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NMR Study of Histidine Metabolism during Alcoholic and Malolactic Fermentations of Wine and Their Influence on Histamine Production

Eva López-Rituerto, Alberto Avenoza, Jesús H. Busto,* and Jesús M. Peregrina*

Departamento de Química, Centro de Investigación en Síntesis Química (CISQ), Universidad de La Rioja, E-26006 Logroño, Spain

ABSTRACT: The metabolic pathways of amino acids play a crucial role in the organoleptic and hygienic quality in wines. In particular, histidine is one of the most studied amino acids of wines due to histamine toxicity in humans, a biogenic amine derived from histidine by enzymatic decarboxylation. The development of new tools to increase knowledge on metabolism that produces histamine in wine is critical. This study investigated by using nuclear magnetic resonance (NMR) spectroscopy the transformation of histidine into histaminol and histamine during alcoholic and malolactic fermentations. The transformations of histidine into histaminol during alcoholic fermentation and into histamine during malolactic fermentation were observed. This paper highlights the importance of selecting lactic acid bacteria for malolactic fermentation to avoid the production of biogenic amines such as histamine.

KEYWORDS: *wine, nuclear magnetic resonance, histidine, histamine, histaminol*

INTRODUCTION

Amino acid content in wine depends on many factors, such as grape berry variety, viticultural techniques, optimal maturation, ammonium content, enological techniques, yeast, bacteria, storage, and aging. Amino acids together with proteins and peptides play an important role as nitrogen sources for yeast and lactic acid bacteria during alcoholic and malolactic fermentations, respectively.[1,2] The term YAN (yeast assimilable nitrogen) represents the optimal nitrogen level for a good performance of fermentation. The amount of nitrogen influences the wine quality. A low level of YAN in grape must leads to low yeast populations and also to the reduction of yeast growth during wine fermentation, thus increasing the risk of "stuck" fermentation and the production of undesirable compounds.[3,4]

Amino acids are precursors of low molecular weight compounds such as fusel alcohols and biogenic amines, which have high impacts on wine quality. Fusel or higher alcohols, containing more than two carbon atoms in their structure, together with their ester derivatives play a critical role in the aromatic complexity of wines.[1,5,6] Yeasts convert amino acids into higher alcohols through the well-known Ehrlich pathway (Figure 1). Biogenic amines are produced by microbial decarboxylation of the corresponding amino acid precursors (Figure 1). These higher alcohols and biogenic amines are formed during fermentative processes, aging, and storage of foods,[7] and they may produce adverse effects in humans in high concentrations, especially when ethanol is present. Biogenic amines may induce headaches, respiratory distress, heart palpitations, hyper- or hypotension, and several allergic disorders.[8] Therefore, studies on the evolution of amino acids during alcoholic and malolactic fermentations should be taken into account to evaluate the quality of wines. However, only studies applying some analytical techniques, mainly HPLC, have been performed to date.[9]

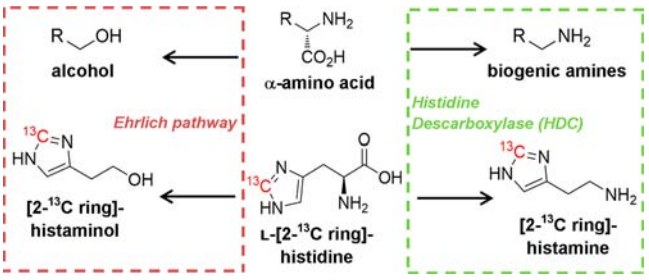

Figure 1. Transformation of amino acids into alcohols by the Ehrlich pathway and into biogenic amines (top) as well as transformation of L-histidine into its higher alcohol and into histamine (bottom).

In this regard, NMR spectroscopy is being applied as an alternative technique for evaluating wine quality. In metabolomics, NMR has emerged as an effective method to investigate metabolic changes[10,11] and then to compare, distinguish, and classify different wines[12−19] or grape berries.[20−22] Consequently, Hong and co-workers used ^{1}H NMR to evaluate the behavior of different yeast and lactic acid bacteria strains[23−25] and, by combining NMR spectroscopy with multivariate statistics, they could classify wines and grape berries according to variety and geographical origin.[26,27] Recently, we have reported the use of quantitative ^{1}H NMR for monitoring and controlling biological processes such as the alcoholic and malolactic fermentations, as well as for wine classification according to vintage, region of production, and winery.[28−30]

