JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 4923–4927 4923
DOI:10.1021/jf904442d

Evidence of Metabolic Transformations of Amino Acids into Higher Alcohols through ^{13}C NMR Studies of Wine Alcoholic Fermentation

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Because the metabolite transformations in wine fermentation processes play a crucial role in the organoleptic and hygienic quality of wines, the nuclear magnetic resonance (NMR) technique is presented as a significant tool to follow metabolic pathways. In this paper, we investigated the transformation of several amino acids into their corresponding higher alcohols during the alcoholic fermentation, showing that the amino acids are totally consumed in the first stages of the process.

KEYWORDS: Wine; nuclear magnetic resonance; yeast; metabolites

INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy has been widely used in the field of agricultural and food chemistry (*1*). NMR is the perfect tool for broad-range profiling of abundant metabolites and for metabolite fingerprinting of extensive sample collections in the agriculture field (*2*). The term metabolomics, defined as the scientific study that seeks an analytical description of complex biological samples and aims to characterize and quantify all of the small molecules in such a sample, could also be applied in the enology world (*3*).

Wine consists of several hundred components presented at different concentrations: Water, ethanol, glycerol, sugars, and organic acids are the major ones. The main and secondary compounds are the result of the biological processes occurring in grapes juice, and their nature and concentration depend on many factors, including grape types and yeasts or bacteria responsible for fermentations (*4*, *5*). NMR spectroscopy of wine has proved to be useful for evaluating the quality of wine, in the verification of wine origin, age, and the effects of adulteration through the site-specific natural isotope fractionation−NMR method (*6*, *7*). The use of high-resolution NMR techniques in the study of wine has attracted the interest of several groups, and as a result, one- and two-dimensional (1D and 2D) NMR experiments have been explored to characterize and to classify a large quantity of wines (*8*−*15*) and grape berries (*16*−*18*). Clark and co-workers have shown the potential of ^1H NMR as a valuable tool in monitoring a commercial fermentation (*19*). On the other hand, some reports used intensities from the ^1H NMR spectra for the quantification of several components in wine (*20*−*22*), and we have used quantitative ^1H NMR for monitoring and controlling several biological processes such as alcoholic and malolactic fermentations (*23*, *24*). Recently, Hong and co-workers used ^1H NMR spectroscopy to investigate the metabolic differences in wines produced from different grape varieties and different regions (*25*), environmental vineyard conditions (*26*), and different lactic acid bacteria (*27*). Applying multivariate statistical analysis, metabolic changes in must during alcoholic fermentation produced by several yeast strains (*28*) and in wines to characterize malolactic fermentation have been investigated (*29*).

Along with ethanol, CO_2, and other minor compounds, fermenting cultures of yeasts produce many low molecular weight flavor compounds that have a strong impact on the quality of the product (*30*). Among these compounds, fusel alcohols (higher alcohols that contain more than two carbon atoms) together with their esters play a crucial role in wine aroma (*31*, *32*). Quantitatively, higher alcohols are the largest group of aroma compounds in alcoholic beverages and are mainly formed by yeast during a fermentative process from grape amino acids via the Ehrlich mechanism (*33*, *34*) (**Figure 1**).

In this way, L-phenylalanine, L-leucine, and L-isoleucine could be transformed into 2-phenylethanol (*35*), 3-methyl-1-butanol (isoamyl alcohol) (*36*), and (*S*)-2-methyl-1-butanol (active amyl alcohol) (*37*), respectively. Consequently, the amino acids that are present in must grape are the major source of higher alcohols (*38*). These alcohols play an important role in the sensory characteristics and quality of wines; therefore, the study of the amino acid profile could be related to the aroma profile of wine (*39*). Recently, several studies have shown the effects of the addition of different concentrations of some amino acids on the generation of aromas (*40*). In this field, some questions could be raised. Are higher alcohols directly generated from the amino acids of grapes? Does the amino acid rate of wines come solely from grapes? With these questions in mind and taking into account that the combination of NMR spectroscopy with the use of isotopically substituted molecules as tracers is a well-established protocol in microbiology (*41*), in this paper, we want to develop this methodology for monitoring the transformation of these amino

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Published on Web 04/06/2010

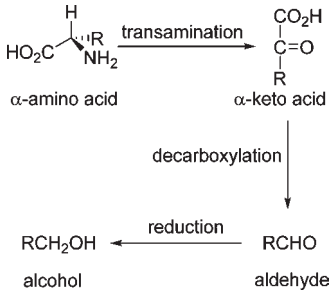

Figure 1. Transformation of amino acids into alcohols by the Ehrlich pathway.

