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A Thorough Study on the Use of Quantitative ^{1}H NMR in Rioja Red Wine Fermentation Processes

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In this study, we focused our attention on monitoring the levels of important metabolites of wine during the alcoholic and malolactic fermentation processes by quantitative nuclear magnetic resonance (qNMR). Therefore, using ^{1}H NMR, the method allows the simultaneous quantification of ethanol, acetic, malic, lactic, and succinic acids, and the amino acids proline and alanine, besides the ratio proline/arginine through fermentation of must of grapes corresponding to the Tempranillo variety. Each ^{1}H NMR spectrum gives direct and visual information concerning these metabolites, and the effectiveness of each process was assessed and compared by carrying out analyses using infrared spectroscopy to ethanol and acetic acid. The quantitative data were explained with the aid of chemometric algorithms.

KEYWORDS: Wine; fermentations; nuclear magnetic resonance; PCA

INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy is a powerful analytical technique and has an outstanding position in the field of complex chemical analysis of agricultural and food products as a potent analytical procedure for chemical characterization (*1*−*3*). The nondestructive nature of NMR is one of its most attractive features, allowing rapid measurements, analysis of samples without laborious sample preparation and the noninvasive study of samples. In addition, NMR is the perfect tool for broad-range profiling of abundant metabolites and for metabolite fingerprinting of extensive sample collections in the agriculture field (*4*). Some examples recently reported include different analyses such as the quality control, authenticity or geographical characterization of coffee (*5*), olive oil (*6*), tomato and orange juices (*7, 8*) and beer (*9*).

Given that the main use of this technique is for structure elucidation, NMR method development has mainly focused on the enhancement of qualitative information, although the quantitative aspects have actually been addressed since the early days of NMR (*10, 11*). In a recent and excellent review (*12*) concerning this topic, Pauli and co-workers introduced the term qHNMR as an abbreviation for proton-specific quantitative NMR and highlighted the enormous potential of qHNMR in the identification, characterization, and discovery of bioactive natural products and its potential uses in the area of metabolome analysis. The routine experimental protocol for qHNMR is illustrated in another excellent report (*13*). It has also been demonstrated that NMR represents a robust method that does not suffer from any significant effect in terms of analyst, instrument, magnetic field strength or experimental parameters (*14*). A simple integration method and chemometric analysis could be used in order to obtain appropriate results for quantification. Partial least-squares (PLS) regression has been successfully used for quantification of components with partially overlapped signals but needs a set of representative samples. An integration method could be correctly used with non-overlapped signals and with careful manual integration (*15*). In the past years, examples of quantitative NMR studies on beverages have increased. These reports include the quantification of organic and amino acids in beer (*16*), quantification of chlorogenic acid (*17*), (−)-epicatechin (*18*), formic acid in apple cider (*19*), quantification of malic and citric acid in fruit juices (*20*), quantification of methanol in a traditional Cypriot spirit (*21*), and quantification of the main organic components of vinegars (*22*).

As an important beverage, wine consists of several hundred components, and these are present at different concentrations, with the major components being water, ethanol, glycerol, sugars and organic acids. The NMR spectroscopy of wine has proven to be useful for assessing wine quality, for example, in the verification of wine origin, age, and the effects of adulteration through the SNIF-NMR method (*23, 24*). In recent years, the use of high-resolution NMR techniques in the study of wine has attracted the interest of several groups, and, as a result, 1-dimensional and 2-dimensional NMR experiments have been

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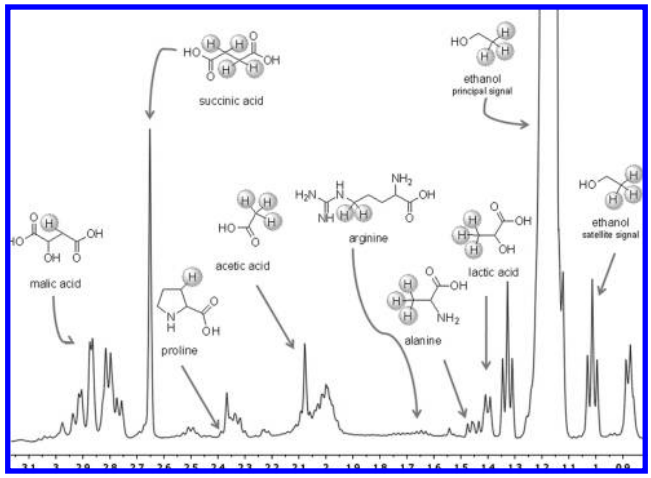

