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Changes in Amine Concentrations during Aging of Red Wine in Oak Barrels

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This investigation studied the evolution of amines in red wines made with Merlot variety, during aging in American oak barrels (*Quercus alba*) and in French oak barrels (*Quercus sessilis*) from the Allier and Nevers regions. From the results obtained it was observed that the evolutions of the amines were similar in all three types of oak woods. Histamine and tyramine were produced at the beginning of the aging process, although they were not accumulated in the wines, probably due to their degradation. Putrescine was the most abundant amine in the wines; its concentration increased to an important extent during aging as it did not undergo degradation. The concentration of cadaverine increased slightly at the first stage of aging and, like putrescine, did not degrade at all. The volatile amines showed slight variations during aging, although in no cases were high accumulations observed in the wines. Dimethylamine and isobutylamine were degraded during storage in the barrels.

KEYWORDS: Volatile amines; biogenic amines; aging in barrels; red wine

INTRODUCTION

High levels of biogenic amines in wine can cause undesirable physiological effects in some susceptible people, especially because of the presence of alcohol and acetaldehyde (*1*). Among these amines histamine is the most toxic and is known to cause headaches, low blood pressure, etc. (*1*−*3*). The effects of histamine can be aggravated by other amines (*4*). Tyramine and phenylethylamine can produce hypertension through the release of noradrenaline and norephedrine, respectively, which are vasoconstrictor substances (*5*). The toxic dose for amines in wine is difficult to establish due to the differing sensitivity of some people to these compounds. However, it has been reported that the concentration of phenylethylamine capable of provoking adverse effects is lower than the toxic dose for histamine or tyramine (*6*). Putrescine and cadaverine, although not toxic in themselves, aggravate the adverse effects of histamine, tyramine, and phenylethylamine, as they interfere with the enzymes that metabolize them (*7*, *8*). Volatile amines do not have a toxic action on the human organism, but they can have a negative effect on wine aroma (*9*). Furthermore, amines with secondary amine groups (spermine, spermidine, dimethylamine, pyrrolidine, etc.) can react with nitrous acid and its salts to form nitroamines, compounds with a known cancerous action.

Biogenic amines originate mainly from the decarboxylation of amino acids (*7*). In fermented beverages such as wine, the reaction is catalyzed by amino acid decarboxylase enzymes produced by microorganisms (*10*). Volatile amines come from amination of non-nitrogen compounds such as aldehydes and ketones (*11*). There are several studies on the production of nonvolatile amines by yeast during alcoholic fermentation (*12*−*14*) and by malolactic bacteria during malolactic fermentation (*1*, *15*, *16*). However, the evolution of amines during wine storage in both bottles and barrels has been less studied, because amines are usually related to food rich in proteins with microbial proteolytic activity. Wine, although not rich in proteins, contains high levels of free amino acids that can be decarboxylated by residual microbial populations to produce the corresponding amines (*17*). Gerbaux and Monamy (*16*) observed an increase in histamine, tyramine, and putrescine in Chardonnay and Pinot Noir wines during aging. Vidal-Carou et al. (*18*) did not find accumulation of histamine or tyramine but did observe a decrease in the concentrations of these amines in wines stored in bottles for 105 days at different temperatures.

The aim of this paper was to study the evolution of amines in red wine stored in oak barrels for 243 days. A Merlot wine fermented in a cellar was used for this purpose. Wine aging was also carried out in a cellar in new American and French oak barrels.

