Vermentino grape berries. Additionally, these results, together with those previously discussed relative to PCA plots, support the hypothesis that the high intrabunch variability could have a prominent discriminating effect over the difference in clone generation or block.

Vegetative Development and Ripening. The experimental procedure described in this work is also suitable for the unbiased investigation on how the metabolic profile of grape berry is changed by physiological events occurring during development. This method could be employed as a new approach to monitor metabolite variations during grape berry growth. As an example, we have performed an analysis on the seven selections of Vermentino, harvested from one block at different dates (August 26, September 8, September 18, and September 23, 2008).

While all seven clone selections were analyzed, we will only discuss here the metabolite concentrations as a function of berry development of a single clone for the sake of brevity and clarity; the biotype SN will be described below. As it is reported in **Figure 5a**, glucose and fructose concentrations show an overall increase during grape berry development as expected. Tartaric acid shows more or less unvarying concentration values during development; conversely the malic acid level is first diluted in the growing berry until it reaches a rather constant value (**Figure 5b**) during the final stages of berry ripening. Citric acid, as described above, is scarcely present in Vermentino, and its concentration has a significant variability among different samples, sometimes showing values under the NMR sensitivity threshold, and therefore it is difficult to find specific trends that characterize its behavior during berry development (**Figure 5d**) (*36*).

Variability in amino acid concentration is in general more noticeable than that in other molecules; this is particularly marked in the case of Vermentino for arginine, proline, glutamine (**Figure 5c**) and isoleucine, valine and leucine (**Figure 5d**). Some amino acids such as arginine and proline are varietal characteristics of the berries but can be also significantly influenced by climate and soil, as shown by previous NMR studies (*14*). Similar general trends have been observed for all the clones analyzed. Subtle peculiarities pertaining to particular clones concern specifically a wider audience in the viticulture and enology fields, grape growers and wine makers and will be explained in more detail elsewhere.

Again, fluctuations in the concentrations of organic acids (especially citric and malic) and amino acids are more marked than other compounds. These trends seem to be in agreement with previous results (vintage period analysis and intrabunch variability).

Sources of Variation. In order to further substantiate the assumptions made previously, especially the existence of a marked intrabunch variability that overcomes variations due to the clone or

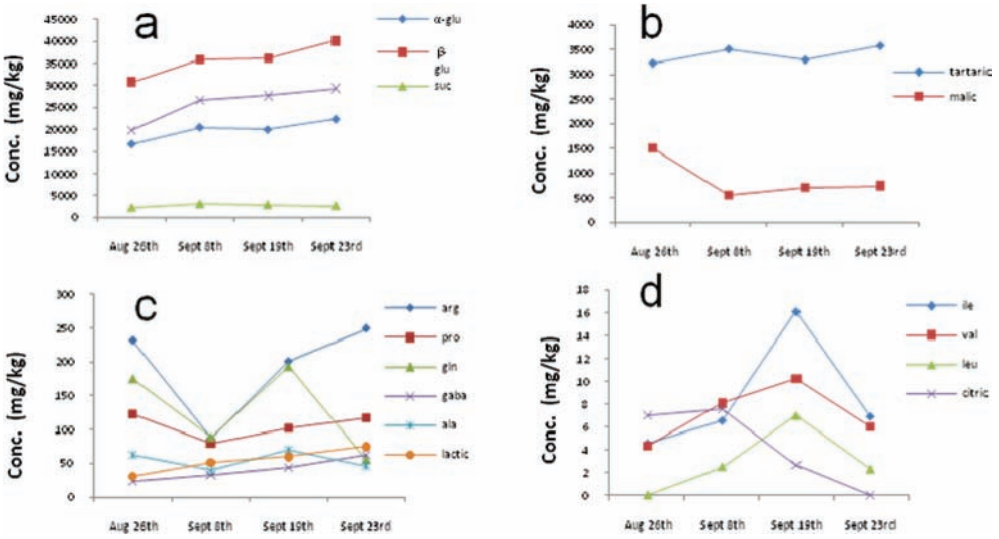

Figure 5. Metabolite concentration of Vermentino berry extract as a function of vegetative development for biotype SN: (**a**) sugars; (**b**) tartaric and malic acids; (**c**) arginine, proline, glutamine, gaba, alanine and lactic acid; (**d**) isoleucine, valine, leucine, citric acid.