Figure 1. Molecules to be analyzed: the protons that were integrated in the ^{1}H NMR are highlighted, and the corresponding signals for the spectrum of ^{1}H NMR (400 MHz) for grape red must (pH = 3) using conventional 1D water presaturation are marked with arrows.

explored in order to characterize and to classify a large quantity of wines (*25*−*33*) and grape berries (*34*−*36*). Only some reports used intensities from ^{1}H NMR spectra for the quantification of several components in wine (*37*−*39*). Recently, Clark and coworkers have shown the potential of ^{1}H NMR as a valuable tool in monitoring a commercial fermentation (*40*).

In our research project related to the use of NMR spectroscopy as a useful tool in wine chemistry, we have recently evaluated the time course of the evolution of malic and lactic acids in the alcoholic and malolactic fermentation of grape must by quantitative ^{1}H NMR spectroscopy using water suppression and an external standard (*41*). This study allows the simultaneous quantification of both organic acids, and the data for malic acid was compared with the data obtained from enzymatic methods.

Moreover, the ^{1}H NMR spectrum contains qualitative and quantitative information of a lot of compounds. Some of these compounds have non-overlapping signals, making possible their integration and direct quantification. Taking into account the preliminary study above-described (*41*), our goal in this paper is the monitoring and the quantification of several compounds in the fermentation process of a commercial wine by qHNMR spectroscopy using water suppression and an external standard. The external standard method was checked by calibration curves, and some data were tested by means of infrared spectroscopy. In particular, the quantification of ethanol, acetic, malic, lactic, and succinic acids, proline, and alanine and the ratio proline/arginine were achieved (**Figure 1**).

MATERIALS AND METHODS

Samples. The time-course evolution of one stainless steel tank (500 HL) from the Bodegas Patrocinio SCL (qualified origin denomination Rioja D.O.Ca. Rioja, Spain) was explored. The tank was filled with must coming from red grapes (Tempranillo grape variety, *Vitis vinifera*) obtained from the Uruñuela region and cultivated in 2006. Destemmed-crushed grapes were homogenized and distributed into the tank, and the chemical composition of the must analyzed by infrared spectroscopy (Foss WineScan FT 120 spectrometer, FTIR interferometer) was as follows: sugar (glucose−fructose), 223 g/L; total acidity, 4.17 g/L (H_2SO_4); pH, 3.47; tartaric acid, 6.10 g/L. Usual treatment involving yeast and SO_2 was performed. When alcoholic fermentation was finished (day 12 from starting the fermentation), the wine from the tank was combined with the wine from another tank (initial composition as follows: sugar, 230 g/L; total acidity, 4.21 g/L (H_2SO_4); pH, 3.56; tartaric acid, 6.20 g/L).

The 28 samples for the period of 207 days were collected from the tank, transported from winery to laboratory and preserved at −25 °C until analysis was carried out. The simplest and fastest method for recording the spectra was used, and this involved two steps. Samples were defrosted, and the pH was measured (pH Meter BasiC Crison) and adjusted to 3 by the dropwise addition of an aqueous 1 M HCl solution in order to fix the chemical shift. A sample of the resulting grape must (0.45 mL) was added to a 5 mm NMR tube together with D_2O (0.05 mL with the addition of the sodium salt of (trimethyl)propanoic-2,2,3,3-d_4 acid (TSP) at the final concentration of 0.01% for the chemical shift calibration).

NMR Spectroscopy. NMR spectra were recorded on a Bruker Avance 400 spectrometer equipped with a 5 mm inverse probe (BBI H-BB Z-GRD). Acquisition of spectra was carried out with TOPSPIN software (version 1.2). Processing was performed with MestRe-C software (4.9.9.9) and MestReMnova (version 5.0) (*42*). The spectrometer was locked onto D_2O in a mixture H_2O-D_2O (9:1), and all the spectra were acquired at 298 K.

