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Content of Biogenic Amines in a Chardonnay Wine Obtained through Spontaneous and Inoculated Fermentations

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This paper describes the content of biogenic amines in wines obtained from a Chardonnay must inoculated with different strains of *Saccharomyces cerevisiae* and in a wine fermented with the indigenous yeasts (control wine). The concentrations of nonvolatile amines and phenethylamine in the wines from the inoculated must were superior to those of the control wine. This was probably due to the fact that consumption of the precursor amino acids of these amines, during fermentation, was also greater in the inoculated samples than in the control sample. Furthermore, from the results obtained it may be said that, at least to some extent, the nonvolatile amines were formed by yeasts during fermentation. The concentrations of dimethylamine, ethylamine, and pyrrolidine (volatile amines) were different for the different wines, although they did not reach concentrations sufficiently high to have any effect on the aroma.

KEYWORDS: Biogenic amines; amino acids; *Saccharomyces cerevisiae* strains; white wine

INTRODUCTION

Biogenic amines are found in wines in variable quantities, and their origin is diverse. Nonvolatile amines and phenethylamine, which is a volatile amine, have their origin in the microbial decarboxylation of amino acids (*1*). Histidine, tyrosine, lysine, and phenylalanine are the precursor amino acids of histamine, tyramine, cadaverine, and phenethylamine respectively; the polyamines putrescine, spermine, and spermidine can be synthesized from ornithine and arginine amino acids (*2*). Volatile amines, with the exception of phenethylamine, can conceivably come from the amination of non-nitrogen compounds such as aldehydes and ketones (*3*).

The presence of high concentrations of histamine, tyramine, and phenethylamine in wine has been related to dietary migraines (*4*−*6*). Putrescine and cadaverine, although not toxic in themselves, intensify the adverse effects of the above-mentioned amines as they interfere with the enzymes that metabolize them (*2*, *7*). Secondary amines (spermine, spermidine, dimethylamine, pyrrolidine, etc.) can react with nitrous acid and its salts to form nitrosamines, compounds of known carcinogenic action (*6*, *8*). On the other hand, volatile amines can have an influence on wine aroma. Due to the acidic pH of wine these amines occur as odorless salts, but in the mouth they are partially liberated and their flavor becomes apparent (*9*).

Several factors affect the content of biogenic amines in wine, such as the kind of soil and nitrogen fertilizer, degree of maturation of the grape, elaboration method of the wine, growth of lactic acid bacteria, residual microbial populations, enological treatments, and yeast strain responsible for fermentation (*10*, *11*). The study of the aminogenic activity of different strains of *Saccharomyces cerevisiae* is important, because this species is widely used in the elaboration of wine in the form of dry active yeast. There have been only a few studies on the influence of yeast on the formation of amines, and they quantified only histamine and compared different species of yeast (*12*−*14*), but not different yeasts belonging to the same species. It is also important to study the relationship between biogenic amines and their precursor amino acids, to get a more thorough understanding of the nitrogen metabolism of yeast and its consequences on the quality of wine. In a recent study (*11*) the concentration of biogenic amines in rosé wines made from a garnacha must inoculated with three commercial yeast strains of *S. cerevisiae* was described. A rosé wine proceeding from the spontaneous fermentation of must was used as a control. In that study it was observed that the rosé wines showed differences in the concentration of amines depending on the inoculated yeast strain. This paper studies the aminogenic activity of the same three yeasts strains of *S. cerevisiae* in the fermentation of a Chardonnay must and the relationship between the content of biogenic amines in the final white wines and the consumption of their precursor amino acids during fermentation. The results are compared with a Chardonnay must fermented by indigenous yeasts.

MATERIALS AND METHODS

Samples and Wine-Making. The must used was *Vitis vinifera* var. *chardonnay*, which had 190 nephelometric units of turbidity (NTU). After treatment with SO_2 (80 mg/L), the must was divided into eight

