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Time Course of the Evolution of Malic and Lactic Acids in the Alcoholic and Malolactic Fermentation of Grape Must by Quantitative ^{1}H NMR (qHNMR) Spectroscopy

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Quantitative NMR can be used to monitor several processes that take place in the transformation of the must of wine grapes. The study described here focused attention on monitoring of the malic and lactic acid levels during the alcoholic and malolactic fermentation processes. The method allows the simultaneous quantification of both acids through a range of 1−3.2 mmol/L. The effectiveness of each process was assessed and compared by carrying out precise analyses using enzymatic methods.

KEYWORDS: Quantitative NMR; wine; grape must; fermentation; malic acid; lactic acid

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*, *2*). The nondestructive nature of NMR is one of its most attractive features and allows rapid measurements, analysis of samples without laborious sample preparation, and the noninvasive study of samples. Some recent examples that have been reported include the analysis of coffee (*3*), olive oil (*4*), and tomatoes (*5*).

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 (*6*, *7*). In a recent and excellent review (*8*) 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 simple integration method and chemometric analysis could be used to obtain appropriate results for quantification. Partial least squares (PLS) regression has been successfully used for the 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 (*9*). Quantitative NMR analysis of other nuclei has also been developed to study gasoline (*10*), the purity of technical grade agrochemicals (*11*), and the analysis of lignins (*12*). In cases when an accurate quantitative determination can be performed, NMR techniques represent a very powerful analytical method not only for structural determination but also for quantitative analysis. Recent developments in this field have provided evidence that NMR can be developed as a precise quantitative tool and a primary ratio analytical method (*13*). Recently, Maniara et al. conducted a detailed metrological comparison between the use of traditional chromatographic techniques and NMR (*14*). 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 (*15*). There are only a few examples in the literature of quantitative NMR studies on beverages, and these include the quantification of organic and amino acids in beer (*9*), the quantification of chlorogenic acid (*16*) and (−)-epicatechin (*17*) in apple cider, and the quantification of methanol in a traditional Cypriot spirit (*18*).

Wines consist of several hundred components that 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 and age and the effects of adulteration (*19*). 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, one-dimensional (1D) (*20*) and two-dimensional (2D) (*21*) NMR experiments have been explored. The use of ^{13}C NMR spectroscopy was introduced in wine analysis by Rapp et al., who showed that ^{13}C NMR can successfully be used for the detection of sugars, alcohols, organic acids, and amino acids (*22*). In addition, Košir et al. used intensities from ^{1}H NMR spectra for the quantification of succinic and acetic acids in wine (*23*).

Given this background, and bearing in mind the excellent versatility of NMR for the study of samples that exhibit a time

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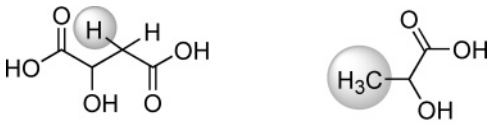

Figure 1. Analyzed molecules. The protons that were integrated in the ^{1}H NMR are highlighted.

course evolution, our goal was to study the transformation of malic acid into lactic acid in alcoholic and malolactic fermentation processes (**Figure 1**). 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 is important in the elaboration of other types of wines such as white wine or rosé wine. One way to reduce the quantities of this acid is to allow the spontaneous growth of lactic acid bacteria, which in turn carry out the malolactic fermentation. In some cases, this conversion into lactic acid could take place during the alcoholic fermentation. Control of this process is therefore essential to obtain a quality wine. In general, the determination of malic acid is carried out by enzymatic methods after derivatization of this acid. This method, although optimized, can suffer from undesirable interference with the sample. In this sense, ^{1}H NMR spectroscopy (*24*) is a noninvasive, rapid, and excellent analytical technique for the monitoring of this transformation and does not involve sample derivatization.