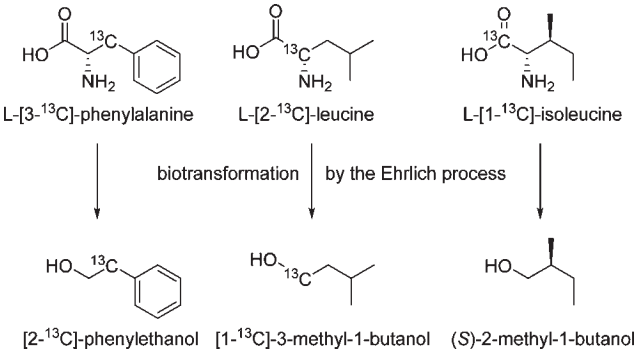

Figure 2. Amino acids and their transformations in alcohols studied in this work.

acids in higher alcohols during the alcoholic fermentation of a must, using both ^{1}H and ^{13}C NMR experiments. In this sense, we could determine the behavior of these substrates in the fermentation process using ^{13}C-labeled amino acids (**Figure 2**).

MATERIALS AND METHODS

Samples. Red grapes (Garnacha grape variety, *Vitis vinifera*) were manually collected in September 2006 in Tudelilla, La Rioja. The chemical composition of the must was as follows: sugar (density), 1.102 g/mL; total acidity, 3.33 g/L (H_2SO_4); pH, 3.24; malic acid, 1.45 g/L; and anthocyanes, 93.3 mg/L. Destemmed−crushed grapes were homogenized. Fermentations were carried out in a sterile Erlenmeyer flask kept in an incubator regulated at 25 °C with activated Uvaferm BC. Alcoholic fermentations were monitored by ^{1}H NMR using a quantitative method (*23*, *24*).

Alcoholic Fermentations. To follow the metabolic transformation of L-phenylalanine, L-leucine, and L-isoleucine during alcoholic fermentation, we added 50 mg of labeled amino acids into 500 mL of grape must to obtain a final concentration of 100 mg/L of L-[3-^{13}C]-phenylalanine (99% ^{13}C, sample A), L-[2-^{13}C]-leucine (99% ^{13}C, sample B), and L-[1-^{13}C]-isoleucine (99% ^{13}C, sample C), respectively. These concentrations are within the range in which amino acids occur in grapes. In all cases, the microvinification processes were carried out in the Erlenmeyer flask using 500 mL of grape juice with the corresponding labeled amino acids (samples A−C) and another one without labeled amino acids (control experiment). Moreover, another microvinification experiment was carried out with the three amino acids simultaneously (sample D). Alcoholic fermentation was carried out under optimal conditions of temperature (25 °C), and samples were collected at different time intervals (0, 8, 24, 32, 48, 56, 72, 96, 120, and 144 h). The alcoholic fermentations were monitored by ^{1}H NMR, and the ethanol signal at 1.17 ppm was initially observed at 32 h 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 the spectra was carried out with TOPSPIN software (version 1.2). Processing was performed with MestReMnova (version 5.2) (*42*). The spectrometer was locked onto D_2O in a mixture H_2O-D_2O (9:1), and all 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 (1D sequence with f1 presaturation) with pl9 (power level for presaturation) at 60 dB, and a flip angle of 90°. The experiments were carried out with automatic tuning and matching (ATM) and with GRADSHIM tools, using the NMR CASE as a NMR sample changer allowing the automatic analysis of several samples. Quantitative ^{1}H NMR was carried out using the methodology previously reported by us in the literature (*23*, *24*).

^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer equipped with a 5 mm probe (BBO BB-1H Z-GRD). An inverse-gated decoupled methodology was used (*43*): Proton decoupling was only applied during the acquisition period. In this case, no polarization transfer from ^{1}H to ^{13}C via NOE took place; therefore, the resulting ^{1}H-coupled ^{13}C spectrum was used for quantitative measurements.

Phase-sensitive gradient-enhanced 2D HSQC (heteronuclear single-quantum correlation) spectra were recorded by using hsqcedgpph pulse program (z filter and selection before t_1 removing the 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 1D files were exported into the MestReMnova program, and prior to carrying out Fourier transformation, an exponential window function was applied to obtain the optimal signal-to-noise ratio (*44*). 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 "Polynomial Fit" function in accordance with the literature (*44*). The signals integration, in both ^{1}H and ^{13}C NMR spectra, was manually carried out. The processing data were achieved twice. The metabolites were assigned by 2D NMR experiments, spiking experiments, and/or information published elsewhere (*23*, *24*).