The combination of ^{13}C NMR spectroscopy with the use of isotopically ^{13}C-labeled molecules as tracers is a well-established protocol for the identification of metabolic pathways in microbiology.[31−33] Although this protocol is very valuable for the identification of metabolites, and hence the metabolic

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pathways, it has not been commonly used for quantification. On this basis, we have recently applied ^{13}C NMR to study the metabolic transformations of some ^{13}C-labeled amino acids into their corresponding higher alcohols during the alcoholic fermentation.[34] Because histidine is present in grape juice and wine, its concentration could be a parameter for quality assesment. Therefore, herein, we have investigated the transformation or metabolism of histidine during alcoholic and malolactic fermentations through ^{1}H and ^{13}C NMR experiments of samples containing ^{13}C-labeled histidine.

According to the Ehrlich pathway, histidine is transformed by yeast into a higher alcohol (Figure 1). However, this alcohol has not been extensively studied even though its presence in wine has been detected by HPLC methods, by Bordiga et al.[35] The same authors indicated the need to study the origin of this alcohol on the basis of previous papers[36,37] showing that yeast and mycobacteria are able to transform histamine into the same higher alcohol. In this regard, a previous study showed that higher alcohols such as 2-phenylethanol, 3-methyl-1-butanol, and (*S*)-2-methyl-1-butanol were produced through the Ehrlich transformation of the corresponding amino acids.[34]

On the other hand, lactic acid bacteria such as *Oenococcus oeni* transform histidine into histamine during the malolactic fermentation. Such transformation has been widely studied from microbiological,[38] biochemical,[39,40] and chemical[41] points of view. Histidine decarboxylase enzyme (HDC) is responsible for the transformation of histidine into histamine (Figure 1). The production of histamine has been recently reviewed by Garcia-Moruno and Muñoz.[42] Histamine is one of the biogenic amines in wine that may cause negative effects on human health. The presence of histamine in wine has a great impact, and there are countries that recommend upper limits for this biogenic amine ranging between 2 and 10 mg/L.[43] Several methods have been developed for the analysis of biogenic amines, including histamine, in foods: thin-layer chromatography (TLC), gas chromatography (GC), a capillary electrophoretic method (CE), and high-performance liquid chromatography (HPLC).[44]

In the current study, we introduce a simple and easy ^{13}C NMR analysis to study the metabolism or transformation of histidine into histamine and histaminol during wine fermentation processes.

MATERIALS AND METHODS

Samples. *Alcoholic Fermentation.* Red grapes (Tempranillo grape variety, *Vitis vinifera*) were manually collected from Uruñuela, La Rioja, Spain. Must chemical composition was as follows: sugar (density), 1.110 g/mL; total acidity, 3.33 g/L (H_2SO_4); pH, 3.35; malic acid, 3.23 g/L. Destemmed and crushed grapes were homogenized, and a prefermentation cold maceration was carried out. Color extraction enzymes were used (750 g/420 hL), and the concentration of free sulfur dioxide was 39 mg/L. Alcoholic fermentations were carried out in sterile Erlenmeyer flasks kept in an incubator regulated at 25 °C with activated Uvaferm BC (Lallemand Bio S.L.).

Malolactic Fermentation. Alcoholic fermentation to produce red wine was carried out at winery and chemical composition as follows: alcoholic degree, 12.4 g/L; total acidity, 4.42 g/L (H_2SO_4); acetic acid, 0.45 g/L; reducing sugar, 2.62 g/L; pH, 3.75; malic acid, 3.41 g/L. Malolactic fermentations were carried out in sterile Erlenmeyer flasks kept in an incubator regulated at 25 °C, activated with commercial preparations of *O. oeni* lactic acid bacteria, Uvaferm Alpha (Lallemand Bio S.L.), which was considered as the activated malolactic fermentation and also with bacterial biomass from the winery, which was considered as the spontaneous malolactic fermentation. Both alcoholic and malolactic fermentations were monitored by ^{1}H NMR using a quantitative method described previously.[28,29]

