

## Formation of Biogenic Amines throughout the Industrial Manufacture of Red Wine

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### ABSTRACT

Changes in biogenic amines (histamine, methylamine, ethylamine, tyramine, phenylethylamine, putrescine, and cadaverine) were monitored during the industrial manufacture of 55 batches of red wine. The origin of these amines in relation to must, alcoholic fermentation, malolactic fermentation, sulfur dioxide addition, and wine aging and the interactions between amines and their corresponding amino acids and pH were statistically evaluated in samples from the same batches throughout the elaboration process. Some amines can be produced in the grape or the musts (e.g., putrescine, cadaverine, and phenylethylamine) or can be formed by yeast during alcoholic fermentation (e.g., ethylamine and phenylethylamine), although quantitatively only very low concentrations are reached in these stages (less than 3 mg/liter). Malolactic fermentation was the main mechanism of biogenic amine formation, especially of histamine, tyramine, and putrescine. During this stage, the increase in these amines was accompanied by a significant decline in their amino acid precursors. Significant correlations between biogenic amine formation and the disappearance of their corresponding amino acids were observed, which clearly supports the hypothesis that malolactic bacteria are responsible for accumulation of these amines in wines. No increase in the concentration of biogenic amines was observed after SO<sub>2</sub> addition and during wine aging, indicating that sulfur dioxide prevents amine formation in subsequent stages.

Wine is an ideal substrate for amine production because its manufacturing process involves available free amino acids, the possible presence of decarboxylase-positive microorganisms, and some favorable environmental conditions (e.g., external pH) that affect the growth of microorganisms and the activity of decarboxylase enzymes (15). The presence of small amounts of biogenic amines in wine and other fermented foods is not usually considered to be a serious health risk (26). Under normal conditions, exogenous amines ingested as part of the diet are absorbed from the food and quickly detoxified in the organism by amine oxidases or through conjugation. However, when normal catabolic routes of amines are inhibited or the amount ingested is large, several physiological changes can occur, such as migraine headaches, nausea, hypo- or hypertension, cardiac palpitations, and anaphylactic shock (25, 26). Histamine and tyramine are the main causes of numerous cases of food intoxication; other amines such as putrescine, cadaverine, and phenylethylamine are also important because they may intensify the undesirable effects of histamine (28). Hence, knowledge of the concentrations of biogenic amines in wines is necessary to assess the health hazards that may arise from drinking wines. Amines are also important in wine from an economical point of view, because they could cause problems in commercial transactions (import and export). Switzerland rejects wines that contain more than 10 mg/liter, and lower maximums have been recommended in

Germany (2 mg/liter), Belgium (5 to 6 mg/liter), and France (8 mg/liter) (2, 13).

Amines in wine may have two different sources; the grapes and other raw materials and the fermentation processes. However, data are complex, and published results are contradictory. In several studies, biogenic amines have been suggested as indicators of hygienic quality or manufacturing practices (30). Some amines such as putrescine and cadaverine are associated with poor sanitary conditions of grapes (14). Several researchers have found that biogenic amines are formed by yeast during alcoholic fermentation (6, 29). Many authors have reported that during malolactic fermentation (MLF), lactic acid bacteria actively produce biogenic amines (12, 16, 22), but other authors have not reported this production (5). Amino acids are the sequential precursors of amines, and as a consequence the higher the content of free amino acids, the higher the probability of biogenic amine production.

In an attempt to prevent the formation of biogenic amines in food in general or in wine in particular, it is necessary to understand the changes that occur in amines at each stage throughout the production of the product on an industrial scale. A structured study was carried out during the industrial elaboration of 55 batches of red wine to investigate the origin and evolution of biogenic amines during alcoholic fermentation (AF), MLF, and a 12-month aging period. Five different Spanish wineries provided 241 samples from two different vintages. Given that the concentration of biogenic amines in wines depends at least partially on the free amino acid content and some extrinsic factors such as pH, we also studied the changes in some of

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the corresponding precursor amino acids and in the pH of the wines.

## MATERIALS AND METHODS

**Winemaking and sample collection.** Red wines were industrially elaborated in five Spanish wine-producing cellars. The samples were taken from 55 stainless steel tanks capable of holding 15,000 liters of wine (28 tanks from the 2001 harvest and 27 from the 2002 harvest). AF was carried out by yeast under controlled temperatures. At the beginning of AF, SO<sub>2</sub> (approximately 100 mg/liter) was added to musts and more (approximately 40 mg/liter) was added just after completion of MLF.