The ^{1}H NMR spectra were recorded with the standard pulse sequence for presaturation of the water signal at 1875 Hz (zgpr with pl9 at 60 dB and flip angle 90°). The spectral window was 10 ppm, and data were collected into 64k data points after 128 scans plus 4 dummy scans. The relaxation delay (d1) was set to 60 s according to suggestions noted in the literature for quantification procedures in order to ensure that all protons were totally relaxed (*43*), and the 90° pulse width was calibrated at 7.1 μs with −1 dB as a power level. All experiments were carried out with a fixed receiver gain in 2300 which was estimate adequate through several tests (not automatic receiver gain function was carried out). The experiments were carried out with automatic tuning and matching (ATM) and with GRADSHIM tools and using the NMR CASE as a NMR sample changer allows the automatic analysis of several samples.

Processing of Spectra. Free induction decay (FID) files were exported into the MestRe-C program, and, prior to carrying out Fourier transformation, an exponential window function was applied in order to obtain the optimal signal-to-noise ratio (*10*). The number of data points in the real part of the spectra was set to 64k. The phase of the spectra was manually corrected by selecting the submenu "Phase Correction", and the baseline was adjusted by the "Multipoint Baseline Correction" function in accordance with the literature (*10*). The integration of signals was manually carried out. The experiments and processing data were achieved twice.

Quantitative NMR Analysis. For qHNMR, it is essential to consider the selection of appropriate postacquisition processing parameters for optimized spectral integration. The integrals taken from the ^{1}H NMR spectra were not subsequently normalized, and the areas of the corresponding signals were calculated with the MestRe-C program as absolute integrals. The spectrum was calibrated with the TSP signal. We observed a methyl group for ethanol, and we developed two integration methods: method A, involving the integration of the principal signal (triplet, ^3J = 7.2 Hz, 3H at 1.18 ppm, without ^{13}C satellites and applying a correction factor), and method B, integrating the right ^{13}C satellite (triplet, ^3J = 7.2 Hz, 3H at 1.01 ppm, and applying a correction factor). Moreover, we detected the following signals: a methyl group for lactic acid (doublet ^3J = 6.9 Hz, 3H at 1.40 ppm), a methyl group for alanine (doublet ^3J = 7.2 Hz, 3H at 1.46 ppm), a δ-methylene group for arginine (multiplet, 2H at 1.60−1.69 ppm), a methyl group for acetic acid (singlet, 3H at 2.06 ppm), one proton of the β-methylene group for proline (multiplet, 1H at 2.29−2.39 ppm), two methylene groups for succinic acid (singlet, 4H at 2.65 ppm), and one diastereotopic proton for malic acid (doublet of doublets ^3J = 8.0 Hz, ^2J = 16.4 Hz, 1H at 2.80 ppm) (**Figure 1**). All chemical shifts are agreement with the literature (*27*, *29*). In order to make the quantitative analysis, we implemented our previously described method, in which succinic acid was used as an external standard without introducing it into the sample (*41*, *44*). More specifically, an experiment was carried out in another NMR tube with a known amount of the reference compound under the same conditions as used in the grape/wine must experiments. It is important to note that all the parameters (number of scans, relaxation delay, receiver gain, and so on) must be the same in both reference and sample experiments. Another experiment was carried out

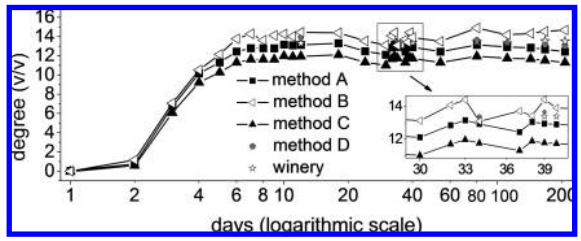

Figure 2. Time-course evolution of ethanol measured by method A (external standard using the area of the principal signal), method B (external standard using the area of the right satellite signal), method C (calibration curve) in degree (v/v), method D (standard ^{1}H NMR), and measured by the winery using the infrared spectroscopy.

with known amounts of the analytes under the same conditions; in other words, we made a synthetic wine in which the concentration of each compound was exactly known. A constant (k) was extracted for all these compounds on the basis of their relationships with the external standard: $k = A_{ES}/A_A \times C_A/C_{ES} \times N_A/N_{ES}$, where A_{ES} is the area of the external standard, A_A the area of analyte, C_A the concentration of analyte, C_{ES} the concentration of external standard, N_A the number of protons for the signal of the analyte and N_{ES} the number of protons for the signal of the external standard. The constant values are: ethanol $k = 0.911$, ethanol satellite $k = 1.05$, acetic acid $k = 0.934$, malic acid $k = 0.978$, lactic acid $k = 0.969$, succinic acid $k = 0.952$, proline $k = 0.966$ and alanine $k = 1.07$. These constants were used to obtain the concentration of the analytes in the grape must or wine sample.