RESULTS AND DISCUSSION

Three commercially available labeled amino acids with influence on the aromatic wine profile, L-phenylalanine, L-leucine, and L-isoleucine, were selected for this study. L-Phenylalanine is an important amino acid in grape juice and works as an important source of nitrogen for yeast. Its concentration range in wines, as reported in the literature, is 2.8−138 mg/L (*33*), and it is involved in the biosynthesis of important metabolites such as flavonoids and related compounds. This amino acid is also involved in the biosynthetic production of resveratrol by means of the phenylpropanoid pathway (*45*, *46*). Because of the interest in this compound, some groups have developed new yeast strains with the ability to produce resveratrol (*47*). In addition, L-phenylalanine is converted into 2-phenylethanol, which is responsible for the roselike aroma of wines (*48*, *49*). On the other hand, L-leucine is an essential amino acid for humans, and the concentration range in wines reported in the literature is 2−160 mg/L (*33*). L-Leucine is transformed following the Ehrlich mechanism into isoamyl alcohol, which provides whiskey, alcohol, harsh, cheese, or herbaceous odors (*48*). Finally, L-isoleucine is also an essential amino acid for humans, and its concentration range in wines is 1−117 mg/L (*33*). L-Isoleucine is transformed, following the Ehrlich mechanism, into active amyl alcohol, which provides winelike and alcohol odors (*48*).

In addition to ^{1}H NMR experiments that allow us to obtain the alcoholic degree value according to our recently published method (*24*), ^{13}C NMR experiments were performed to address the monitoring of ^{13}C-labeled amino acids. Previously, ^{13}C NMR experiments were carried out with the amino acids to identify the chemical shifts for the ^{13}C-labeled atoms, setting at 39.2 ppm for L-[3-^{13}C]-phenylalanine, at 56.2 ppm for L-[2-^{13}C]-leucine, and at 176.8 ppm for L-[1-^{13}C]-isoleucine. The ^{13}C NMR spectra at the initial time (0 h) for all samples showed several peaks corresponding to principal compounds with natural abundance carbons (sugars, organic acids, etc.) along with the signals corresponding to ^{13}C-labeled L-phenylalanine in sample A, ^{13}C-labeled L-leucine for sample B, and ^{13}C-labeled L-isoleucine for sample C