To study the changes in biogenic amine content during vinification, we obtained a total of 241 samples from the 55 tanks or batches during the following stages of elaboration: 1, musts (17 samples); 2, just after AF (54 samples); 3, during MLF (42 samples); 4, just after MLF (44 samples); 5, after MLF and 5 days after addition of SO<sub>2</sub> (39 samples); 6, after 6 months of aging in oak barrels (30 samples); and 7, after 12 months of aging (6 months in oak barrels and another 6 months in bottles) (15 samples). The samples from stages 3 (during MLF) and 4 (just after MLF) correspond to wines with a 60% of initial L-malic acid content and an L-malic acid content of <0.1 g/liter, respectively.

At each sampling time, must and wine samples were collected and immediately frozen until analysis. Each assay was performed at least in duplicate.

**Determination of biogenic amines.** Biogenic amines were analyzed by reverse-phase (RP) high-pressure liquid chromatography (HPLC) according to the method described by Marcobal et al. (18). A liquid chromatograph consisting of a Waters 600 Controller programmable solvent module (Waters, Milford, Mass.), a WISP 710B autosampler (Waters), and an HP 1046-A fluorescence detector (Hewlett Packard, Palo Alto, Calif.) was used. Chromatographic data were collected and analyzed with a Millennium32 system (Waters). The separations were performed on a Waters Nova-Pak C18 column (150 by 3.9 mm inside diameter, 60 Å, 4 µm) with a matching guard cartridge of the same type. Samples were submitted to an automatic precolumn derivatization reaction with *o*-phthalaldehyde before injection. Derivatized amines were detected using the fluorescence detector (excitation wavelength of 340 nm and emission wavelength of 425 nm). Samples were filtered (0.45-µm-pore-size filter; Millipore, Bedford, Mass.) and then directly injected in duplicate onto the HPLC system. All reagents used were HPLC grade.

**Determination of amino acids.** Amino acids were analyzed by RP-HPLC using a liquid chromatograph (Waters). Samples were submitted to automatic precolumn derivatization with *o*-phthalaldehyde in the presence of 2-mercaptoethanol. Solvents and gradient conditions were as described by Moreno-Arribas et al. (21). Separations were performed on a Waters Nova-Pak C18 column (150 by 3.9 mm inside diameter, 60 Å, 4 µm) and the same type of precolumn. Detection was done with a fluorescence detector (HP 1046-A; λ excitation = 340, λ emission = 425). Millennium32 software was employed for chromatographic control and data acquisition.

**Determination of pH.** pH values were determined with a 601-I pH meter (Orion Research, Beverly, Mass.).

**Statistical methods.** The statistical methods used for the data analysis were a one-way analysis of variance with a Fisher least significant difference (LSD) range test for means comparison, Student's *t* test for paired samples, and Pearson and Spearman rank

TABLE 1. Summary statistics for the biogenic amines, amino acids, and pH in must and wine samples

Measure	Must samples (n = 17)					Wine samples (n = 224)				
	Mean	SD	Minimum	Maximum	No. (%) of positive samples	Mean	SD	Minimum	Maximum	No. (%) of positive samples
<b>Amine</b>										
Histamine	0.00	0.00	0.00	0.00	0	4.46	5.70	0.00	25.00	97 (43.7)
Methylamine	0.65	0.89	0.00	2.21	7 (41.2)	0.97	1.03	0.00	5.00	66 (29.5)
Ethylamine	1.27	0.83	0.00	2.23	13 (76.5)	2.02	1.99	0.00	7.92	37 (16.5)
Tyramine	0.00	0.00	0.00	0.00	0	3.13	4.42	0.00	19.00	151 (67.4)
Phenylethylamine	2.01	2.39	0.00	6.44	9 (52.9)	2.25	3.83	0.00	16.26	74 (33.0)
Putrescine	2.35	3.27	0.00	12.33	8 (47.1)	6.05	10.60	0.00	55.00	98 (43.8)
Cadaverine	2.58	3.90	0.00	12.90	9 (52.9)	2.02	3.36	0.00	14.00	114 (50.9)
<b>Amino acid</b>										
Histidine	23.38	5.42	12.99	33.23		4.60	4.71	0.00	22.49	
Tyrosine	21.39	6.02	10.78	31.88		3.99	3.20	0.00	14.12	
Arginine	119.0	67.92	53.98	278.2		30.10	33.23	0.00	210.2	
Ornithine	13.43	6.90	0.00	28.11		7.62	7.82	0.00	44.92	
pH	3.64	0.11	3.43	3.78		3.77	0.13	3.37	3.99	

correlation coefficients between biogenic amines and amino acids and pH. The STATISTICA program for Windows, version 7.1 was used for data processing (StatSoft, Inc., Tulsa, Okla., www.statsoft.com.).