In addition, and in order to corroborate the results obtained by the use of an external standard, we performed the calibration curves for two principal compounds such as ethanol (integrating the principal signal without ^{13}C satellites) and acetic acid. Five NMR tubes were prepared with the same synthetic wine above-described with different and known amounts of ethanol and acetic acid (in the range of concentrations that there is in the must and wine), and the experiments were repeated three times for each tube. The equation for ethanol is $A = 40369C + 27782$, $R^2 = 0.999$, and for acetic acid $A = 28505C + 1518.51$, $R^2 = 1.00$, where A is absolute area and C is concentration in g/L.

Statistical Analysis. Principal component analysis (PCA) was carried out using the SPSS version 14 statistical package. PCA was used to evaluate the importance of the seven different wine metabolites, whose concentrations were obtained from quantitative NMR.

RESULTS AND DISCUSSION

Initially, we wished to evaluate the time-course evolution of ethanol in the fermentation process, the most important compound after water and the main product of alcoholic fermentation (*45*). A wine strength is expressed in terms of ethanol content or the percentage of ethanol by volume. In this case, we considered the data obtained using the external standard, taking into account the principal signal (method A), the right ^{13}C satellite signal (method B), and the equation found by the calibration curve (method C). **Figure 2** shows the progression of the alcoholic fermentation using the three methods previously commented on. When we consider the principal signal, the area was corrected with a factor of 100/98.9, in order to include the ^{13}C satellites. When we consider the right ^{13}C satellite signal, the area was corrected with a factor of 100/0.55. The graphics are showed in g/L and in alcoholic degree (v/v). By the other side, the data were compared with those obtained by the winery using infrared spectroscopy (Foss WineScan FT 120 spectrometer). Because the ethanol concentrations in the samples measured with both techniques were very similar, we could not use the simple correlation to assess that both methods provide comparable results. However, paired t test of the results showed that the qHNMR values were not significantly different from those obtained by infrared spectroscopy.

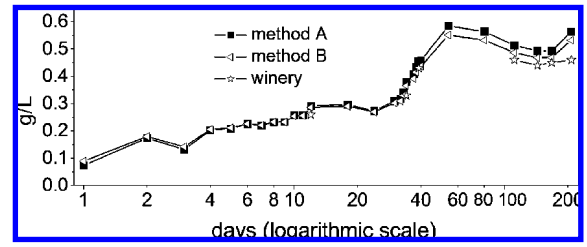

Figure 3. Time-course evolution of acetic acid measured by method A (external standard), by method B (calibration curve), and by the winery using infrared spectroscopy in g/L.

On the other hand, there is a practical and rapid method for the quantification of ethanol degree in beverages. Following the literature (*46*), we recorded a standard ^{1}H NMR experiment with a zg pulse program, with relaxation delay of 5 s, and we compared the corresponding integrals between water and ethanol signals, allowing us the rapid determination of ethanol concentration (method D). This method is only applicable to the measure of the water/ethanol ratio.

Alternatively, volatile acidity in wine is a highly important physicochemical parameter, to be monitored by analysis throughout the winemaking process (*45*). Acetic acid, the principal component of volatile acidity—around 97%—is mainly formed during yeast fermentation as a result of the side reaction of acetaldehyde oxidation. Acetic acid is also formed by *Acetobacter spoilage* in aerobic conditions. We analyzed the time-course evolution of acetic acid taking into account the data obtained using the external standard and the data found by the calibration curve method (**Figure 3**). These concentrations expressed in g/L were compared with those obtained by the winery using infrared spectroscopy (Foss WineScan FT 120 spectrometer), obtaining a good correlation with a slope of 0.938 and R^2 of 0.94. Again, paired t test of the results showed that the qHNMR values (method A) were not significantly different from those obtained by infrared spectroscopy.