Alcoholic Fermentation. To follow the metabolic transformation of L-histidine during alcoholic fermentation, we added 40.9 mg of labeled L-histidine into an Erlenmeyer flask with 500 mL of grape must to obtain a final concentration of 81.0 mg/L of L-[2-^{13}C ring]-histidine (99% ^{13}C, sample A), and 41.0 mg of nonlabeled histidine was added into an Erlenmeyer flask with 500 mL of grape must to obtain a final concentration of 81.2 mg/L (sample B). Moreover, we carried out another experiment in an Erlenmeyer flask with 500 mL of grape must without addition of histidine (sample C). Alcoholic fermentation was carried out under optimal conditions of temperature (25 °C), and samples were collected at different time intervals (0, 9, 21.5, 26, 30, 34, 46, 50, 54, 58, 71.5, 78, 84.5, 98, and 105.5 h). The alcoholic fermentations were monitored by ^{1}H NMR, and the ethanol signal at 1.17 ppm was initially observed at 30 h for all samples.

Spontaneous Malolactic Fermentation. To follow the metabolic transformation of L-histidine during malolactic fermentation, we added 20.0 mg of labeled L-histidine into 250 mL of wine (activated with bacterial biomass from the winery) to obtain a final concentration of 79.2 mg/L of L-[2-^{13}C ring]-histidine (99% ^{13}C, sample A) and 19.0 mg of nonlabeled histidine to obtain a final concentration of 75.2 mg/L (sample B). Furthermore, we carried out another fermentation without addition of histidine (sample C). Spontaneous malolactic fermentation was induced under optimal conditions of temperature (25 °C), and samples were collected at different time intervals (1, 4, 7, 10, 15, and 60 days). Malolactic fermentations were monitored by ^{1}H NMR, and the transformation of malic acid (2.83 ppm) into acid lactic (1.40 ppm) was observed for all samples.

Activated Malolactic Fermentation. To follow the metabolic transformation of L-histidine during the malolactic fermentation with commercial lactic acid bacteria *O. oeni*, we added 39.4 mg of labeled L-histidine into 500 mL of wine (activated with Uvaferm Alpha, Lallemand Bio S.L.) to obtain a final concentration of 78.0 mg/L of L-[2-^{13}C ring]-histidine (99% ^{13}C, sample A) and 41.0 mg of nonlabeled histidine to obtain a final concentration of 81.0 mg/L (sample B). In addition, we carried out another experiment without addition of histidine (sample C). Activated malolactic fermentation was carried out under optimal conditions of temperature (25 °C), and samples were collected at different time intervals (1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, 11, 13, 16, 19, 21, 23, 27, 30, 33, and 37 days). Malolactic fermentations were monitored by ^{1}H NMR, and the transformation of malic acid (2.83 ppm) into acid lactic (1.40 ppm) was observed for all samples.

NMR Spectroscopy. ^{1}H 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 MestReNova (version 7.0.2).[45] The spectrometer was locked onto D_2O in a mixture H_2O/D_2O (9:1), and all spectra were acquired at 298 K.

^{1}H NMR spectra were recorded with the standard pulse sequence for presaturation of the water signal at 1875 Hz; zgpr (1D sequence with f1 presaturation) with pl9 (power level for presaturation) at 60 dB and a flip angle of 90°. Experiments were carried out with automatic tuning and matching (ATM) and with GRADSHIM tools, using the NMR CASE as a NMR sample changer allowing automatic analysis of several samples. Quantitative ^{1}H NMR was carried out using the methodology previously reported.[28,29]

^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer equipped with a 5 mm inverse probe (PABBO BB-1H/D Z-GRD). Inverse gated decoupled methodology was used.[46] Proton decoupling was applied only during the acquisition period. In this case, no polarization transfer from ^{1}H to ^{13}C via NOE takes place; therefore, the resulting ^{1}H-coupled ^{13}C spectrum should be used for quantitative measurements.

Phase-sensitive gradient-enhanced 2D heteronuclear single-quantum correlation (HSQC) spectra were recorded by using hsqcedgpph pulse program (z filter and selection before t_1 removing decoupling during acquisition) with CNST2 $J(^{13}C,^{1}H)$ = 145 Hz [heteronuclear scalar $J(^{13}C,^{1}H)$ coupling].