## RESULTS AND DISCUSSION

**Biogenic amines and amino acids in musts and wines.** Table 1 gives the concentration of biogenic amines and amino acids in the samples of must and wine analyzed (mean, standard deviation, and minimum and maximum values). There is little information available concerning the presence of amines in musts. In contrast with previous studies in which histamine was detected before AF (4, 29), we did not find histamine or tyramine in any of the analyzed musts, even in musts rich in histidine. These results suggest that these amines are mainly fermentative. Only low concentrations of the other amines studied were detected; the most abundant were putrescine, cadaverine, and phenylethylamine, with mean concentrations of 2.35, 2.58, and 2.01 mg/liter, respectively. These results suggest that these amines are mainly associated with the grape or the must, in agreement with previous data (4). Ethylamine appeared in the largest number of must samples (76.5%), whereas phenylethylamine, cadaverine, putrescine, and methylamine were detected in only 52.9, 52.9, 47.1, and 41.2% of the must samples, respectively.

The highest mean concentrations of the biogenic amines in the wines were recorded for putrescine, histamine, tyramine, and phenylethylamine (in descending order of magnitude; Table 1). Tyramine, cadaverine, putrescine, and histamine were the most prevalent amines, detected in 67.4, 50.9, 43.8, and 43.7% of wine samples, respectively. The lowest concentrations were recorded for phenylethylamine, methylamine and ethylamine. These data are similar to those reported for other Spanish (12) and French (27) wines. Histamine has often been associated with intoxication of food (10). Wine, after fish and cheese, is probably one of the foods most commonly associated with this effect. Nevertheless, although no correlation has been established in wine between the concentration of biogenic amines and the appearance of clinical signs of intoxication, the highest histamine concentration in the batches of Spanish red wines analyzed here was below the threshold of toxicity suggested for other foods (2).

To acquire data on the factors that influence the quantity of amines in wines, the concentration of four amino acids (histidine, tyrosine, arginine, and ornithine) and the pH were also determined for the wines (Table 1). These amino acids are precursors of histamine (histidine), tyramine (tyrosine), and putrescine (arginine and ornithine), the major amines in the analyzed wines. Arginine was found in the highest concentrations in musts and wines, as expected, with mean concentrations of 119.0 and 30.10 mg/liter, respectively, followed by ornithine, with mean concentrations of 13.43 and 7.62 mg/liter, respectively. The lower concentrations were found for histidine (23.38 and 4.60 mg/liter, respectively) and tyrosine (21.39 and 3.99 mg/liter, respectively). The pH values ranged from 3.37 to 3.99.