MATERIALS AND METHODS

Samples and Vinification. The grape used was *Vitis vinifera* cv. Merlot (2001 vintage), grown in the vineyards of the Navarra region, in northern Spain. Vinification of the wine was carried out in a cellar. The grapes were destemmed and crushed, and alcoholic fermentation was made in stainless steel tanks at 27 °C and taken to dryness; the duration of the fermentation was 6 days. Maceration was carried out during fermentation, with periodic pumping over. The wine underwent malolactic fermentation during 54 days before aging in barrels. The

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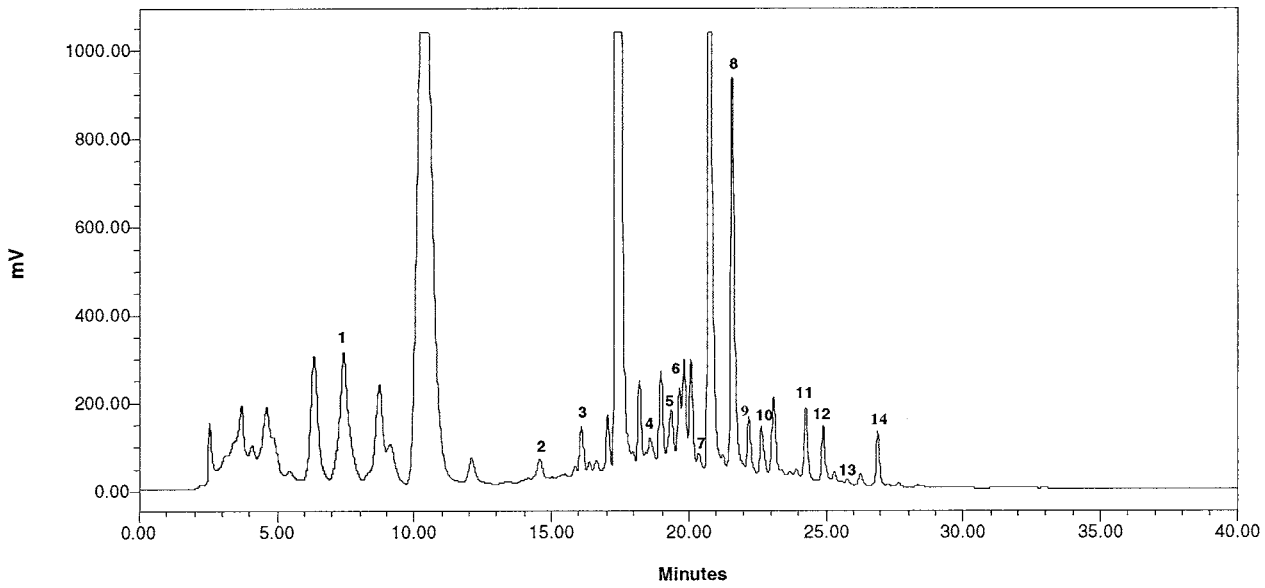

Figure 1. Example of a chromatogram of a red wine with the addition of 2 mg/L of each amine. Peaks: (1) histamine; (2) dimethylamine; (3) ethylamine; (4) pyrrolidine; (5) isopropylamine; (6) tyramine; (7) diethylamine; (8) putrescine; (9) isobutylamine; (10) cadaverine; (11) phenylethylamine + spermidine;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

wine was filtered in a horizontal-tray filter with diatomaceous earth (or kieselguhr) precoats (Della Toffola) and was subsequently aged during a period of 243 days in new barrels of American oak (*Quercus alba*) and French oak from the Allier and Nevers regions (*Quercus sessilis*). All of the barrels were made at the Intona cooperage (Navarra, Spain). For the manufacture of the barrels, the wood was naturally seasoned for 36 months and all of the barrels were submitted to a medium toasting. Samples to be analyzed were taken from the barrels after 30, 60, 90, 120, 175, and 243 days of aging. In all cases the wine was put into two barrels of each type of oak; consequently, all experiments were made in duplicate. One representative sample was taken from each of the barrels, and the amines from each sample were analyzed in duplicate. Results presented in the figures are shown with their standard deviations and are the arithmetic mean of four analyses.