In view of the above results and the good regression between qHNMR versus classical enzymatic method published by us ($y = 0.0198 \pm 0.046 + 0.9965x \pm 0.029$ with $R^2 = 0.9969$) (*41*), the qHNMR offers a solid method for metabolite quantification in wine. So, we extended the study toward other interesting compounds using the external standard method. Taking into account that the determinations of malic and lactic acids are crucial in order to obtain a good wine (*47*), we decided to monitor their evolutions in the fermentation process. Low levels of malic acid are considered to be a prerequisite for the commercial production of some red wines (0.4−0.5 g/L is desirable for some of these), and the adjustment of this acid rate is important in the elaboration of other types of wine such as white or rose wines. One way to reduce the quantities of this acid is to allow the spontaneous growth of lactic acid bacteria, which in turn carries out malolactic fermentation. In some cases, this conversion into lactic acid could take place during the alcoholic fermentation. Control of this process is therefore essential in order to obtain a quality wine.

The graph in **Figure 4** represents the time-course evolution of malic and lactic acids in mol/L, and from these data it is possible to compare the formation of lactic acid from malic acid in the wine. The data after 40 days illustrates finished malolactic fermentation with completed transformation of malic acid into lactic acid.

Alanine is another main amino acid found in must, and it can be a source for pyruvic acid (2-ketopropionic acid), acetaldehyde, and ethanol by the Ehrlich pathway (*48*, *49*).

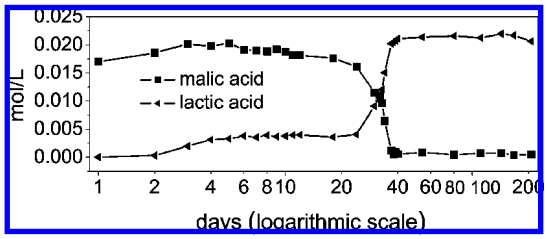

Figure 4. Time-course evolution of malic and lactic acid measured by external standard in mol/L.

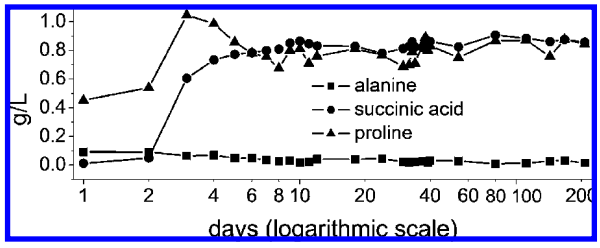

Figure 5. Time-course evolution of succinic acid, alanine, and proline measured by external standard in g/L.

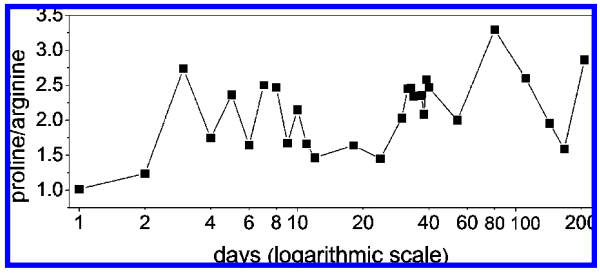

Figure 6. Time-course evolution of the proline/arginine ratio.

Pyruvic acid is an important compound in winemaking due to its ability to bind SO_2 and to react with phenols. The concentration of alanine in the tank decreased 0.84 mmol/L in 40 days (**Figure 5**). This fact is according with its importance as a major source of yeast-assimilable nitrogen (YAN) for yeast. Succinic or 1,4-butanedioic acid is an organic acid afforded during alcoholic fermentation due to the action of the yeasts, and their concentrations in wine are close to 1 g/L. By this reason, it can be used to evaluate the end of the alcoholic fermentation. This acid is produced by all living organisms, and it is involved in lipid metabolism and in the Krebs cycle, together with fumaric acid (*48*). After alcoholic fermentation, the concentration of succinic acid in the tank was 0.89 g/L. When malolactic fermentation was finished, the concentration was 0.85 g/L, keeping stable until the last day (**Figure 5**).