EXPERIMENTAL PROCEDURES

Samples. The time course of the evolution of six tanks from the Dinastia Vivanco winery (La Rioja, Spain) was explored. The first tank of red grapes (Tempranillo grape variety, *Vitis vinifera*) was obtained from the Briones region and was not treated in any way. The second tank of red grapes (Tempranillo grape variety, *V. vinifera*) obtained from the Tudelilla region was treated with lactic bacteria (Oenos, *Leuconostoc oenos*, viniflora) in order to develop the correct malolactic fermentation. The third tank of white grapes (Viura grape variety, *V. vinifera*) obtained from the Briones region had *Saccharomyces cerevisiae* yeast and Polycase added to preserve the white wine against oxidation or bacterial action. The fourth tank was composed of red grapes to obtain a rosé wine, and the fifth tank was composed of red grapes obtained from the San Asensio region. The last sample was obtained of red grapes (Garnacha grape variety, *V. vinifera*) from the experimental winery of the University of La Rioja and was treated with lactic bacteria (*Penococus oeni*, alpha Lallemand). The samples were collected directly at intervals of 2 days, transported from winery to laboratory, and preserved at −25 °C until analysis was carried out. The option of freeze-drying the samples and dissolving them in D_2O prior to recording the ^{1}H NMR spectra was rejected because the high content of sugars in the grape must would not allow sufficient elimination of water. Therefore, the simplest and fastest method for recording the spectra was used, and this involved two steps. First, the pH of the grape must sample was measured (pH-meter BasiC Crison) and adjusted to 3 by the dropwise addition of aqueous 1 M HCl to fix the chemical shift. The grape must from the experimental winery of the University of La Rioja was directly measured without the frozen process. A sample of the resulting grape must (0.6 mL) was added to the NMR tube together with D_2O (0.1 mL). Malic and lactic acid standards were purchased from Aldrich and were used for 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 XWIN-NMR software (version 3.5). Processing was performed with MestRe-C software (*25*) (version 4.1.7). The spectrometer was locked onto H_2O−D_2O, and all spectra were acquired at 25 °C.

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 was set to 60 s according to suggestions noted in the literature for quantification procedures to ensure that all protons were totally relaxed (*26*), and the 90° pulse width was calibrated at 7.1 μs with −1 dB as a power level. The experiments were carried out with automatic tuning and matching (ATM) and with GRADSHIM tools.

Processing of Spectra. Free induction decay (FID) files were exported into the MestRe-C program and, prior to Fourier transformation, an exponential window function (lb factor 0.01 Hz, after trying 0, 0.2, 0.5, and 1 Hz) was applied to obtain the optimal signal-to-noise ratio (*6*). 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 (*6*).

Quantitative NMR Analysis. We selected the quantification by single integration because quantification by PLS needs a set of