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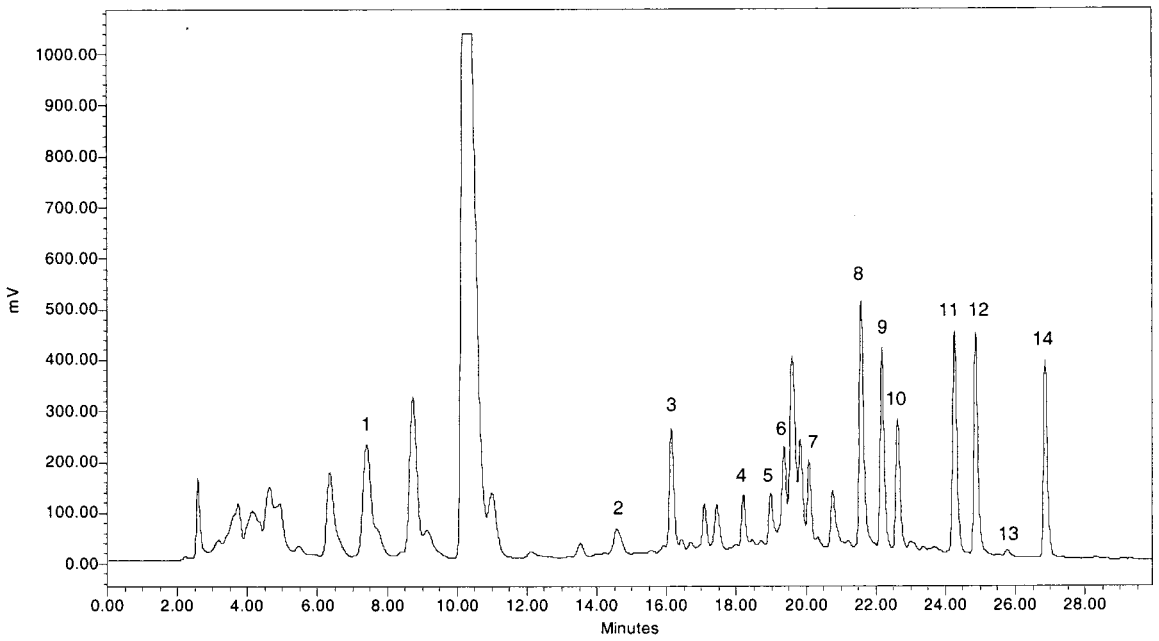

Figure 1. Example chromatogram of a white wine with the addition of 1 mg/L of each biogenic amine. This chromatogram has been processed with the analytical procedure described. Peaks: (1) histamine; (2) dimethylamine; (3) ethylamine; (4) pyrrolidine; (5) isopropylamine; (6) tyramine; (7) diethylamine; (8) putrescine; (9) isobutylamine; (10) cadaverine; (11) phenethylamine + spermidine; (12) amylamine; (13) spermine; (14) hexylamine.

aliquots of 5.0 L. Six aliquots were inoculated with active dry yeast *S. cerevisiae* var. *cerevisiae* (two with Na33 strain, two with ICV D47 strain, and two with ICV K1M strain). The two remaining aliquots were exclusively fermented by indigenous yeasts (control sample). Na33 strain was selected by the Estación de Viticultura y Enología de Navarra (a government research center) from must originating from the Navarra region (northern Spain). With regard to killer character, this strain possesses a neutral phenotype. ICV D47 and ICV K1M strains were selected by the Department of Microbiology from Montpellier Institut Coopératif du Vin (ICV). ICV D47 strain comes from the French region of Côtes du Rhône, whereas ICV K1M comes from the French region of Languedoc; these two strains show killer K2 phenotype. Na33, ICV D47, and ICV K1M strains were commercialized by Lallemand (Madrid, Spain). Strains were inoculated in the must in a proportion of 0.2 g of active dry yeast/L of must. To do this, 1.25 g of dry yeast was rehydrated in a sterile flask in 12.5 mL of distilled water with 0.125 g of sucrose (number of viable cells per gram $\geq 2 \times 10^9$); it was kept in this medium for 30 min at 35 °C. The must was inoculated with mixing in order to get a homogeneous distribution. Fermentations were carried out in modular bioreactors of 5.0 L (Gallenkamp, Leicestershire, U.K.) at a controlled temperature of 20 ± 2 °C. The recently obtained wines were racked off the lees and did not undergo any malolactic fementation.

Polymerase Chain Reaction (PCR). This technique was used to identify Na33, ICV D47, and ICV K1M strains and check their predominance in the fermentation of inoculated musts. To do so, 5 mL samples of must were taken in the last phase of fermentation (density = 1.02 g/mL) and in the wine obtained. These samples were centrifuged at 5000 rpm for 3 min, the supernatant was eliminated, and the sediment was resuspended in 5 mL of sterilized water. It was centrifuged again, and the sediment was mixed with 1 mL of glycerol at 30% v/v for keeping at −40 °C. PCR analyses were carried out in the Sigmo Laboratory of Nantes (France). The method used was that of Ness et al. (*15*). In this method, using customized oligonucleotides, some regions of the yeast genome between δ elements are amplified to give an "amplified sequence polymorphism" characteristic of the strains. According to the PCR analysis, Na33, D47, and K1M strains predominated over indigenous yeasts in all of the inoculated samples.