Table 2. Metabolite Concentrations for the Biotype RN[a]

	total			block 1			block 2			block 3		
	μ	σ	σ/μ	μ	σ	σ/μ	μ	σ	σ/μ	μ	σ	σ/μ
ile	6.0	4.1	0.7	3.5	4.2	1.2	7.4	3.7	0.5	7.2	4.7	0.7
val	3.5	3.3	1.0	2.3	4.0	1.7	5.1	3.3	0.6	3.0	3.3	1.1
leu	5.0	5.5	1.1	3.2	1.0	0.3	4.7	8.2	1.7	7.0	6.6	0.9
lactic	61.2	26.5	0.4	62.3	24.2	0.4	72.2	32.2	0.4	49.2	28.2	0.6
ala	49.7	24.7	0.5	47.3	42.3	0.9	49.3	23.5	0.5	52.4	9.2	0.2
arg	152.1	133.7	0.9	94.9	164.4	1.7	247.0	109.1	0.4	114.3	109.4	1.0
pro	80.8	26.3	0.3	77.5	39.6	0.5	67.7	16.7	0.2	97.3	15.1	0.2
gln	110.3	75.5	0.7	151.9	103.5	0.7	125.5	48.5	0.4	53.5	44.1	0.8
malic	702.1	415.6	0.6	681.0	502.5	0.7	749.0	299.3	0.4	676.3	586.3	0.9
citric	3.7	5.3	1.4	5.0	8.7	1.7	6.0	2.0	0.3	0.0	0.0	
gaba	7.2	8.1	1.1	6.5	5.7	0.9	9.2	10.7	1.2	6.0	10.4	1.7
fruct	26555.4	4106.2	0.2	27099.4	2761.3	0.1	24233.3	5975.2	0.2	28333.3	3292.9	0.1
sucr	1367.5	721.4	0.5	1699.7	1334.5	0.8	1250.0	153.9	0.1	1152.7	146.8	0.1
tart	2750.8	1257.6	0.5	2442.3	532.6	0.2	3506.7	859.7	0.2	2303.3	2000.8	0.9
β glu	35242.2	5975.0	0.2	36693.2	4360.0	0.1	30600.0	7754.4	0.3	38433.3	3592.1	0.1
α glu	19667.9	3574.0	0.2	20370.4	2880.8	0.1	17166.7	4834.6	0.3	21466.7	2107.9	0.1

[a] Mean concentration value (μ) of the metabolites, STDV (σ) and RSD (σ/μ) related to each block or to the total of samples.

the block, metabolite mean concentration (μ), standard deviations STDV (σ) and RSD (σ/μ) were calculated for each clone. The data discussed below are relative to the vintage period.

First, the block effect was evaluated comparing concentration values from berries of the same clone taken from three different blocks with data concerning the total of samples for that clone. **Table 2** shows results for biotype RN, as a representative example. On the left columns of the table are reported metabolite concentration values (μ, σ and σ/μ) determined collecting all RN samples' data together (9 berries analyzed, three per each block, referred to as total), while the right part illustrates results corresponding to single blocks separately. It can be easily seen that for each metabolite RSD numerical values related to each single block are always higher than (or at least comparable to) those associated with all berries together, indicating that block effect is less significant than intrabunch variability. For example, RSD for glutamine from the third block is 0.8, while for samples sum (total) is 0.7; for alanine it is 0.9 in the first block against 0.5, etc. Furthermore interclone variability was estimated in a similar way, comparing every clone selection by pair per time. In **Table 3** is reported the example of the RN−640 couple: the two series of columns on the right correspond to RN (9 measurements) and 640 (9 measurements) respectively, while the left part refers to collected metabolite data of two clones together (18 measurements). Even in this case intrabunch differences are larger than interclone variability (intraclone RSD values are higher than or at least similar to interclone ones).