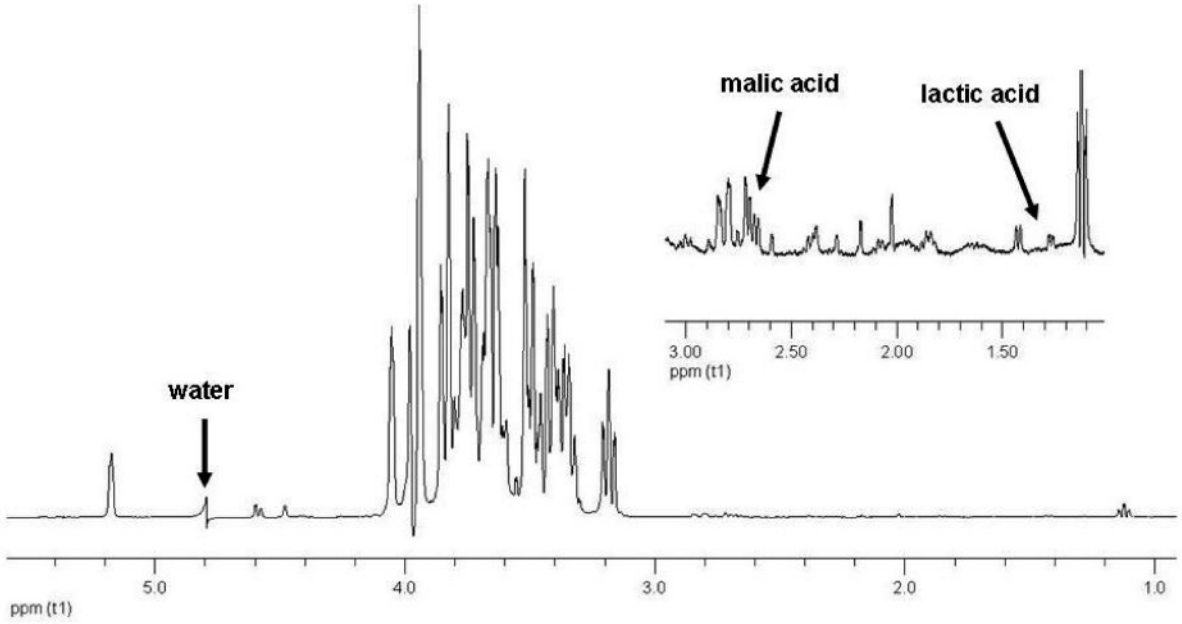

Figure 2. Spectrum of ^{1}H NMR (400 MHz) for grape red must (pH 3) using 1D Excitation Sculpting with expansion of the working region.

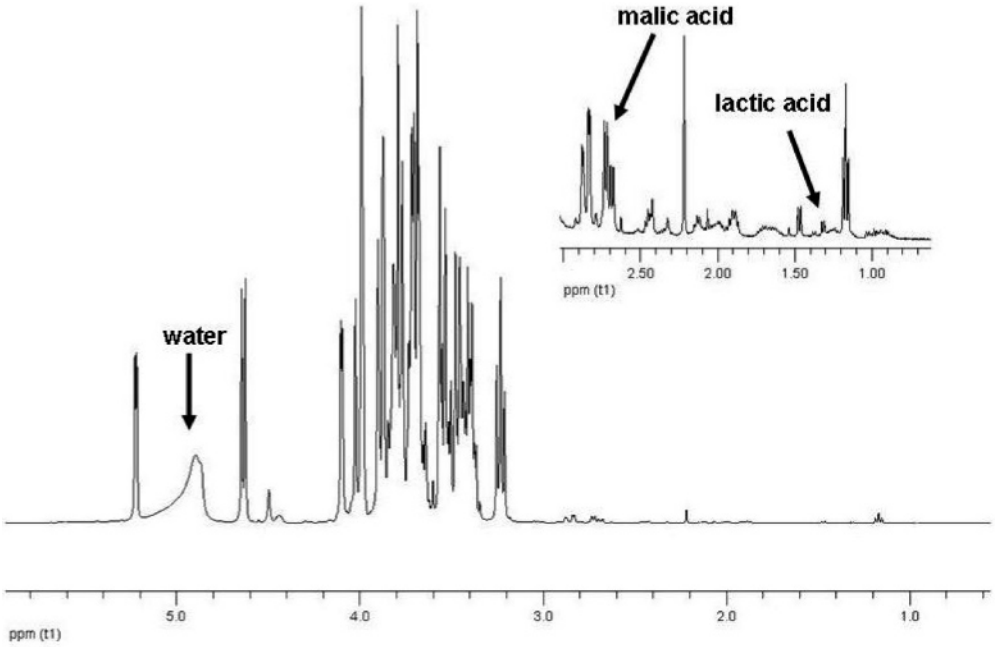

Figure 3. Spectrum of ^{1}H NMR (400 MHz) for grape red must (pH 3) using conventional 1D water presaturation with an expansion of the working region.

representative samples. 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 in the MestRe-C program as absolute integrals. We observed one diastereotopic proton for malic acid (doublet of doublets $^3J = 8.0$ Hz, $^3J = 16.4$ Hz, 1H at 2.70 ppm) and a methyl group for lactic acid (doublet $^3J = 7.2$ Hz, 3H at 1.32 ppm) (**Figure 1**). There are other signals for malic acid, but the region of the spectum for this doublet of doublets is sufficiently clear for its analysis. Succinic acid was used as an external standard without introducing it into the sample (*27*). 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 of the parameters (ns, d1, rg, ...) must be equivalent in the experiments on both the reference and sample. Individual experiments were carried out with known amounts of L-malic acid and L-lactic acid. A constant (k) was extracted for both the L-malic acid and L-lactic acid 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. This constant was used to obtain the concentration of malic or lactic acid in the grape must or wine sample. The accuracy of the measurements was determined with four different mixtures that contained known amounts of malic and lactic acids. To evaluate the matrix effect, we added malic and lactic acid to the grape must sample. Measurements and processing of spectra of both original and enriched samples were carried out under the same conditions, and a matrix effect of the high sugar concentration was not detected. Accordingly, 11 mg of malic acid (0.087 mmol) was added in a sample (1 mL of must with 0.026 mmol of malic acid), and the global result gives a corresponding 0.11 mmol/L.