HPLC Analysis of Biogenic Amines and Amino Acids. Fifteen amines were studied (putrescine, cadaverine, histamine, tyramine, phenethylamine, spermidine, spermine, amylamine, hexylamine, dimethylamine, ethylamine, diethylamine, isopropylamine, isobutylamine, and pyrrolidine). The method used to determine biogenic amines is described in Torrea and Ancín (*11*) and is based on the method of Busto et al. (*16*). Samples were cleaned by ultrafiltration with a Millipore Ultrafree MC cartridge. Subsequently, precolumn derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) from AccQ·Fluor reagent kit (Waters) was carried out. Analyses of the derivatized amines were performed with a Waters high-pressure liquid chromatograph (Waters, Milford, MA) equipped with two 510 pumps, a 717 Plus autosampler, and a 474 fluorescence detector, using 250 and 395 nm as excitation and emission wavelengths, respectively. Maxima 820 software was employed for chromatographic control. The amount of sample injected was 10 μL. A reversed phase column (300 mm × 3.9 mm i.d.) was used, with a stationary phase of dimethyloctadecylsilyl bonded to amorphous silica. The column was set at 65 °C. The compositions of the mobile phases were as follows: phase A, solution of sodium acetate trihydrate (140 mM) (Scharlau, Barcelona, Spain) and triethylamine (17 mM) (Aldrich, Madrid, Spain), with pH adjusted to 5.05 with phosphoric acid (85%) (Merck, Darmstadt, Germany); phase B, methanol (Scharlau). The program used was as follows: initial isocratic elution at 80% phase A and 20% phase B for 5 min, followed by linear gradient elution from 20 to 80% phase B up to 25 min. Phenethylamine and spermidine could not be separated, and they were quantified as a single peak. As an example, the chromatogram of a white wine with the addition of 1 mg/L of each biogenic amine, analyzed under the conditions discussed above, is shown in **Figure 1**. Standards were added to allow for a better visualization of the peaks on the chromatogram.

Analysis of amino acids was performed with a Waters high-pressure liquid chromatograph, using a 486 UV−vis detector at 254 nm. The Pico·Tag method used is described in Ancín et al. (*17*).

Enological Parameters. Enological parameters are described by the Office International de la Vigne et du Vin (*18*).

Results shown in the tables are the arithmetic mean of six replicates, because the experiments were carried out in duplicate and three analyses were made for each sample. Analysis of variance was used to assess the significance of the treatment means in the results of **Tables 1**, **2**, and **4**. Differences between treatment means were compared using the LSD at the 0.05 probability level. Results in **Table 3**, which arise from the differences in the data obtained from must and from wine, are given with their 95% confidence interval.

Table 1. Enological Parameters of Wines[a]

	control	Na33	D47	K1M
pH	3.62 a	3.47 b	3.58 c	3.54 d
total acidity[b] (g/L)	4.1 a	4.5 b	5.0 c	4.5 b
volatile acidity[c] (g/L)	0.90 a	0.18 b	0.55 c	0.38 d
ash (g/L)	2.3 a	2.1 a	2.3 a	1.6 a
alcohol (v/v %)	11.3 a	12.7 b	12.3 c	12.2 c

[a] Means within the same row followed by different letters are significantly different ($p < 0.05$). [b] As g/L tartaric acid. [c] As g/L acetic acid.

Table 2. Concentration of Nonvolatile Amines (Micrograms per Liter) in Wines[a]

	control	Na33	D47	K1M
putrescine	3074 a	3745 b	3910 b	3888 b
spermine	766 a	1482 b	1137 c	1575 b
Phe + Spd[b]	2070 a	2896 b	2485 c	2518 c
histamine	210 a	362 b	309 c	360 b
tyramine	<180	<180	<180	<180
cadaverine	<120	<120	<120	<120

[a] Means within the same row followed by different letters are significantly different ($p < 0.05$). [b] Phenethylamine and spermidine.