Processing of Spectra. Free induction decays (FIDs) from one-dimensional files were exported into the MestReNova software and, prior to carrying out Fourier transformation, an exponential window function was applied to obtain an optimal signal-to-noise ratio.[47] The number of data points in the real part of spectra was set to 64K. Spectra phases were manually corrected by selecting the submenu "Phase Correction", and baselines were adjusted by the "Polynomial Fit" function in accordance with the literature.[46] Signal integration, in both ^{1}H NMR and ^{13}C NMR spectra, was manually carried out, and processing data were achieved twice. Metabolites were assigned by two-dimensional NMR experiment, spiking experiments, and/or information published elsewhere.[28,29]

RESULTS AND DISCUSSION

Histidine is an essential amino acid for humans, and the concentration range in grape juice is 5−197 mg/L;[4] in all experiments of the current study, the quantity of histidine added was therefore within this range. In NMR experiments, the ^{13}C NMR chemical shift of the L-[2-^{13}C ring]-histidine was observed at 137.0 ppm and the proton attached to this carbon at 8.66 ppm in the ^{1}H NMR spectrum. Notably, chemical shifts of histidine protons substantially depend on pH. Thus, the imidazole ring of the histidine is protonated at the pH of wine, modifying the proton chemical shifts around 1 ppm[30] compared to the chemical shifts of histidine at a neutral pH.[48]

Alcoholic Fermentation. Three different processes (samples A, with ^{13}C-labeled histidine; B, with histidine; C, without histidine) were monitored using ^{1}H NMR to evaluate the consumption of histidine through alcoholic fermentation process and the influence of the labeled amino acid in these processes. Histidine was quantified by the methodology developed in our group,[28,29] which allows a simultaneous quantification of several compounds in wine by ^{1}H NMR spectroscopy. The histidine transformation was similar for samples A and B, with the added amino acid disappearing at 105.5 h. Moreover, we could conclude that yeast does not discriminate between labeled and nonlabeled amino acids (Figure 2A).

Although we visualized by ^{1}H NMR the disappearance of histidine during alcoholic fermentation, we did not know the nature of the metabolites generated. For this purpose, in subsequent experiments, we used isotopically labeled histidine. The ^{13}C NMR spectrum at initial time (0 h) showed a peak at 137.0 ppm, corresponding to labeled C2 carbon of histidine aromatic ring (Figure 2B, spectrum a). When alcoholic fermentation began, this peak decreased quickly, disappearing at 84.5 h after initiation of alcoholic fermentation. At 71.5 h, the spectrum showed two different peaks (Figure 2B, spectrum b), the histidine signal and a new one located at 136.0 ppm, corresponding to a novel ^{13}C-labeled compound derived from labeled histidine. This new compound was assigned to the higher alcohol 2-(1*H*-imidazol-4-yl)ethanol (histaminol). The assignment of histaminol was confirmed by spiking with commercial histaminol and by two-dimensional (2D) heteronuclear multi bond correlation (HMBC) spectroscopy, between two bonded ^{1}H−^{13}C atoms, as shown in Figure 3. In the spectrum at 105.5 h (Figure 2B, spectrum c), we were able to observe the total disappearance of the representative L-[2-^{13}C ring]-histidine signal and its complete transformation to labeled [2-^{13}C ring]-histaminol, likely following an Ehrlich process (Figure 1). All of these spectra were compared with those obtained from the control sample C, in which we did not observe any ^{13}C signals (Figure 2B, spectrum d). Time course evolution of labeled metabolites together with progress of the main parameter in wine, such as ethanol, is shown. According to our previous works, histidine was completely transformed into histaminol in the beginning of the alcoholic fermentation, when the alcoholic degree is nearly 5 v/v (Figure 2C). No other metabolites associated with the Ehrlich pathway (α-keto acids and/or aldehydes derived from histidine or histamine) were detected in any spectra. Considering the data reported, it is reasonable to think that histamine in wine does not originate from the metabolism of histidine in the alcoholic fermentation process but in malolactic fermentation, depending on bacteria used. Therefore, we concluded that the production of histaminol in wine was likely a consequence of the metabolism of histidine by yeast.