Anova. In order to exploit differences among the measures due to the bunch, to the clone, or to both, we performed an Anova-two factors analysis for each molecular species. We used the following model:

$$X_{jkl}^{\mathrm{mol}} = \mu^{\mathrm{mol}} + \alpha_j^{\mathrm{mol}} + \beta_k^{\mathrm{mol}} + \gamma_{jk}^{\mathrm{mol}} + \Delta_{jkl}^{\mathrm{mol}} \quad (1)$$

where X_{jkl}^{mol} denotes the random variables, the subscripts j and k refer to the clones and to the bunches respectively, while l indicates the replicate. The superscript *mol* refers to the molecular species and X_{jkl}^{mol} denotes the measurements.

With μ^{mol} we indicate the total average, α_j^{mol} and β_k^{mol} are the contributes to X_{jkl}^{mol} due to the clone factor and to the bunch factor respectively, $\gamma_{jk}^{\mathrm{mol}}$ represents their interactions and $\Delta_{jkl}^{\mathrm{mol}}$ are random independent variables, with zero mean and variance σ^{mol} which may depend on the particular molecular species.

Table 3. Mean Concentration Value of the Metabolites (μ), STDV (σ) and RSD (σ/μ) Related to Each of the Two Selected Clones, RN and 640, Compared to the Same Values of the Total

	total			RN			640		
	μ	σ	σ/μ	μ	σ	σ/μ	μ	σ	σ/μ
ile	6.9	3.4	0.5	6.0	4.1	0.7	7.8	2.5	0.3
val	2.6	2.6	1.0	3.5	3.3	1.0	1.7	1.4	0.8
leu	6.8	4.9	0.7	5.0	5.5	1.1	8.6	3.7	0.4
lactic	70.2	21.6	0.3	61.2	26.5	0.4	79.3	10.0	0.1
ala	58.4	21.6	0.4	49.7	24.7	0.5	67.2	14.4	0.2
arg	160.6	102.8	0.6	152.1	133.7	0.9	169.1	66.5	0.4
pro	98.0	31.2	0.3	80.8	26.3	0.3	115.3	26.6	0.2
gln	112.9	66.7	0.6	110.3	75.5	0.7	115.5	61.1	0.5
malic	912.4	398.0	0.4	702.1	415.6	0.6	1122.7	253.9	0.2
citric	4.3	4.7	1.1	3.7	5.3	1.4	4.9	4.2	0.9
gaba	10.5	8.9	0.9	7.2	8.1	1.1	13.8	8.9	0.6
fruct	28580.0	8479.0	0.3	26555.4	4106.2	0.2	30604.6	11255.7	0.4
sucr	1566.9	882.4	0.6	1367.5	721.4	0.5	1766.3	1022.2	0.6
tart	3563.3	1577.2	0.4	2750.8	1257.6	0.5	4375.8	1489.6	0.3
β glu	38771.3	8785.6	0.2	35242.2	5975.0	0.2	42300.5	10014.8	0.2
α glu	22220.0	4292.5	0.2	19667.9	3574.0	0.2	24772.2	3424.2	0.1

We adopt the following constraints:

$$\sum_j \alpha_j^{\mathrm{mol}} = 0 \tag{2}$$

$$\sum_k \beta_k^{\mathrm{mol}} = 0 \tag{3}$$

$$\sum_j \gamma_{jk}^{\mathrm{mol}} = 0 \tag{4}$$

$$\sum_k \gamma_{jk}^{\mathrm{mol}} = 0 \tag{5}$$

where the total variation has four terms,

$$v = v_{\mathrm{r}} + v_{\mathrm{c}} + v_{\mathrm{i}} + v_{\mathrm{e}} \tag{6}$$

due respectively to: the clone and bunch factors, their interaction, and to the residual error.

With this model we formulate the following statistical hypothesis:

$$\mathrm{H}_0^{(1)}\text{: all clones are identical} \rightarrow \alpha_j^{\mathrm{mol}} = 0 \tag{7}$$

$$\mathrm{H}_0^{(2)}\text{: all bunches are identical} \rightarrow \beta_k^{\mathrm{mol}} = 0 \tag{8}$$

$$\mathrm{H}_0^{(3)}\text{: no clone} - \text{bunches interactions} \rightarrow \gamma_{jk}^{\mathrm{mol}} = 0 \tag{9}$$

We found no significant differences among the clones and the bunches for any molecular species, with very few exceptions. We report the *p*-values only for some molecular species. Proline ($p = 0.0017$), isoleucine ($p = 0.019$), malic acid ($p = 0.021$) and leucine (0.024).