In this context, proline is the most abundant amino acid in wines. About 30% to 85% of total amino acids content is proline. Millery et al. (*47*, *50*) showed a close correlation between the proline concentration and the ripeness of the grapes (IM). In particular, the correlations between the logarithm of the proline concentration and the ripeness in two Champagne grape varieties have a good regression coefficient according this formula: Log [proline] = a[IM] + b. Proline therefore appears to be a marker for ripeness, consequently, the proline rate increases markedly about two weeks before the grapes are picked (*46*, *48*). On the other hand, proline and its hydroxylated derivatives have been cited as discriminating among wines made from different grape varieties in diverse areas (*51*). This is based on the fact that proline is not usually metabolized by yeast during fermentation. Moreover, some studies revealed that there were higher concentrations of proline in water-deficit treated plants. During alcoholic fermentation we observed the increase in the first days

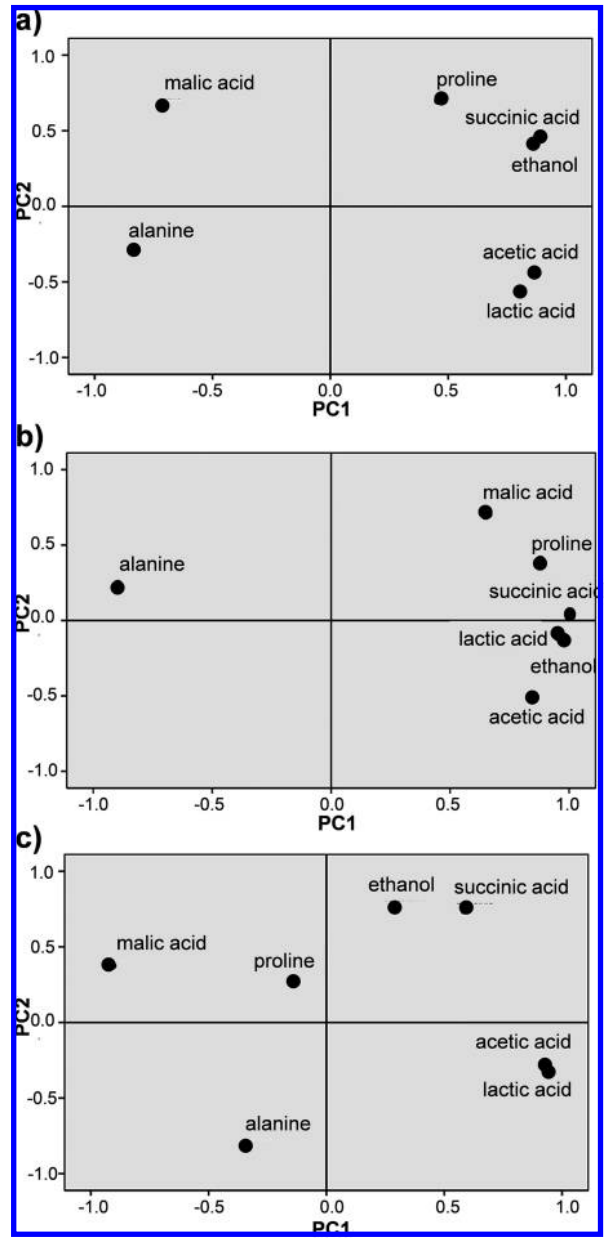

Figure 7. PCA loadings plot derived from the ^{1}H NMR data: (**a**) alcoholic and malolactic fermentation, (**b**) alcoholic fermentation, and (**c**) malolactic fermentation.

(1.03 g/L). However the time-course evolution presented another minimum in the eighth day (0.73 g/L). From this point, the proline concentration fluctuates between 0.85 g/L and 0.93 g/L for the last day.