RESULTS AND DISCUSSION

Enological Parameters of Wines. The pH of the wines (**Table 1**) was found to be close to the superior limit of the range (3.1−3.6) recommended by Amerine and Ough (*19*) to obtain a wine with good organoleptic characteristics and safe from any contaminating bacteria. The volatile acidity from wines coming from inoculated must was <0.6 gHAc/L, a limit given by Peynaud (*20*) so that the sensorial quality is not altered to any great extent. In the control wine, the value of volatile acidity was above this limit. Alcoholic degree was lower in the control wine than in the other wines (**Table 1**).

Concentration of Nonvolatile Amines in Wines. These results are shown in **Table 2**. The pair phenethylamine + spermidine will be discussed in this section despite the fact that phenethylamine is a volatile amine. Tyramine and cadaverine concentrations were below that of the method detection limit in all samples (180 μg/L for tyramine and 120 μg/L for cadaverine).

Putrescine was the most abundant amine in all wines (**Table 2**). This result coincides with that of other authors, who have found that this amine is the major one in must (*21*, *22*) and in wine (*9*, *23*, *24*). The concentration of putrescine was lower in the control wine than in the wines coming from the inoculated must. In the inoculated samples this amine showed a similar concentration (**Table 2**). During the fermentation of the control sample, the consumption of arginine, the precursor amino acid of putrescine, was inferior to that observed in the inoculated samples (**Table 3**). Consequently, the lower concentration of putrescine in the control wine could be related to the low consumption of arginine, its precursor, during fermentation. In the inoculated samples the consumptions of arginine were similar in all cases. Ornithine, also a precursor of putrescine, was excreted during fermentation in all of the samples (**Table 3**).

The concentrations of spermine and phenethylamine + spermidine were lower in the control wine than in the wines fermented by selected yeasts (**Table 2**). There were differences between the inoculated samples, depending on the yeast that predominated during fermentation. The concentration of spermine was lower in the wine fermented by D47 yeast than in the other two wines; the concentrations of phenethylamine + spermidine were similar in the wines fermented by D47 and K1M strains and lower than those found in the wine fermented by the NA33 strain. Arginine and phenylalanine were consumed in a higher quantity during the fermentation of the inoculated must than during the fermentation of the control sample (**Table 3**). Consequently, just as occurred with putrescine, the lower concentration of spermine and phenethylamine + spermidine in the control wine would be related to the low consumption of their precursor amino acids. In the inoculated samples, although there were differences in the concentration of these amines, the consumptions of arginine and phenylalanine were similar in all of them (**Table 3**). Although the toxicological limits of phenethylamine are not known with precision, Soufleros et al. (*24*) stated 3 mg/L as the limit beyond which the wine might provoke negative physiological effects. Therefore, according to this value, our wines would not present toxicological problems due to phenethylamine, because the sum of phenethylamine and spermidine never surpassed this value.

Table 3. Use of Amino Acids during Fermentation (+ Utilization, − Excretion) and Concentrations (Milligrams per Liter) in Must (M) and Wines (W)[a]

	control	Na33	D47	K1M
arginine	+114 ± 22 M: 422 W: 308	+390 ± 13 M: 422 W: 32	+393 ± 13 M: 422 W: 29	+397 ± 13 M: 422 W: 25
ornithine	−2.6 ± 0.4 M: 1.9 W: 4.5	−1.1 ± 0.3 M: 1.9 W: 3.0	−7 ± 1 M: 1.9 W: 8.9	−3.3 ± 0.4 M: 1.9 W: 5.2
phenylalanine	+5 ± 1 M: 18 W: 13	+14 ± 1 M: 18 W: 4	+13 ± 1 M: 18 W: 5	+15 ± 1 M: 18 W: 3
histidine	−8 ± 4 M: 45 W: 53	+40 ± 2 M: 45 W: 5	+33 ± 2 M: 45 W: 12	+40 ± 2 M: 45 W: 5
tyrosine	+5 ± 1 M: 18.2 W: 13.2	+16.6 ± 0.8 M: 18.2 W: 1.6	+11.7 ± 0.9 M: 18.2 W: 6.5	+16.3 ± 0.8 M: 18.2 W: 1.9
lysine	−1.8 ± 0.3 M: 2.0 W: 3.8	−3.5 ± 0.6 M: 2.0 W: 5.5	−4.0 ± 0.6 M: 2.0 W: 6.0	−1.4 ± 0.4 M: 2.0 W: 3.4

[a] Results are given with their 95% confidence intervals.