Enzymatic Analysis. The malic acid was measured in the winery according to the standard enzymatic assay method using malate, with the production of NADH (Boëhringer test) and control by ultraviolet absorption at 340 nm using an enzymatic kit (NOVAKIT SL ref 01NK0025) (*28*).

RESULTS AND DISCUSSION

Several experiments were developed that involved the addition of known amounts of malic and lactic acids to the must matrix in order to select the accuracy acquisition parameters. Several relaxation delay times from 5 to 100 s were evaluated, and the best result was found for a value of d1 = 60 s. Other gradient-based water suppression methods were also evaluated including 1D WATERGATE (p3919gp pulse program) (*29*) and

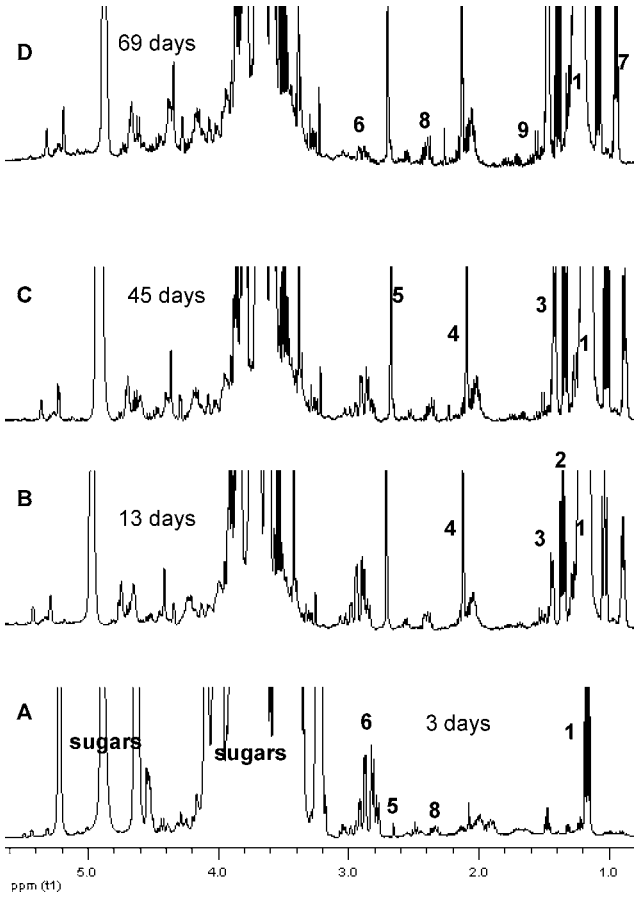

Figure 4. Time course of the evolution of wine in alcoholic and malolactic fermentations by ^{1}H NMR spectra (400 MHz) for grape red must (pH 3). Peaks: 1, ethanol; 2, ethanol satellites; 3, lactic acid; 4, acetic acid; 5, succinic acid; 6, malic acid; 7, 2,3-butanediol; 8, proline; 9, alanine.