Arginine is an important nitrogen source for yeasts due to its catabolism by arginase enzyme to form L-ornithine and urea, which, under appropriate conditions, ultimately forms glutamate and ammonium. Some authors explain that the differential accumulation of proline and arginine by different grape varieties provides a characteristic index based on the ratio of the two amino acids (*48*). This index, which reflects the proportion of nonassimilable (proline) to assimilable nitrogen (arginine), provides a useful indication of the likely nutritional value of the grape must of a particular cultivar to yeast. Arginine and proline concentrations can vary by a factor of 10 to 15, depending on the variety: for example, from 300 to 4600 mg/L for proline. The proline/arginine ratio is relatively constant from one vintage to another in the same grape variety (*47*). Indeed, a more detailed analysis of concentrations of each amino acid,

Table 1. Summary of Data in g/L Obtained by qHNMR[a]

	method A													
	ethanol		acetic acid		malic acid		lactic acid		succinic acid		proline		alanine	
day	g/L	RSD	g/L	RSD	g/L	RSD	g/L	RSD	g/L	RSD	g/L	RSD	g/L	RSD
1	0.376	18.7	0.0739	17.8	2.28	9.70	0.00348	53.5	0.0123	12.6	0.452	14.2	0.0923	11.7
2	5.87	20.7	0.174	19.8	2.49	10.3	0.0293	24.5	0.0497	15.8	0.539	16.1	0.0915	19.2
3	53.1	0.440	0.132	5.07	2.70	1.65	0.180	5.25	0.606	1.88	1.05	5.40	0.0649	8.00
4	80.3	2.32	0.202	8.33	2.65	6.84	0.283	9.31	0.733	5.70	0.986	8.14	0.0689	11.7
5	89.3	2.42	0.209	1.89	2.72	7.19	0.298	12.6	0.773	6.40	0.858	8.84	0.0480	16.8
6	98.2	3.43	0.225	0.918	2.56	2.59	0.343	5.88	0.785	2.40	0.780	11.0	0.0485	11.5
7	101	0.580	0.220	11.0	2.55	4.21	0.322	10.7	0.799	3.27	0.755	12.4	0.0344	24.0
8	101	1.02	0.232	3.86	2.53	2.43	0.355	20.8	0.809	2.85	0.675	15.4	0.0282	11.4
9	101	2.42	0.233	12.3	2.57	7.08	0.332	14.7	0.852	5.07	0.800	9.06	0.0327	40.3
10	104	3.30	0.256	1.62	2.52	6.45	0.342	14.1	0.865	5.65	0.812	9.25	0.0193	33.0
11	103	1.88	0.258	4.33	2.44	4.29	0.359	10.3	0.847	1.20	0.710	14.6	0.0240	18.5
12	104	1.12	0.291	10.8	2.43	4.89	0.361	12.7	0.832	2.96	0.760	9.36	0.0432	23.8
18	105	2.33	0.296	8.99	2.36	6.74	0.324	9.20	0.829	5.29	0.811	11.8	0.0404	32.8
24	98.4	2.72	0.273	8.99	2.16	5.18	0.368	4.20	0.781	2.52	0.768	12.8	0.0444	13.0
30	95.6	7.60	0.310	4.31	1.54	6.79	0.822	9.77	0.814	4.24	0.687	7.72	0.0245	90.1
32	101	1.16	0.318	3.91	1.48	4.03	0.906	4.19	0.828	3.56	0.700	7.29	0.0196	33.2
33	104	0.966	0.340	1.71	1.29	7.66	1.09	8.57	0.860	5.74	0.791	7.28	0.0223	77.2
34	102	1.03	0.380	5.66	0.869	8.84	1.36	6.19	0.824	3.19	0.711	2.57	0.0226	23.1
37	98.1	4.46	0.408	3.41	0.154	7.09	1.82	1.55	0.821	0.659	0.846	8.11	0.0279	9.05
38	103	0.807	0.435	0.700	0.0735	4.96	1.84	3.17	0.849	1.73	0.890	1.12	0.0298	32.3
39	102	1.27	0.456	5.55	0.124	5.55	1.87	6.17	0.867	2.72	0.801	5.68	0.0198	21.4
40	102	1.42	0.458	1.60	0.0746	12.6	1.90	3.37	0.862	1.32	0.832	3.66	0.0312	9.35
54	98.1	1.31	0.585	6.29	0.118	7.56	1.92	5.99	0.825	0.884	0.750	3.13	0.0294	37.3
80	104	2.16	0.564	5.22	0.0631	7.17	1.94	3.89	0.907	3.35	0.868	10.6	0.00943	23.0
111	102	2.29	0.513	4.02	0.0982	17.9	1.91	0.41	0.884	2.84	0.871	10.7	0.0138	53.3
143	101	3.11	0.493	4.20	0.103	14.3	1.98	2.20	0.861	7.44	0.758	25.5	0.0289	13.5
167	100	0.907	0.493	1.59	0.0495	44.6	1.95	4.93	0.876	1.56	0.876	2.59	0.0320	32.4
207	98.1	1.71	0.563	1.99	0.0680	42.1	1.86	0.389	0.858	1.77	0.845	6.48	0.0150	41.1