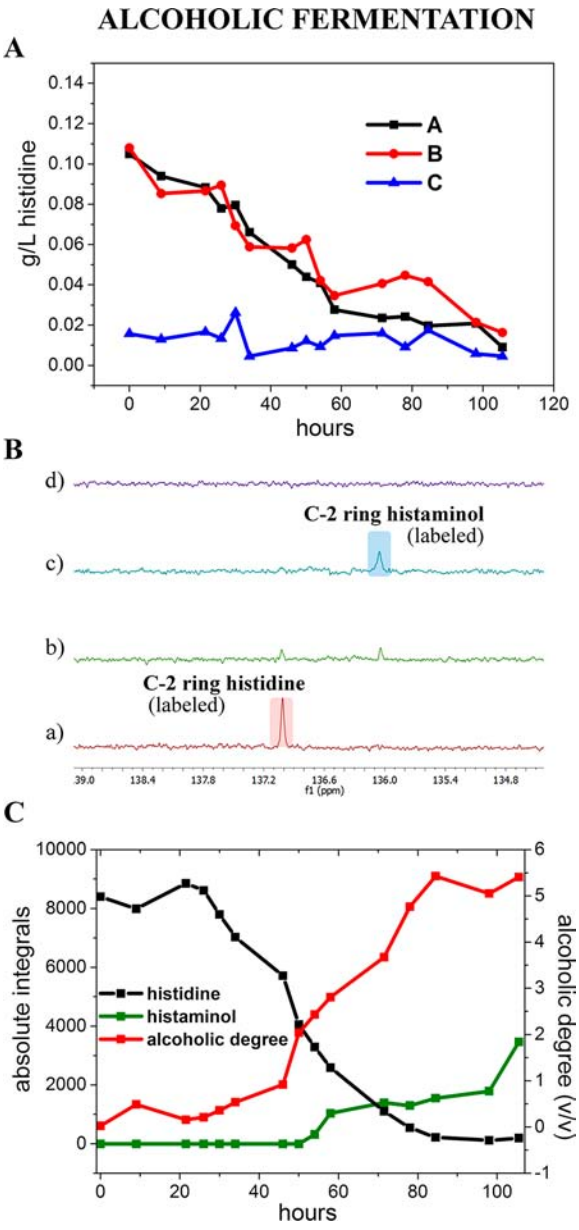

Figure 2. (A) Time course evolution of L-histidine (by ^{1}H NMR, g/L) in samples A, with ^{13}C-labeled histidine; B, with histidine; and C, without histidine. (B) ^{13}C NMR spectra for monitoring transformations of L-histidine into histaminol: (a) sample A at 0 h; (b) sample A at 71.5 h; (c) sample A at 105.5 h; (d) sample C (control) at 105.5 h. (C) Time course evolution of L-histidine, histaminol (by ^{13}C NMR, absolute integrals), and alcoholic degree (by ^{1}H NMR, v/v).

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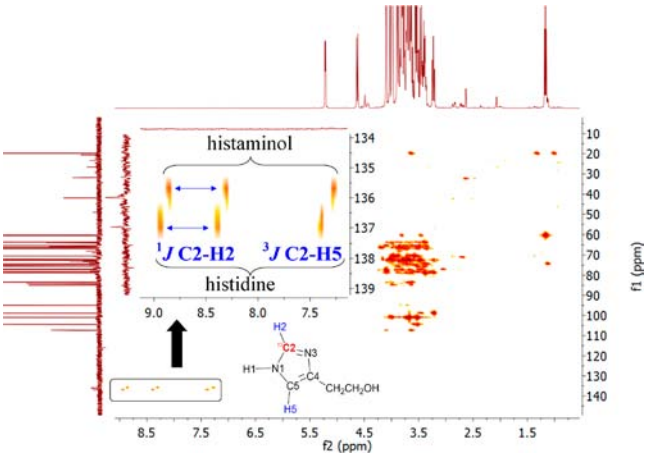

Figure 3. HMBC for sample A at 71.5 h.

Spontaneous Malolactic Fermentation. The other essential metabolic process in wine elaboration is malolactic fermentation by lactic acid bacteria. In most cases, these bacteria are present in the tanks in which fermentation takes place, and this process can occur spontaneously without the addition of more bacteria by indigenous strains. Taking into account the different metabolic processes that occur in malolactic fermentation, it is necessary to study the behavior of histidine in these processes. The ^{13}C NMR spectrum at initial time (day 1) showed a peak at 137.0 ppm corresponding to labeled histidine (Figure 4A, spectrum a). When malolactic fermentation began, this peak decreased quickly, and at day 4 two adjacent peaks (Figure 4A, spectrum b) were observed in spectrum, consisting of the labeled histidine at 137.0 ppm and the labeled histamine at 136.9 ppm. To corroborate this assignment, we compared this value with that obtained in the ^{13}C NMR experiment carried out on commercially available histamine. In the spectrum at day 15 (Figure 4A, spectrum c), we were able to observe the presence of histamine, but not histaminol or other metabolites associated with histidine (Figure 4A). Time course evolution of labeled metabolites was checked by ^{13}C NMR during malolactic fermentation, and transformation of malic acid into lactic acid was monitored by ^{1}H NMR. Both progressions are described in Figure 4B (^{13}C NMR) and 4C (^{1}H NMR), showing that the transformation of histidine by lactic acid bacteria took place simultaneously with transformation of malic acid. The complete transformations of histidine and malic acid were observed around 15 days. At the same time, the histamine and lactic acid were appearing. In view of these results, the presence of large quantities of histidine at the beginning of malolactic fermentation could likely be the main source of production of histamine in wine.