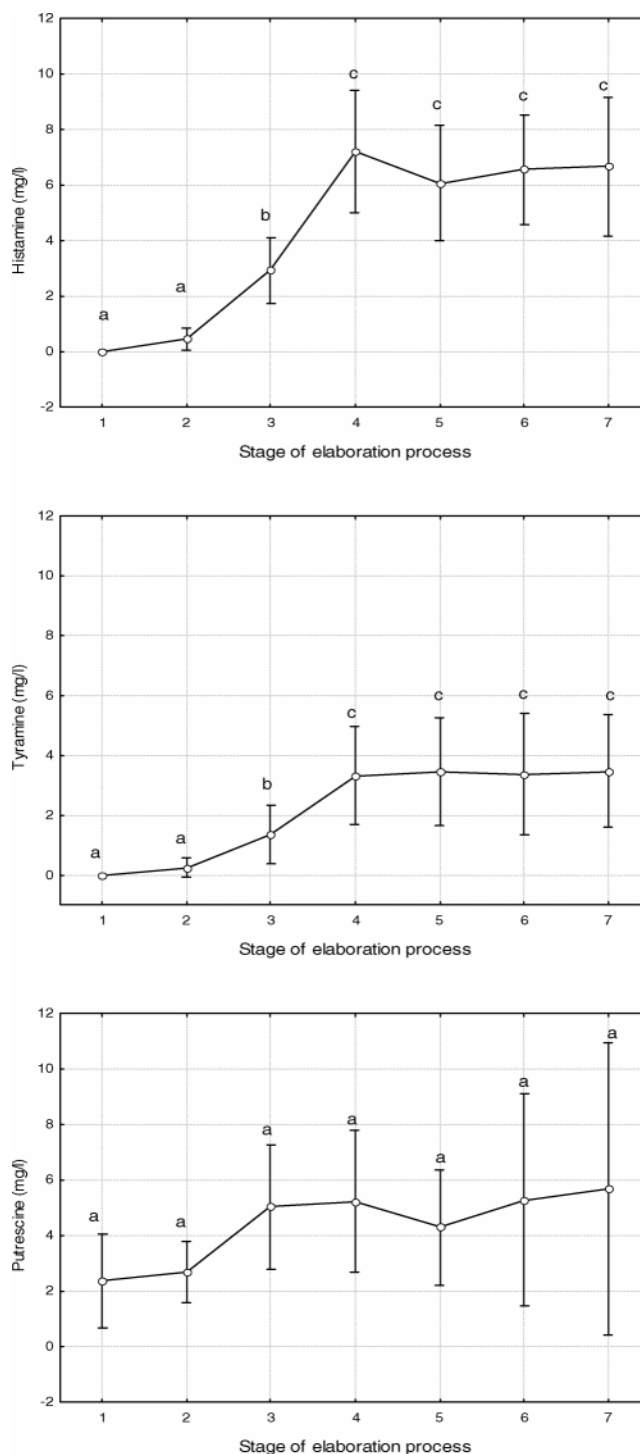


FIGURE 1. Means and 95% confidence intervals for concentrations of histamine, tyramine, and putrescine during the seven stages of the elaboration process: 1, must; 2, after AF; 3, during MLF; 4, just after MLF; 5, after MLF and addition of  $SO_2$ ; 6, after 6 months of aging; 7, after 12 months of aging. Means with the same letter are not significantly different according to Fisher's LSD test.

**Changes in biogenic amines during the winemaking process.** Figures 1 and 2 give the means and 95% confidence intervals for the concentrations of biogenic amines in wine samples at the seven different stages of the elaboration process (before and after AF, during MLF, after MLF,