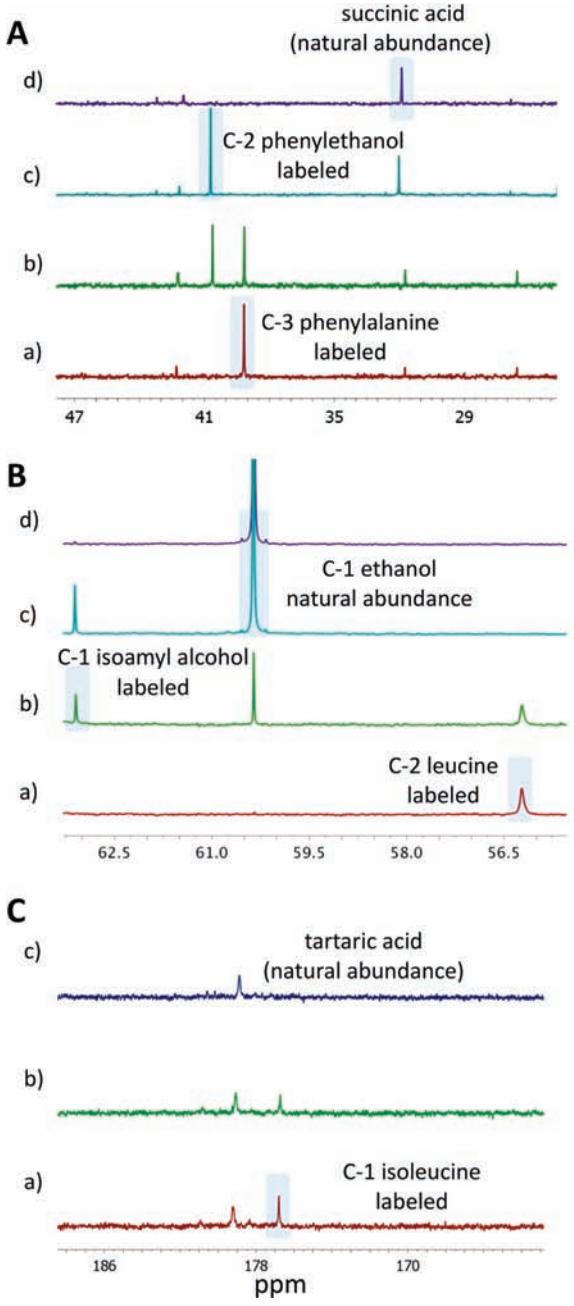

Figure 3. ^{13}C NMR spectra for monitoring the transformations of amino acids in higher alcohols. (**A**) L-Phenylalanine: sample A at (a) 0, (b) 48, and (c) 144 h and (d) control sample at 144 h. (**B**) L-Leucine: sample B at (a) 0, (b) 48, and (c) 144 h and (d) control sample at 144 h. (**C**) L-Isoleucine: sample C at (a) 0, (b) 32, and (c) 72 h. The vertical scaling is the same for all spectra.

(**Figure 3**). Obviously, these ^{13}C-labeled signals are missing in the control sample spectrum at the same time.

These signals kept their intensities for the first 32 h in samples A and B and for the first 24 h in sample C, and when the alcoholic fermentations began, the intensity of these signals was quickly reduced. At 48 h, in samples A and B, we could observe two new signals, which were not observed in the spectra of the control samples at the same time (**Figure 3**). These signals at 40.6 ppm for sample A and at 63.1 ppm for sample B were assigned to the C-2 of 2-phenylethanol and to the C-1 of isoamyl alcohol, respectively. To corroborate this assignment, we compared these values with those obtained in ^{13}C NMR experiments carried out on commercially available 2-phenylethanol and isoamyl alcohol. In

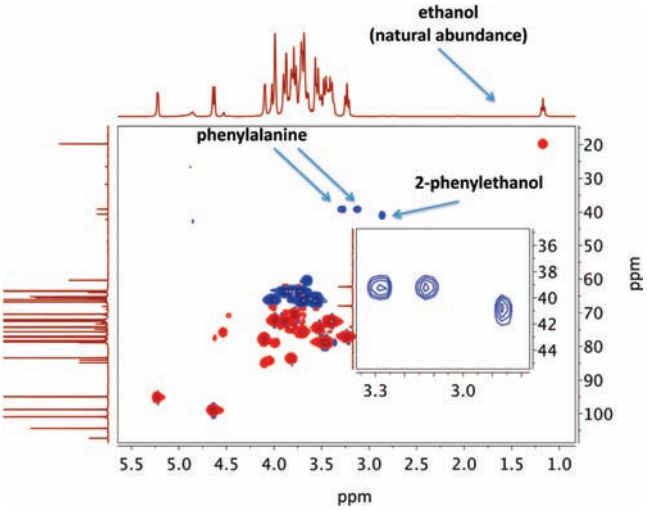

Figure 4. HSQC edited (blue cross-peaks are CH_2, and red cross-peaks are CH or CH_3) for sample A at 48 h.

addition, in the case of 2-phenylethanol, we confirmed the identification of this metabolite by 2D HSQC NMR experiments, which allowed us to obtain a 2D heteronuclear chemical shift correlation map between directly bonded $^{1}H-^{13}C$ atoms (**Figure 4**).

For sample A, in the spectrum at 144 h, we were able to observe the total disappearance of the representative L-[3-^{13}C]-phenylalanine signal and the complete transformation to labeled [2-^{13}C]-phenylethanol (**Figure 3**) among the major products in natural abundance such as ethanol, succinic acid, acetic acid, malic acid, etc. A comparable situation showed the spectrum of sample B at 144 h, the disappearance of the representative leucine signal, and the presence of ^{13}C-labeled isoamyl alcohol. In sample C, the amino acid L-[1-^{13}C]-isoleucine is labeled at the carboxylic group, and through the Ehrlich process, this carbon is transformed to afford $^{13}CO_2$, and as a result, active amyl alcohol is not detected in this experiment. However, at 72 h, we were able to observe the lack of the representative isoleucine signal and only the presence of tartaric acid in natural abundance (**Figure 3**) among the major products in natural abundance such as ethanol, succinic acid, acetic acid, malic acid, etc.