Activated Malolactic Fermentation. In this section, we attempt to determine if the use of an appropriate selection of lactic acid bacteria could control the production of histamine. We used *O. oeni* lactic acid bacteria commercialized as Uvaferm Alpha. Transformation of L-[2-^{13}C ring]-histidine was investigated by ^{13}C NMR analysis to evaluate its metabolic process during the malolactic fermentation induced by commercial *O. oeni*. Unlike the previous case, in ^{13}C NMR spectra, the histidine signal was the only one observed in all cases; no histamine or histaminol signals were detected (Figure 5A). To confirm that malolactic fermentation had taken place correctly, transformation of malic acid into lactic acid was followed by ^{1}H NMR (Figure 5B). Once malolactic fermentation was

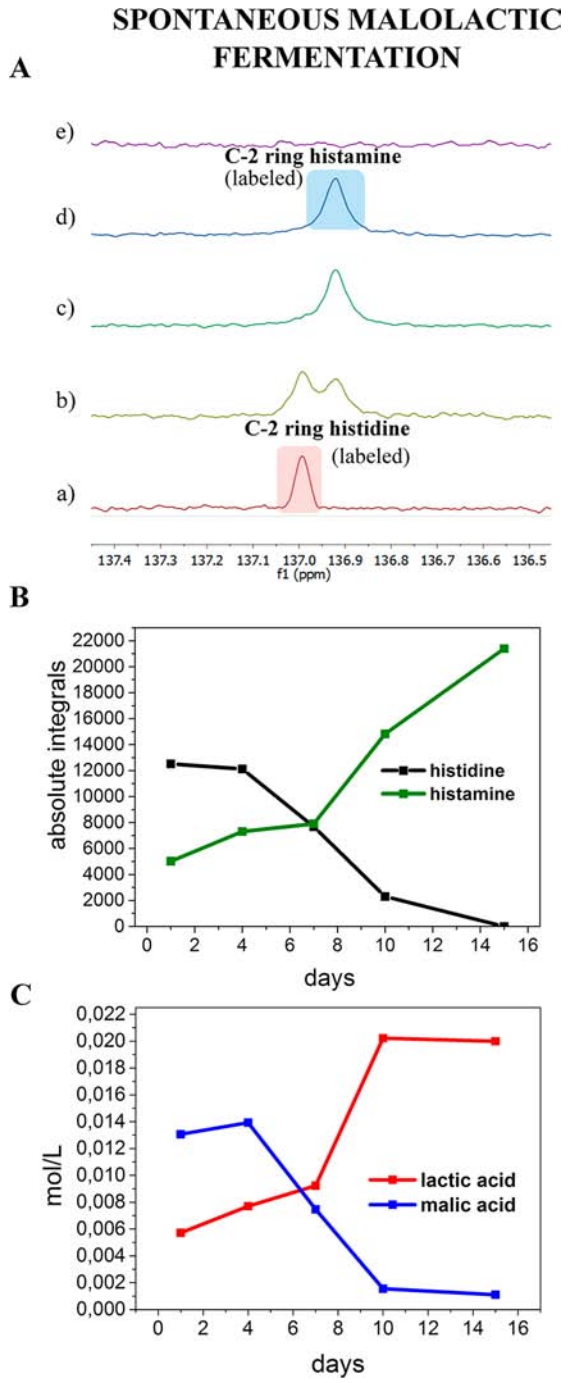

Figure 4. (A) ^{13}C NMR spectra for monitoring transformations of L-histidine into histamine for samples A, with ^{13}C-labeled histidine, and C, without histidine: (a) sample A at 1 day; (b) sample A at 4 days; (c) sample A at 15 days; (d) sample A at 60 days; (e) sample C at 60 days. (B) Time course evolution of histidine and histamine in sample A by ^{13}C NMR (absolute integrals). (C) Time course evolution of malic and lactic acids in sample A by ^{1}H NMR (mol/L).