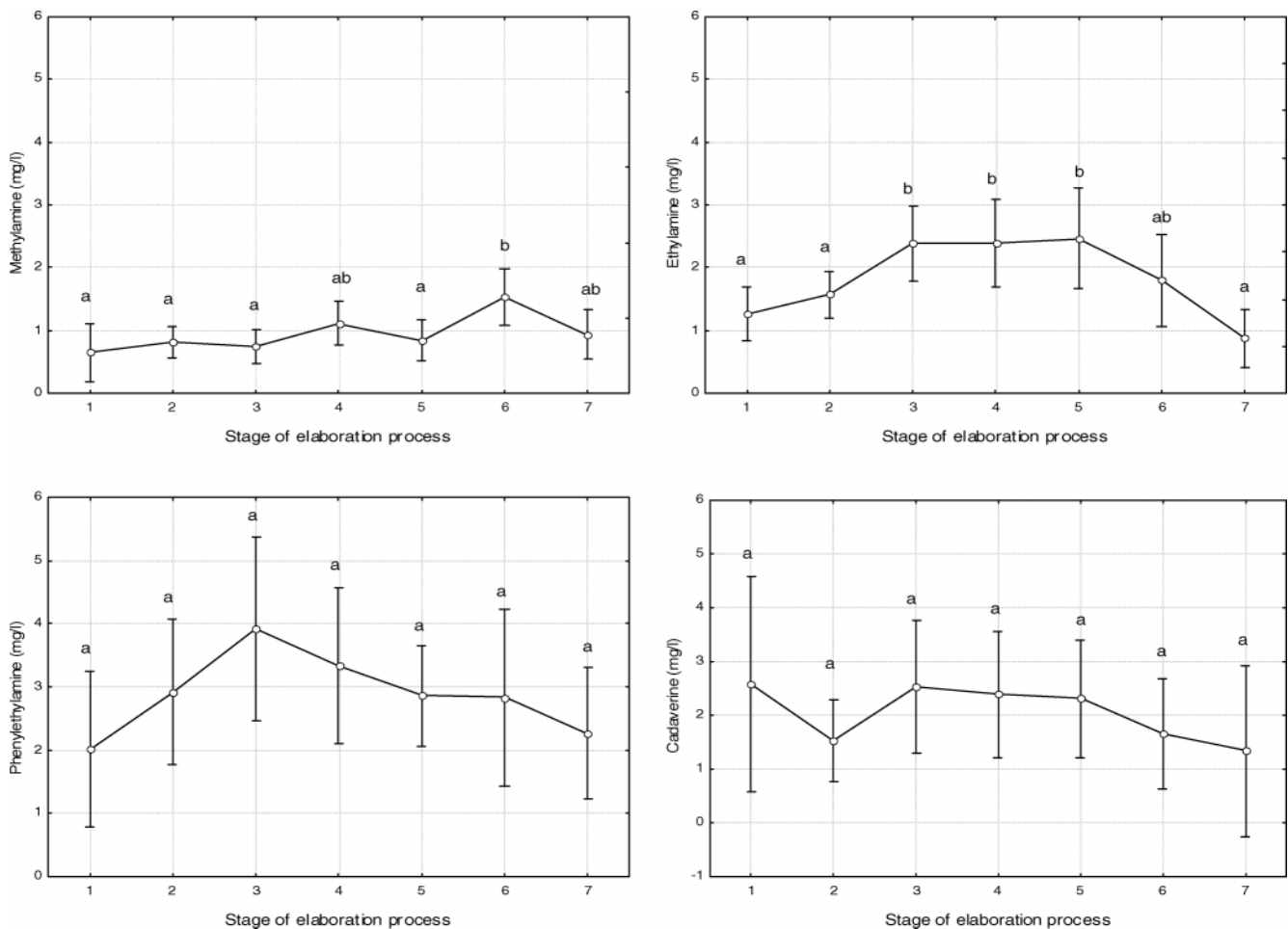


FIGURE 2. Means and 95% confidence intervals for concentrations of methylamine, ethylamine, phenylethylamine, and cadaverine during the seven stages of the elaboration process: 1, must; 2, after AF; 3, during MLF; 4, just after MLF; 5, after MLF and addition of  $\text{SO}_2$ ; 6, after 6 months of aging; 7, after 12 months of aging. Means with the same letter are not significantly different according to Fisher's LSD test.

after MLF and  $\text{SO}_2$ , and after 6 and 12 months of aging). A one-way analysis of variance was applied to compare the means for these seven stages. When significant differences among the means were found, Fisher's LSD multiple range tests were applied to determine which means were significantly different from which others (Figs. 1 and 2). Significant changes were observed for the concentrations of histamine and tyramine (Fig. 1) and methylamine and ethylamine (Fig. 2) throughout winemaking ( $P < 0.05$ ).

In general, for most of the samples no remarkable increases in the concentrations of biogenic amines were seen during AF (Figs. 1 and 2). The mean concentrations of amines reached after this step were less than 3 mg/liter (0.5 mg/liter for histamine, 0.8 mg/liter for methylamine, 1.6 mg/liter for ethylamine, 0.24 mg/liter for tyramine, 2.9 mg/liter for phenylethylamine, 2.7 for putrescine, and 1.5 mg/liter for cadaverine).

A more suitable evaluation of the changes that take place between two steps of the elaboration process can be obtained by considering the differences in the concentrations of amines in wines from the same batch. The results obtained are shown in Table 2 with the results of Student's *t* test for paired samples. Mean differences in amine con-

centrations for samples from the same 17 batches of wines taken before AF (stage 1, Table 2) and after AF (stage 2, Table 2) indicate that the only significant increases were recorded for phenylethylamine (3.64 mg/liter) and ethylamine (0.49 mg/liter) ( $P < 0.05$ ) during this stage. Other authors (3) also found increases in these amines after AF, although the concentrations detected were low, as are those in the present study. These results are consistent with those of previous studies, in which histamine, tyramine, or putrescine production was not observed in 50 yeast strains, including *Saccharomyces cerevisiae* and other non-*Saccharomyces* yeasts, but some of these yeasts varied in their capacity to form phenylethylamine (6). Therefore, yeast does not appear to be the origin of most of the amines found in wines.