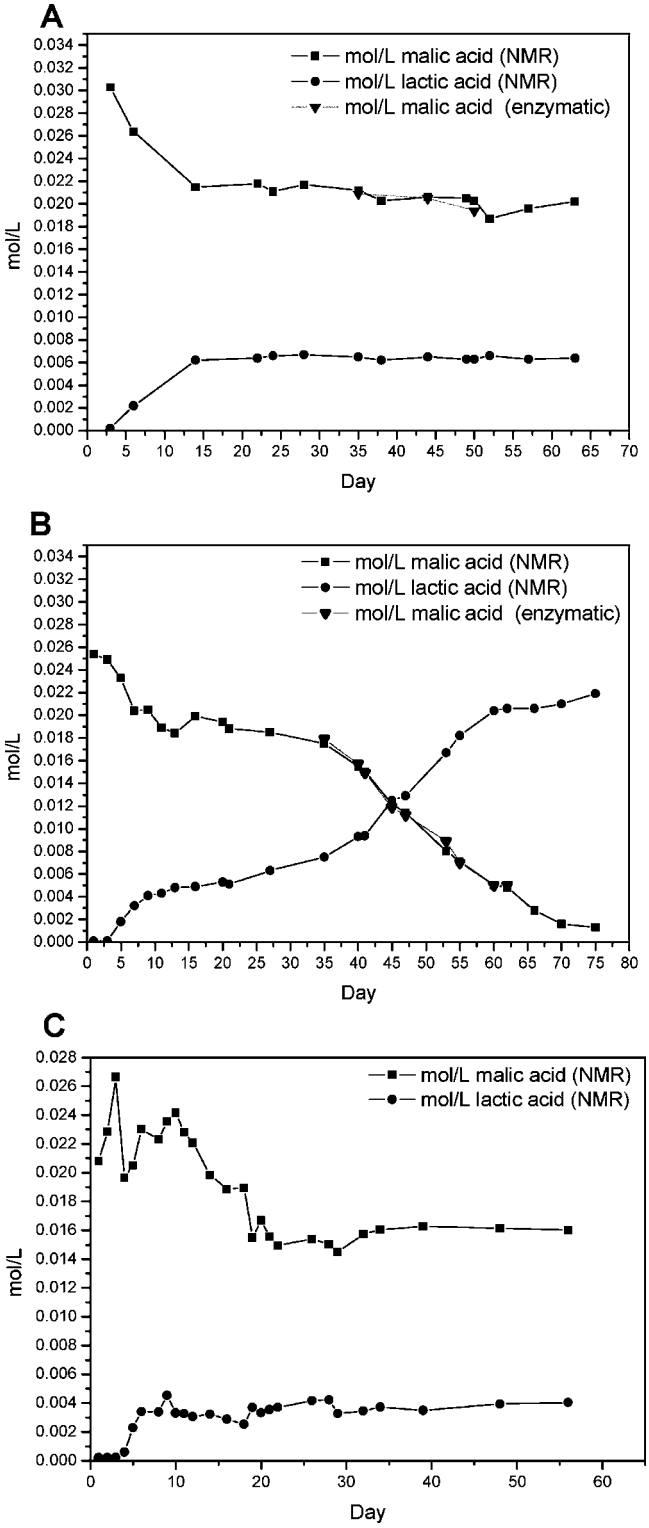

Figure 5. Time course of the evolution of malic and lactic acids by ^{1}H NMR spectroscopy (400 MHz) and enzymatic method: (**A**) red wine from Briones; (**B**) red wine from Tudelilla; (**C**) white wine from Briones.

1D Excitation Sculpting using W5 pulse train (zggpw5 pulse program) (*30*). Water suppression with the latter pulse sequence is excellent, as can be seen in **Figure 2**, but the pulse distorts the integrations of signals when they are compared with known concentrations of malic and lactic acids samples.

The spectrum of grape must obtained using a simple presaturation pulse program to give smaller water suppression but correct integrations is shown in **Figure 3**. Several internal standards reported in the literature (*8*) were used, but these were