Histamine was found at a low concentration in all wines, and its content was higher in the wines from the inoculated must than in the control wine (**Table 2**). In the inoculated samples the concentration of histamine was lower in the wine fermented by D47 strain than in the other two wines. Histidine, the precursor amino acid of histamine, was consumed during fermentation in the inoculated samples, whereas in the control sample it was excreted (**Table 3**). It is likely that this difference in the use of histidine resulted in different concentrations of histamine depending on whether the must had been inoculated. In the inoculated samples the consumption of histidine was lower in the D47 sample than in the other two samples (**Table 3**). Just as occurred with phenethylamine, the toxic dose of histamine is difficult to establish, because its toxicity depends on the effectiveness of the diamine oxidase enzyme (DAO), the activity of which varies considerably among different individuals (*2*). On this point, authors simply recommend intervals of concentration that should not be surpassed. Daeschel (*25*) and Soufleros et al. (*24*) considered that wines with concentrations of histamine between 8 and 20 mg/L showed toxic effects if they were consumed in important quantities. All

Table 4. Concentration of Volatile Amines (Micrograms per Liter) in Wines[a]

	control	Na33	D47	K1M
amylamine	<100	<100	<100	<100
hexylamine	<100	<100	<100	<100
dimethylamine	245 a	235 a	192 b	189 b
ethylamine	680 a	1000 b	930 c	812 d
diethylamine	<125	<125	<125	<125
isopropylamine	<180	<180	<180	<180
isobutylamine	<120	<120	<120	<120
pyrrolidine	832 a	331 b	239 c	249 c

[a] Means within the same row followed by different letters are significantly different ($p < 0.05$).

of the wines analyzed in our study showed concentrations of histamine much lower than those levels considered to be dangerous (**Table 2**). Tyramine was not detected in any wine (**Table 2**), despite the fact that its precursor, tyrosine, was consumed during fermentation (**Table 3**). Cadaverine was not detected either in any wine (**Table 2**), and its precursor, lysine, was excreted in all samples during the fermentation process (**Table 3**).

In vinification of garnacha must inoculated with the same three yeasts (*11*), the wine fermented with the Na33 strain showed a lower concentration of biogenic amines than the control sample, and wines obtained with D47 and K1M killer strains showed the highest concentrations of these substances. However, in vinification of white wine the control sample always showed concentrations of biogenic amines lower than those of the inoculated wines. One difference between white and rosé vinification is that in the former there does not exist any contact between must and skin, so that the yeast population is usually lower. This was probably the reason the nitrogen metabolism in the uninoculated Chardonnay must was slowed. That is to say, few amino acids were consumed and few biogenic amines were produced. Consequently, from these data it may be assumed that yeast formed nonvolatile amines during fermentation.

Concentration of Volatile Amines in Wines. Table 4 shows the concentrations of volatile amines in wines from both the inoculated must and the uninoculated must. From among all volatile amines analyzed, only dimethylamine, ethylamine, and pyrrolidine were present in all wines at a level above the detection limit for this method. The concentrations of these three amines were low, and some difference could be found depending on the yeast involved in the fermentation. The content of dimethylamine was slightly inferior in the wines inoculated with D47 and K1M strains. Ethylamine was present in a slightly higher concentration in the inoculated samples than in the control sample. However, pyrrolidine was found at a higher concentration in the control sample than in the inoculated ones. Given that volatile amines do not come from the microbial decarboxylation of amino acids, the differences in their concentrations were probably due to their different use by the yeasts as sources of nitrogen.

In the literature search we have not found anything on the levels of sensorial impact of volatile amines in wine. In one of the few studies carried out on alcoholic beverages, Palamand et al. (*26*) observed a negative effect on the aroma of beer with 50 μg/L dimethylamine and 2000 μg/L ethylamine. In our samples ethylamine was <2000 μg/L in all cases, whereas the level of dimethylamine was somewhat >50 μg/L in all of the wines. It is likely that the sensorial effect of volatile amines in our wines was low. This assumption comes from the fact that the threshold levels of organoleptic impact on wine are higher than those of beer, due to the greater content of ethanol and other volatile compounds such as esters, terpenes, and higher alcohols (*27*). The low concentrations of dimethylamine and pyrrolidine, and the absence of diethylamine are also important from the point of view of the health of the consumer, because these three amines can react with nitrites and produce nitrosamines, compounds of a known carcinogenic action (*6*). The results of volatile amines coincide with those found in rosé wine fermented by the same yeast strains used in this present study (*11*), and the levels of these amines were also very low and varied slightly depending on the yeast strain involved in fermentation.

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