completed, the histidine level was stable. Therefore, we could establish that histidine was not metabolized by lactic commercial bacteria Uvaferm Alpha. In addition, during malolactic fermentation the appearance of histamine was not observed; neither were other ^{13}C-labeled metabolites such as histaminol. The metabolism of histidine during malolactic fermentation with various strains of lactic acid bacteria remains for further study. This protocol could be very useful to analyze

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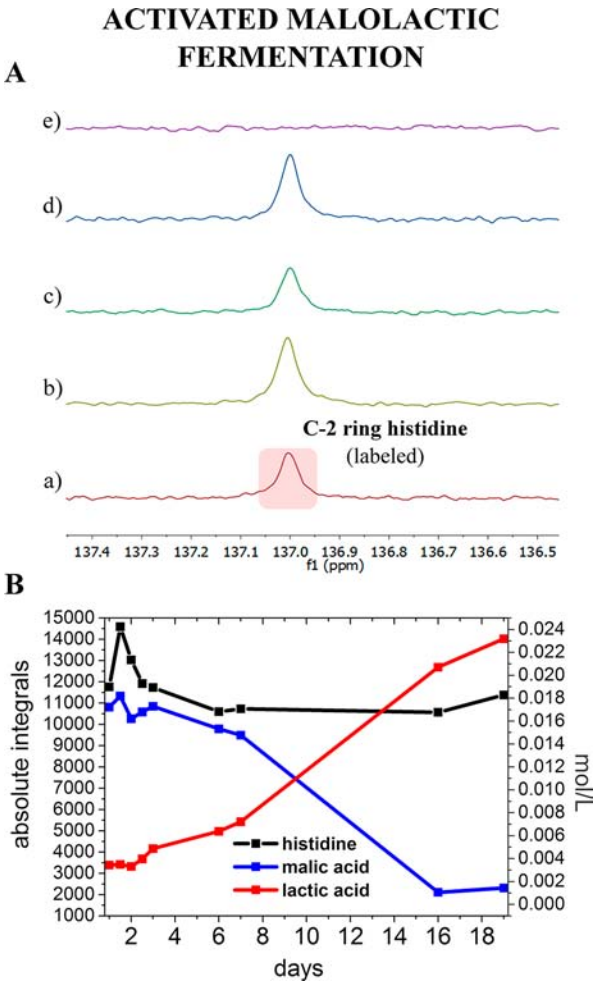

Figure 5. (A) ^{13}C NMR spectra for monitoring transformations of L-histidine into histamine for samples A, with ^{13}C-labeled histidine, and C, without histidine; (a) sample A at 1 day; (b) sample A at 4 days; (c) sample A at 16 days; (d) sample A at 37 days; (e) sample C at 37 days. (B) Time course evolution of histidine (by ^{13}C NMR, absolute integrals) and malic and lactic acids in sample A by ^{1}H NMR (mol/L).

bacteria strains to be used in the corresponding fermentations, minimizing the concentration of histamine.

In conclusion, NMR spectroscopy has been confirmed as an important tool in the evaluation of the metabolic pathway of histidine during alcoholic and malolactic fermentations in wine. Using this methodology, transformation of histidine was monitored during these processes. In the beginning of the alcoholic fermentation, transformation of histidine into histaminol was observed. Moreover, the transformation of histidine into histamine during malolactic fermentation may depend on strains of lactic acid bacteria, highlighting the importance of lactic acid bacteria selection for malolactic fermentation to avoid the production of biogenic amines such as histamine. The use of certain bacteria as Uvaferm Alpha allowed a drastic decrease in histamine concentration. Therefore, NMR analysis with the labeled amino acids could be a useful analytical tool to evaluate their metabolism during wine fermentation.

AUTHOR INFORMATION

Corresponding Authors

*(J.H.B.) Fax: +34 941 299621. E-mail: hector.busto@unirioja.es.

*(J.M.P.) Fax: +34 941 299621. E-mail: jesusmanuel.peregrina@unirioja.es.

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Notes

The authors declare no competing financial interest.

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