Correlation Analysis: Scatter Plots. Scatter plot analysis allows indentifying correlations among metabolite concentrations in grapes of different clones, regions of the vineyard, and replicates. We have calculated correlations between couples of data, meaning between the seven clones, three regions of the vineyard, and three repetitions of each sample resulting in correlation and *p*-value matrices made of 63 rows per 63 columns (covering all possible combinations). **Figure 6** shows four under-matrices, extrapolated from the correlation matrix, describing a number of combination examples. A high correlation is found in general for all clones (with the exception of VCR1 and VCR2, which in few cases show small differences), as it can be seen in the top left corner under-matrix referring to the first block and repetition. Moreover, it appears that the correlation is strictly linked to high-concentrated species, e.g. sugars. The correlation calculated on the minor compounds (bottom left corner under-matrix) shows that less concentrated species discriminate more between clones (i.e., show lower correlation values). The correlation matrix points out that within the same region of the vineyard, for the same clone, the internal variability between repetitions is non-negligible and can represent a discriminating factor. The corresponding under-matrix (top right corner in **Figure 6**) actually illustrates clearly this phenomenon; for example RN shows a higher correlation value with CAPVS12 (0.9995), with respect to the value with another RN sample of the same bunch (0.9985). Finally the region of the vineyard (the block effect) does not influence correlation between clones (bottom right corner under matrix in **Figure 6**, block 1 vs block 3).

Summarizing, scatter plots and correlation analyses seem to validate previous results and assumptions made about the masking effect of fluctuations in the concentrations of metabolites within the same bunch with respect to clone-to-clone differences and to the block influence. These analyses pinpoint the intra-bunch variability as a major source of data variation, indicating that some amino acids and enologically relevant organic acids play a crucial role in characterizing the metabolic profile of each Vermentino grape berry. The main sources of variability in the seven selections of Vermentino can be ascribed to the differences between berries of the same bunch, although clones and biotypes differ considerably for morphological and genetic features.

In conclusion, in this work a detailed protocol is described for extracting polar metabolites from grape berries and for their analysis by means of nuclear magnetic resonance spectroscopy. This method pays particular attention to avoiding postharvest enzymatic activity, oxidations and unwanted fermentations, thus guaranteeing the correspondence between the analytical determination and the metabolic status of the sample at the harvest. The present work differs from previous similar studies on grape berry in that metabolomic analysis has been carried out here on unaltered extracts due to the careful experimental procedure followed for sample preparation. Sample homogenization and extractions with solvents rapidly lead to must, and corresponding

Block 1 repetition 1

	Capvs3	Cap. 12	RN	SN	VCR1	VCR2	640
Capvs3	1	0.9996	0.9993	0.9991	0.9996	0.9995	0.9999
Cap.12	0.9996	1	0.9992	0.9996	0.9998	1	0.9998
RN	0.9993	0.9992	1	0.9989	0.9998	0.9991	0.9991
SN	0.9991	0.9996	0.9989	1	0.9993	0.9998	0.9995
VCR1	0.9996	0.9998	0.9998	0.9993	1	0.9997	0.9996
VCR2	0.9995	1	0.9991	0.9998	0.9997	1	0.9998
640	0.9999	0.9998	0.9991	0.9995	0.9996	0.9998	1

Block 1 repetition 1 with repetition 2

	Capvs3	Cap.12	RN	SN	VCR1	VCR2	640
Capvs3	0.9931	0.9989	0.9984	0.9986	0.8592	0.9973	0.9980
Cap.12	0.9957	0.9998	0.9995	0.9997	0.8497	0.9989	0.9993
RN	0.9935	0.9985	0.9989	0.9988	0.8529	0.9965	0.9982
SN	0.9970	0.9991	0.9990	0.9993	0.8377	0.9988	0.9995
VCR1	0.9945	0.9993	0.9994	0.9994	0.8529	0.9977	0.9988
VCR2	0.9961	0.9997	0.9994	0.9996	0.8471	0.9990	0.9994
640	0.9946	0.9991	0.9987	0.9989	0.8529	0.9981	0.9986