Throughout MLF, a general increase was found in the concentrations of amine in the wines studied (Figs. 1 and 2). This increase was observed for most of the amines, especially histamine and tyramine in the samples taken in stage 3, i.e., during MLF. These findings indicate that the biosynthesis of decarboxylase amino acid enzymes can take place when the bacterial population is at the end of the growth phase and during the exponential phase, in agree-



TABLE 2. Mean differences in biogenic amine and amino acid concentration between different stages of the elaboration processes for wines from the same batches

Content	Differences (mg/liter) between stages <sup>a</sup> :				
	2 - 1 (n = 17)	4 - 2 (n = 44)	5 - 4 (n = 29)	6 - 5 (n = 20)	7 - 6 (n = 14)
<b>Amine</b>					
Histamine	0.18	6.68 <sup>b</sup>	0.07	0.45	-0.64
Methylamine	0.35	0.20	-0.17	0.20	-0.79 <sup>b</sup>
Ethylamine	0.49 <sup>b</sup>	0.95 <sup>b</sup>	0.27	-0.71	-1.84 <sup>b</sup>
Tyramine	0.06	3.05 <sup>b</sup>	0.48	0.15	-1.71
Phenylethylamine	3.64 <sup>b</sup>	-0.07	-0.14	-0.15	-2.88 <sup>b</sup>
Putrescine	-0.35	6.41 <sup>b</sup>	0.14	0.65	0.29
Cadaverine	0.13	0.68 <sup>b</sup>	0.00	-0.05	-1.07
<b>Amino acid</b>					
Histidine	-13.50 <sup>b</sup>	-4.16 <sup>b</sup>	-1.67 <sup>b</sup>	-1.16 <sup>b</sup>	-0.25
Tyrosine	-15.21 <sup>b</sup>	-0.95	-0.45	-0.90	-0.79
Arginine	-46.81 <sup>b</sup>	-20.03 <sup>b</sup>	-3.16 <sup>b</sup>	-3.85	-3.48 <sup>b</sup>
Ornithine	-5.21 <sup>b</sup>	1.08	0.89	-2.36 <sup>b</sup>	-0.20

<sup>a</sup> Stages: 1, must; 2, after AF; 4, after MLF; 5, after MLF and addition of SO<sub>2</sub>; 6, 6 months of aging; 7, 12 months of aging.

<sup>b</sup> Significant differences ( $P < 0.05$ ) between the two stages as determined with Student's *t* test for paired samples.

ment with recent previous in vitro studies (23). Considering the differences in amine concentrations alone in the 44 batches of samples taken before and immediately after MLF (stages 2 and 4, respectively, in Table 2) and using Student's *t* test for paired samples, we found that this increase was significant ( $P < 0.05$ ) for most of the amines except methylamine and phenylethylamine (Table 2). Higher increments were detected for the amines histamine, tyramine, and putrescine, which after MLF reached concentrations up to 14 times greater than those detected in the same wines after AF, with mean values of 7.2, 3.3, and 5.2 mg/liter, respectively (Fig. 1). These data are similar to those reported by other authors. For example in French wines, histamine, tyramine, and putrescine increased during MLF but to variable extent (3, 27). In other studies, only the formation of histamine (11, 12) or tyramine (29) was associated with lactic acid bacteria and MLF or wine storage. In contrast, other authors have stated that biogenic amines, in particular histamine, decrease during MLF (5). The fact that wine lactic acid bacteria have a variable capacity to produce biogenic amines from amino acids explains the variability in the data of biogenic amine production obtained by different authors (8, 12, 16, 17, 20, 22).

After MLF, wine is sulfited to eliminate yeast and bacteria that are no longer desirable. Sulfur dioxide does not completely stop all the biochemical reactions triggered by bacteria, such as amino acid decarboxylation (24). Therefore, changes in biogenic amines were also studied after the completion of MLF and the addition of SO<sub>2</sub> (stages 4 and 5, respectively, Table 2). No significant differences for any amine were observed in this step. Although some of the analyzed wines had higher pH values (>3.5), which could reduce the efficacy of the SO<sub>2</sub> (15), the results suggest that the amount of SO<sub>2</sub> added to the wines studied seemed to have an inhibitory effect of biogenic amine formation by bacteria. These findings also encouraged the more rapid ad-

dition of SO<sub>2</sub> immediately after malic acid degradation to eliminate lactic acid bacteria and to prevent the formation of biogenic amines.