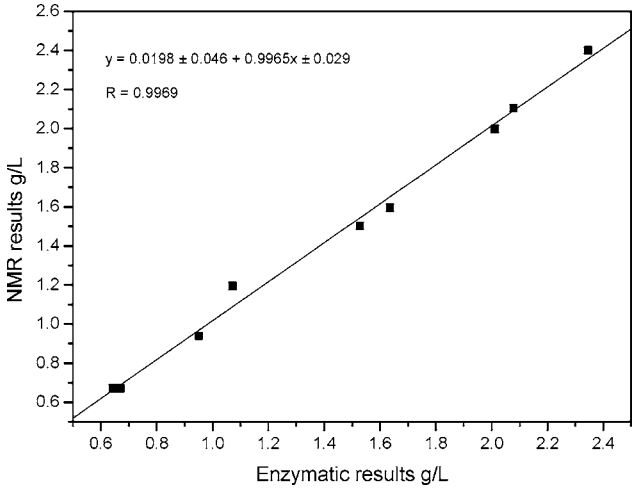

Figure 6. Concentrations of malic acid determined by qHNMR (^{1}H NMR spectroscopy at 400 MHz) versus concentrations determined by the enzymatic method for red wine from Tudelilla.

ruled out for a variety of reasons. Aromatic compounds such as 1,3,5-benzenetricarboxylic acid gave a signal that was far removed from the protons under investigation, and the phase or baseline cannot efficiently be corrected. On the other hand, the signals for compounds such as 1,3,5-trioxane and succinic acid overlap the signals in the areas of interest in the spectra. However, a procedure (*27*) has recently been described in which separate but identical precision tubes are used for analytes and for readily prepared external standards. This method enables the direct measurement of analyte concentrations without contaminating the sample or signal. For this reason, we decided to use an external reference in a different NMR tube with the same acquisition and processing parameters as used in the experiments.

The evolution of red wine from Tudelilla, as determined by ^{1}H NMR spectroscopy after 3, 13, 45, and 69 days from the start of the alcoholic fermentation process, is shown in **Figure 4** as spectra **A**−**D**, respectively. Spectrum **A**, recorded on the sample 3 days after collection of the grape, showed the principal components of the must are the sugars (both fructose and glucose), malic acid, several amino acids, and a small amount of ethanol due to the alcoholic fermentation. Spectrum **B** (13 days) shows a considerable increase in the amount of ethanol as well as acetic acid, which appears in a significant concentration. After 45 days (spectrum **C**), the alcoholic fermentation is complete, and the signals due to ethanol and its satellites are present in the spectrum. Acetic and succinic acids increase in concentration, as expected, and lactic acid appears to the detriment of malic acid. It can be seen from spectrum **D** (69 days) that the malolactic fermentation in the wine has concluded and, subsequently, the malic acid concentration is stable (integration of doublet of doublets at 2.7 ppm). Other compounds that are clearly evident in these spectra are proline, alanine, tartaric acid, glycerol, 2,3-butanediol, and other minor components such as leucine or isoleucine. It should also be emphasized that these experiments permit quantification of other products to be carried out as well. The chemical shifts of compounds are in agreement with the literature (*15*).

The graphs in **Figure 5** represent the time course of the evolution of malic and lactic acids in moles per liter, and from these it is possible to compare the formation of lactic acid from malic acid in the wine. The triangular marks correspond to the analysis of malic acid by the enzymatic method. **Figure 5A** represents the evolution for a red wine without any lactic