Block 1 Minor Compound

	Capvs3	Cap. 12	RN	SN	VCR1	VCR2	640
Capvs3	1	0.7803	0.7100	0.7250	0.8791	0.6163	0.3270
Cap.12	0.7830	1	0.9136	0.9090	0.8292	0.8577	0.6652
RN	0.7100	0.9136	1	0.9770	0.8009	0.8484	0.6527
SN	0.7251	0.9090	0.9977	1	0.8319	0.8703	0.6780
VCR1	0.8791	0.8292	0.8009	0.8319	1	0.8905	0.7126
VCR2	0.6163	0.6163	0.8577	0.8485	0.8703	1	0.9340
640	0.3270	0.3270	0.6652	0.6780	0.7126	0.9340	1

Block 1 repetition 1 with Block 3 repetition 1

	Capvs3	Cap. 12	RN	SN	VCR1	VCR2	640
Capvs3	0.9992	0.9986	0.9989	0.9982	0.9980	0.8927	0.9977
Cap.12	0.9999	0.9996	0.9992	0.9995	0.9992	0.8989	0.9992
RN	0.9990	0.9990	0.9982	0.9984	0.9969	0.8925	0.9976
SN	0.9992	0.9996	0.9978	0.9994	0.9988	0.9032	0.9992
VCR1	0.9996	0.9995	0.9989	0.9991	0.9981	0.8970	0.9984
VCR2	0.9998	0.9996	0.9989	0.9995	0.9992	0.8999	0.9992
640	0.9993	0.9990	0.9988	0.9987	0.9986	0.8973	0.9984

Figure 6. Graphical representation of four under-matrices extrapolated from the main correlation matrix showing correlation values between clones from the same block (top left corner); between repetition (repetition 1 and 2 from block 1, top right corner); between blocks (blocks 1 and 3, bottom right corner) and between clones calculated only for minor compounds excluding sugars (block 1, bottom left corner).

extracts are certainly affected by enzymatic and yeast activity. The influence of different sample preparation methods on ammonia, free amino acids and yeast assimilable nitrogen has been recently examined for Pinot Noir grapes (*37*). The metabolic profile of a clonal selection of Vermentino grapes has been presented here for the first time; in particular five clones and two biotypes have been described. The method turned out to be suitable for characterizing the dynamics of biosynthesis and catabolism of secondary metabolites as a function of berry development and ripening. The influence of several factors on the concentration of secondary metabolites in grapes has been pointed out by statistical analysis of the experimental results; in particular, the intrabunch variability was found to be relevant compared to the difference between different clones or regions of the vineyard where the vines are cultivated. Environmental factors mainly influencing these effects are suggested to be the exposure to sun, wind and drought condition during ripening. The amount and quality of light distribution in the canopy influence berry sugar, organic acids and amino acids content, by affecting the whole plant photosynthetic capacity. Previous works have explored the influence of microclimate on metabolic profiles of Merlot grape homogenates (*38*), and the effects of climate and soil have been found to be more discriminating than that of cultivar (*39*).

Interestingly, PCA analysis on NMR data of berry extracts at the vintage period groups the samples into only two well-defined clusters in the scores plot PC1/PC2. Metabolic profiles of extracts from berries belonging to the same bunch place the samples in both clusters, ruling out a net clone-induced diversity in the metabolisms of different clones of Vermentino berries. The environmental factors, indeed, are crucial in characterizing the molecular fingerprint and metabolism of each grape berry and the rapid changes of metabolite concentrations, especially for minor compounds such as amino acids arginine, proline, gaba and some organic acids as citric and malic can be easily and simultaneously monitored by NMR. Remarkably, the effect of the environmental variables on the metabolism of grape berry is more visible by comparing different berries of the same bunch than different vines situated in different blocks of the vineyard or genetically dissimilar clones. As amino acids are believed to be important precursors of aromatic compounds in wine, their evolution due to metabolic pathways in which they are involved should be well-known or at least measurable. The sensitivity and rapidity of this metabolic response of the vine to exposure to light, wind and drought condition, regardless of clone selection, should be taken into account when defining the *terroir*. Minor compounds, evidenced in our NMR investigation of Vermentino berries, have been found to be subject to rapid changes in concentration, merely following environmental stimuli in the bunch and regardless of the clone selection and position of the vine within the vineyard.

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