[a] The repeatability of the method was evaluated through the relative standard deviation (RSD). RSD was calculated for each compound from triplicate consecutive analytical runs and was considered satisfactory for analyses.

especially arginine and proline, shows that the proline rate increases markedly about two weeks before the grapes are picked. In the time-course evolution of the proline/arginine ratio in this study, we observed an initial value of 1.08 (**Figure 6**). During alcoholic fermentation the ratio varied between 3.37 and 1.18. After malolactic fermentation, with high levels of arginine (YAN amino acid) consumed, the ratio was 3.73. This ratio decreased to 1.75 in the last day.

In order to understand the key processes that occur during alcoholic and malolactic fermentations, multivariate data analysis was carried out using principal component analysis (PCA). The 90% of the sample variance can be explained with the first two principal components. The variations in concentrations of ethanol, proline, alanine, and acetic, malic, lactic, and succinic acids during the fermentation processes are significant for the two principal components. The loadings plot of these two factors extracted from the analyte correlation matrix is shown in **Figure 7a**. The first factor (PC1) exhibits high positive loadings for succinic, lactic, and acetic acids as well as for ethanol concentration and negative loadings for malic acid and alanine. Nevertheless, malic acid and proline concentrations are significant in the two factors (PC1 and PC2). These results describe a clear tendency: the increase of concentrations of ethanol and succinic, lactic, and acetic acids and the decrease of alanine and malic acid concentrations during the fermentation processes. In contrast, the second factor (PC2) indicates different rates of consumption of malic acid and alanine.

With the idea of studying the variations of the analytes in terms of concentrations during each type of fermentation (alcoholic and malolactic), two similar PCA analyses were carried out, the first one during the initial 7 days and the second one during the rest of the days (8 to 207 days). In this way, in the PCA analysis corresponding to the alcoholic fermentations (first 7 days), the first two principal components accounted for over 94% of the sample variance and the loadings plot is shown in **Figure 7b**. The first factor (PC1) shows high positive loadings for all analytes except alanine, which has a high negative loading. Only malic and acetic acids are significant for the second factor (PC2). The most important conclusions extracted from this analysis involve the increase of ethanol, proline and succinic and lactic acids along with the decrease of alanine during alcoholic fermentation.

The PCA analysis corresponding to the malolactic fermentation is showed in **Figure 7c**, and the 76% of the sample variance is explained with the first two principal components. Acetic and lactic acids show high positive loadings for PC1 while malic acid has a high negative loading. The second principal component (PC2) serves to show the increase of ethanol and succinic acid and the decrease of alanine during this type of fermentation. In contrast to that observed in the above PCA analyses, the proline concentration is not significant for either of the two principal factors.

From the comparison of the three PCA analyses, important conclusions could be assessed. The most important increase of proline, succinic acid and ethanol is produced in the alcoholic fermentation. The major consumption of alanine is produced in the alcoholic fermentation. While the malic acid concentration scarcely decreases in the alcoholic fermentation, an increase of lactic acid concentration is observed. The most important enhancement of acetic and lactic acids along with a high decrease of malic acid is observed in the malolactic fermentation. On the other hand, it is important to notice that similar results were obtained by Clark et al. (*40*) for ethanol, acetic acid, succinic acid, lactic acid and malic acid.

In conclusion, we have demonstrated the enormous potential of ^{1}H NMR as a tool for monitoring and controlling several

biological processes such as alcoholic and malolactic fermentations. The study has been developed in a commercial Rioja red wine from Tempranillo grapes, using as the best method external standard for quantification. **Table 1** shows the summary of all these data, which were used, through principal component analysis, to explain the behavior of the fermentation processes. In the future, we want to make more automatic the process in order to obtain high quality quantitative information for principal compounds.

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