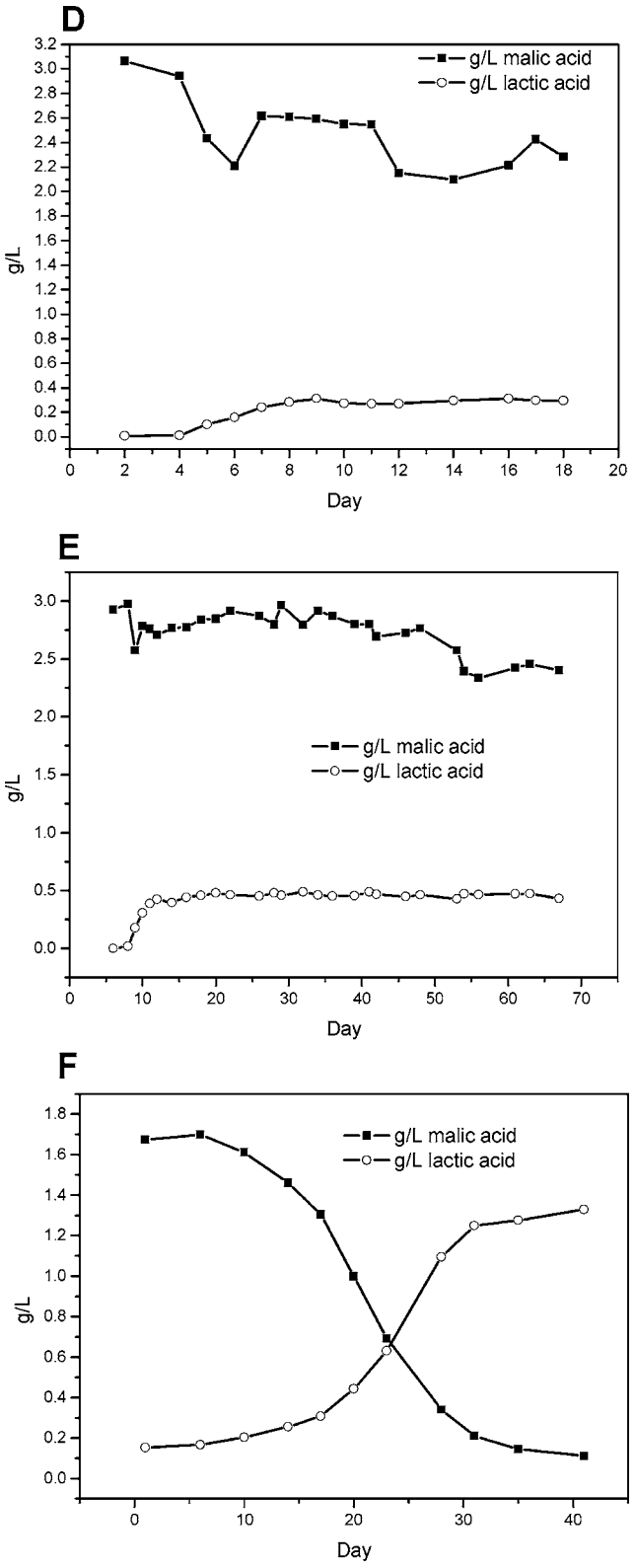

Figure 7. Time course of the evolution of malic and lactic acids in grams per liter by ^{1}H NMR spectroscopy (400 MHz): (**D**) rosé wine; (**E**) red wine from San Asensio; (**F**) red wine from the experimental winery of the University of La Rioja.

bacteria and was stopped. The malolactic fermentation in this case is slow. **Figure 5B** shows the evolution for the second tank, in which lactic bacteria were added—once again, the triangular marks show the analysis of malic acid by the enzymatic method. The evolution of both acids is clear, and on the 45th day the concentrations of malic and lactic acid are identical. **Figure 5C** shows the evolution for a white wine in which the malolactic fermentation process is monitored; this was stopped when the malic acid concentration was ≈2 g/L.

It is important to note that the 12 measurements carried out by enzymatic methods are in excellent agreement with the corresponding 12 measurements from the qHNMR method. The linear regression of the qHNMR results versus classical enzymatic method in grams per liter is shown in **Figure 6** and gives the following equation: $y = 0.0198 \pm 0.046 + 0.9965x \pm 0.029$ with $R = 0.9969$.

On the other hand, **Figure 7** shows the evolution of malic and lactic acids for the other three tanks of wines expressed in grams per liter. **Figure 7D** illustrates the evolution of a rosé wine. **Figure 7E** shows a red wine for which the fermentation process has been stopped with the purpose of continuing during the spring. Finally, **Figure 7F** illustrates a completed malolactic fermentation with inversion of quantities of malic and lactic acids. In this last case, the samples were collected and measured directly by NMR.

In conclusion, we have developed a versatile and noninvasive qHNMR method for the rapid analysis of malic and lactic acids in the alcoholic and malolactic fermentation processes of several wines. These results are consistent with those obtained by using the classical enzymatic analysis of malic acid. The principal advantage of the NMR method is the minimal sample preparation protocol because the method does not require any preconcentration, separation, or derivatization reaction. The study of other compounds and the possibility of actually carrying out the fermentation process "in the NMR tube" is currently in progress, and it could be applied to other similar fermentation processes.

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