When we observed the time−course evolution of the labeled metabolites together with the progress of the main parameters in wine such as ethanol (**Figure 5** as alcoholic degree), we noticed that the rapid consumption of labeled phenylalanine, leucine, and isoleucine corresponded to the beginning of alcoholic fermentation. In this context, leucine and isoleucine are consumed when the alcoholic degree reaches nearly 1% (v/v), and phenylalanine is consumed when the alcoholic degree is close to 3% (v/v). As a result, it is important to notice that phenylalanine, leucine, and isoleucine are completely consumed in the first stage of the alcoholic fermentation. Moreover, and in a concomitantly way, the disappearance of labeled phenylalanine and leucine agreed with the emergence of 2-phenylethanol and isoamyl alcohol, respectively. No other metabolites associated with the Ehrlich mechanism (α-keto acids and/or aldehydes derived from L-[3-^{13}C]-phenylalanine, L-[2-^{13}C]-leucine, and L-[1-^{13}C]-isoleucine) were detected in any spectra. Metabolites produced by catabolic processes of other amino acids were not found. In this way, the presence of phenylethylamine from the decarboxylative process of the amino acid was not observed, consistent with the fact that biogenic amines are produced in malolactic fermentation and not in alcoholic fermentation (*50*).

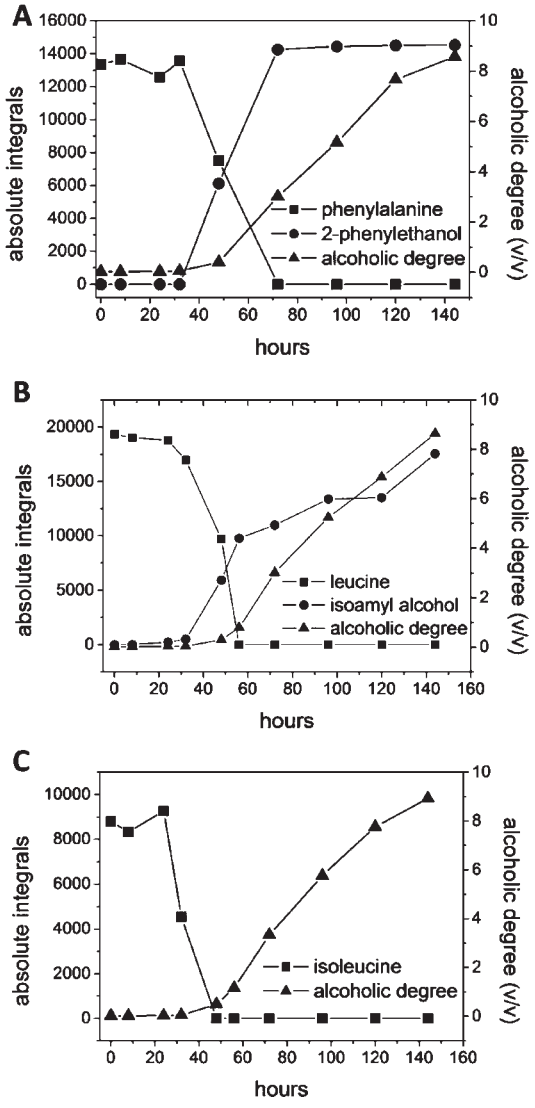

Figure 5. Time−course evolution of amino acids, higher alcohols (by ^{13}C NMR), and alcoholic degree (by ^{1}H NMR). (**A**) Phenylalanine, 2-phenylethanol (absolute integrals), and alcoholic degree (v/v) for sample A. (**B**) Leucine, isoamyl alcohol (absolute integrals), and alcoholic degree (v/v) for sample B. (**C**) Isoleucine (absolute integrals) and alcoholic degree (v/v) for sample C.

On the other hand, sample D (fermentation carried out with the three amino acids) shows a similar pattern to the other samples with a single amino acid; nevertheless, fermentation occurs slightly more rapidly due to the increase of the total nitrogen content.

In conclusion, NMR is becoming an indispensable tool to follow the catabolic pathway of amino acids during the alcoholic fermentation of wine. In the present study, three amino acids, phenylalanine, leucine, and isoleucine, are completely consumed in the early stages of alcoholic fermentation. Moreover, we have established that the phenylalanine and leucine amino acids are transformed into the corresponding higher alcohols during alcoholic fermentation through the Ehrlich process. Therefore, amino acids originating from grapes can affect higher alcohols in wine during wine fermentation. In addition, we could conclude that the amino acids studied in this work, which were present before fermentation, were completely consumed, and as a result of this, the amino acids found in wine could come from other pathways. In future works, we hope to expand this study to additional amino acids and other metabolites to provide and understand the behaviors of these compounds in wine-making processes including the alcoholic and malolactic fermentations.

ACKNOWLEDGMENT

We thank Belén Ayestarán (Universidad de La Rioja) for providing the samples of must.

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Received for review September 16, 2009. Revised manuscript received February 16, 2010. Accepted March 29, 2010. E.L.-R. thanks the Universidad de La Rioja for a predoctoral grant.