In contrast with previous results (9, 12), the increase in histamine concentration was not observed during storage of any of the batches of wine in the present study. Changes in histamine and tyramine were similar; i.e., they reached a maximum after MLF and became stable thereafter (Table 2 and Fig. 1). For these two amines, the concentrations reached at the end of the period of aging were of the same order as those detected after MLF. A reduction in the contents of ethylamine and phenylethylamine and to a lesser extent methylamine was detected during the 12 months of storage, and this reduction was more evident after the wine was bottled (Table 2). This decrease may be due at least in part to the different treatments used in the wineries in the steps before bottling, mainly treatment with amine-absorbing clarifiers (5). However, these results are also consistent with the possibility that the microorganisms present in wine metabolize these amines and use the resulting compounds as a source of nitrogen (1), similar to the process previously identified in other food products (7).

**Changes in amino acids during the winemaking process.** Figure 3 give the means and 95% confidence intervals for concentrations of amino acids at the seven stages of the elaboration process (before and after AF, during MLF, after MLF, after MLF and SO<sub>2</sub>, and after 6 and 12 months of aging). A one-way analysis of variance was applied to compare the mean concentrations of amino acids at each of the different stages, and as expected a significant decrease in the concentration of all amino acids during AF was observed (Table 2). This decrease was due to the development of yeasts that metabolize must amino acids during AF. A significant decrease in histidine and arginine concentrations was also observed after MLF. Histidine is the amino acid

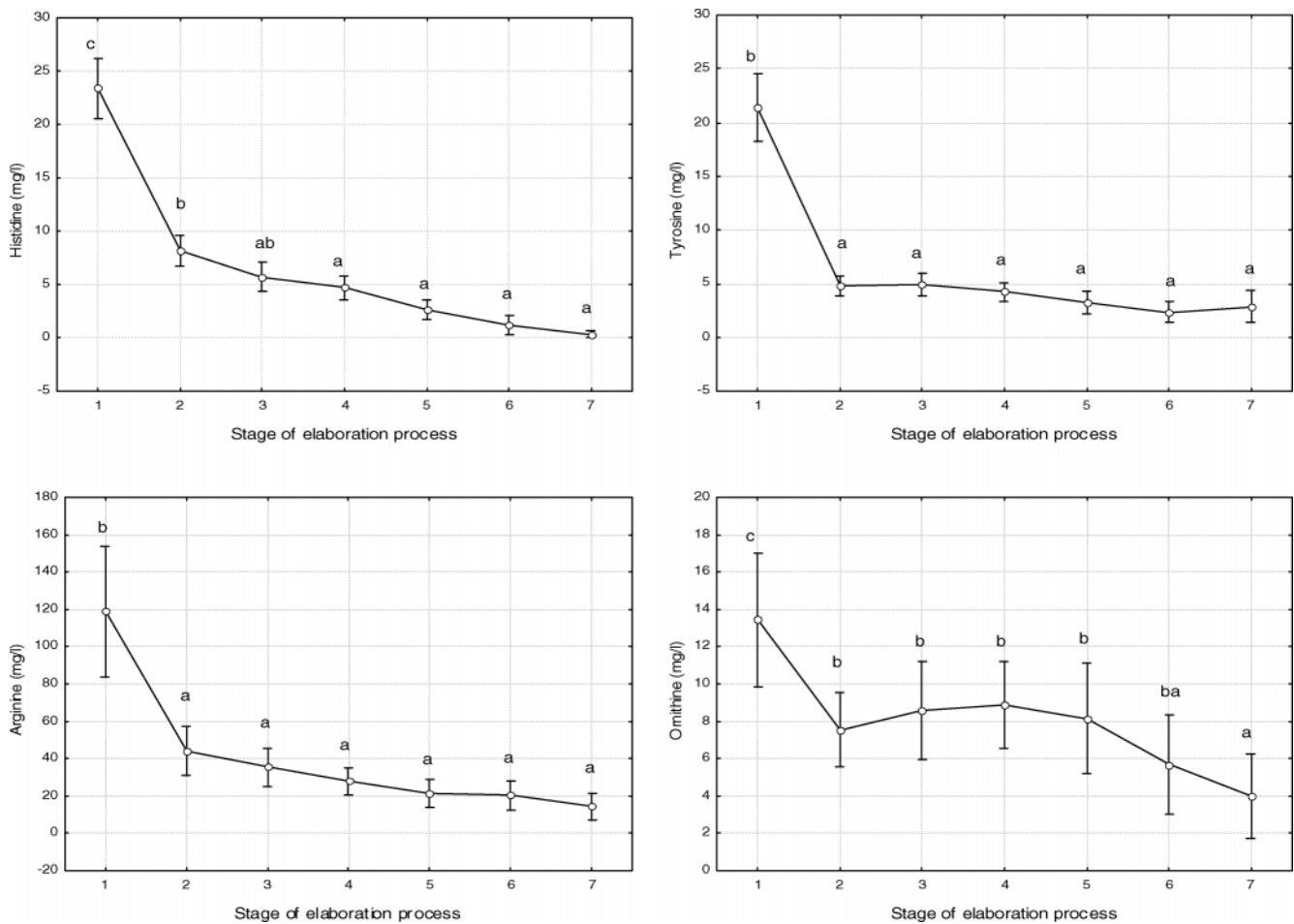


FIGURE 3. Means and 95% confidence intervals for concentrations of the amino acids histidine, tyrosine, arginine, and ornithine during the seven stages of the elaboration process: 1, must; 2, after AF; 3, during MLF; 4, just after MLF; 5, after MLF and addition of  $SO_2$ ; 6, after 6 months of aging; 7, after 12 months of aging. Means with the same letter are not significantly different according to Fisher's LSD test.

precursor of histamine; its concentration in the analyzed wines decreased significantly ( $P < 0.05$ ) during MLF, in agreement with the significant increase of this amine. Putrescine may be formed by two different metabolic pathways: the direct decarboxylation of ornithine or the decarboxylation of arginine to agmatine followed by the removal of urea from agmatine by agmatine ureohydrolase. A slight decrease in arginine concentration was observed after MLF, and this decrease was accompanied by a slight increase in ornithine concentrations (Fig. 3) by the transformation of arginine into ornithine that takes place as a consequence of lactic acid bacteria metabolism (19). During wine maturation

in barrels and bottles, there was a slight drop in the concentrations of the four amino acids, although this decrease was significant only for ornithine.

**Relationship between biogenic amines and amino acids and pH before and after MLF.** The relationship between amines and their corresponding amino acid precursors is given in Table 3 as Pearson correlation coefficients. Spearman rank correlation coefficients, which are less sensitive to outliers than are Pearson coefficients, between each pair of variables also were calculated. The pH data for the wines were also included in these correlations.

TABLE 3. Significant ( $P < 0.05$ ) Pearson (with Spearman rank) correlation coefficients between biogenic amines, amino acids, and pH values in the 224 wine samples

	Histidine	Tyrosine	Arginine	Ornithine	pH
Histamine	-0.26 (-0.39)	-0.15 (-0.21)			0.16 (0.24)
Methylamine			(0.21)		0.28 (0.31)
Ethylamine		0.20 (0.21)	(0.28)		
Tyramine	-0.23 (-0.36)	(-0.27)			
Phenylethylamine		0.42 (0.27)			
Putrescine		(-0.13)		0.17 (0.24)	0.40 (0.38)
Cadaverine					0.20 (0.20)

Significant negative nonzero correlations ( $P < 0.05$ ) were obtained for histamine and histidine, histamine and tyrosine, tyramine and histidine, and tyramine and tyrosine. These results clearly indicate the correlation between the formation of the amines histamine and tyramine and the disappearance of the corresponding amino acids histidine and tyrosine and suggest that the formation of both amines depends on the same factors. A positive correlation was also observed between putrescine and its amino acid precursor ornithine and especially between putrescine and pH. Significant correlations between histamine, methylamine, and cadaverine and pH were also found, indicating that pH clearly affects the accumulation of biogenic amines in wines, in agreement with previous data (12).

These results indicate that some amines can be produced in the grape or the must (putrescine, cadaverine, and phenylethylamine) or can be formed by yeasts during AF (ethylamine and phenylethylamine), although quantitatively only very low concentrations are reached during these early stages. The fact that the increase in the concentration of histamine, tyramine, and putrescine during MLF coincides with the decrease in amino acid precursors and that the formation of these amines was significantly correlated with the disappearance of their corresponding amino acids clearly support the hypothesis that malolactic bacteria are mainly responsible for the accumulation of these amines in wines. Sulfur dioxide, as a result of the reduction of lactic acid bacteria, prevents the biochemical formation of biogenic amines; no increase in biogenic amine concentrations was observed after the addition of SO<sub>2</sub> and during wine aging. The only changes observed during wine aging were the decreases in the amines methylamine, ethylamine, and phenylethylamine, which could be due to chemical or biological degradation. Careful monitoring of the vinification process, especially in critical stages, could be highly effective for preventing the action of microorganisms with a capacity to produce biogenic amines and thus controlling the presence and concentration of these compounds during the elaboration of wine.

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