Preparation of Sample and HPLC Analysis of Amines. To determine amines, the method described by Torrea et al. (*19*) was followed. Samples were cleaned by ultrafiltration with a Millipore Ultrafree MC cartridge. Next, precolumn derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) was carried out by adding 40 μL of a borate buffer solution to a 20 μL sample. Then 40 μL of AQC was added. Reagents were from an AccQ-Fluor reagent kit (Waters, Milford, MA). Analyses of the derivatized amines were performed with a Waters high-pressure liquid chromatograph (Waters) equipped with two 510 pumps, a 717 Plus autosampler, and a 474 fluorescence detector, using 250 and 395 nm as the excitation and emission wavelengths, respectively. Millenium 32 software was employed for chromatographic control. The amount of sample injected was 10 μL. A reverse-phase column (300 mm $\times$ 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 amines studied were histamine, tyramine, putrescine, cadaverine, spermidine, spermine, phenylethylamine, isobutylamine, ethylamine, isopropylamine, amylamine, hexylamine, dimethylamine, pyrrolidine, and diethylamine. An individual stock standard solution of 2 g/L of each amine was prepared in HPLC grade methanol (Scharlau, Barcelona, Spain) and stored in darkness at 4 °C. Solutions for further studies were prepared by diluting these stock standard solutions with water purified using a Milli-Q system (Millipore, Bedford, MA). Standards were supplied by Aldrich (Gillingham, U.K.). Phenylethylamine and spermidine could not be separated and were quantified as a single peak. As an example, the chromatogram of a red wine with the addition of 2 mg/L of each 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. The elution volumes for each compound were as follows: histamine, 7.62 mL; dimethylamine, 14.55 mL; ethylamine, 16.09 mL; pyrrolidine, 18.59 mL; isopropylamine, 19.35 mL; tyramine, 19.65 mL; diethylamine, 20.36 mL; putrescine, 21.59 mL; isobutylamine, 22.18 mL; cadaverine, 22.64 mL; phenylethylamine plus spermidine, 24.27 mL; amylamine, 24.89 mL; spermine, 25.77 mL; hexylamine, 26.88 mL.

Table 1. Enological Parameters in Wine after Malolactic Fermentation (Young Wine) and after 243 Days of Aging in American and French Oak Barrels

	Young wine	American oak	Allier oak	Nevers oak
pH	3.30 ± 0.02	3.46 ± 0.01	3.45 ± 0.01	3.44 ± 0.01
total acidity[a] (g/L)	5.7 ± 0.1	6.1 ± 0.1	6.1 ± 0.1	6.0 ± 0.1
volatile acidity[b] (g/L)	0.42 ± 0.01	0.69 ± 0.01	0.64 ± 0.01	0.64 ± 0.01
free SO_2 (mg/L)	12 ± 2	5 ± 1	4 ± 1	4 ± 1
ash (g/L)	1.7 ± 0.4	1.5 ± 0.3	2.0 ± 0.5	1.3 ± 0.2
alcohol (v/v %)	14.8 ± 0.4	14.6 ± 0.4	14.6 ± 0.3	14.6 ± 0.4

[a] As tartaric acid. [b] As acetic acid.

The precision of the method was calculated from the coefficient of variation (CV), and the results fluctuated between 0.8 and 9.4% for the different quantified amines. To calculate the CV, six derivatizations were carried out from a wine and the concentrations of all the amines were determined in each derivatized sample in duplicate. To examine the accuracy of the method, the recovery index was used. A known amount of each amine was added to a previously analyzed wine sample, and all of the amines were quantified. This procedure was carried out for two different concentrations of the added amine standard. The recovered quantity was calculated from the difference between the measured concentration after the addition of the amines and the initial, endogenous concentration. The recovery index varied between 73 and 105% depending on the different amines analyzed.

Enological Parameters. Enological parameters are described by the Office International de la Vigne et du Vin (*20*). The results of these parameters for the wine after malolactic fermentation and after 243 days of storage in the barrels are shown in **Table 1**.

RESULTS AND DISCUSSION

Evolution of Biogenic Amines during Aging of Wine in Oak Barrels. Histamine was formed at the beginning of the aging period in the three types of barrels; its maximum

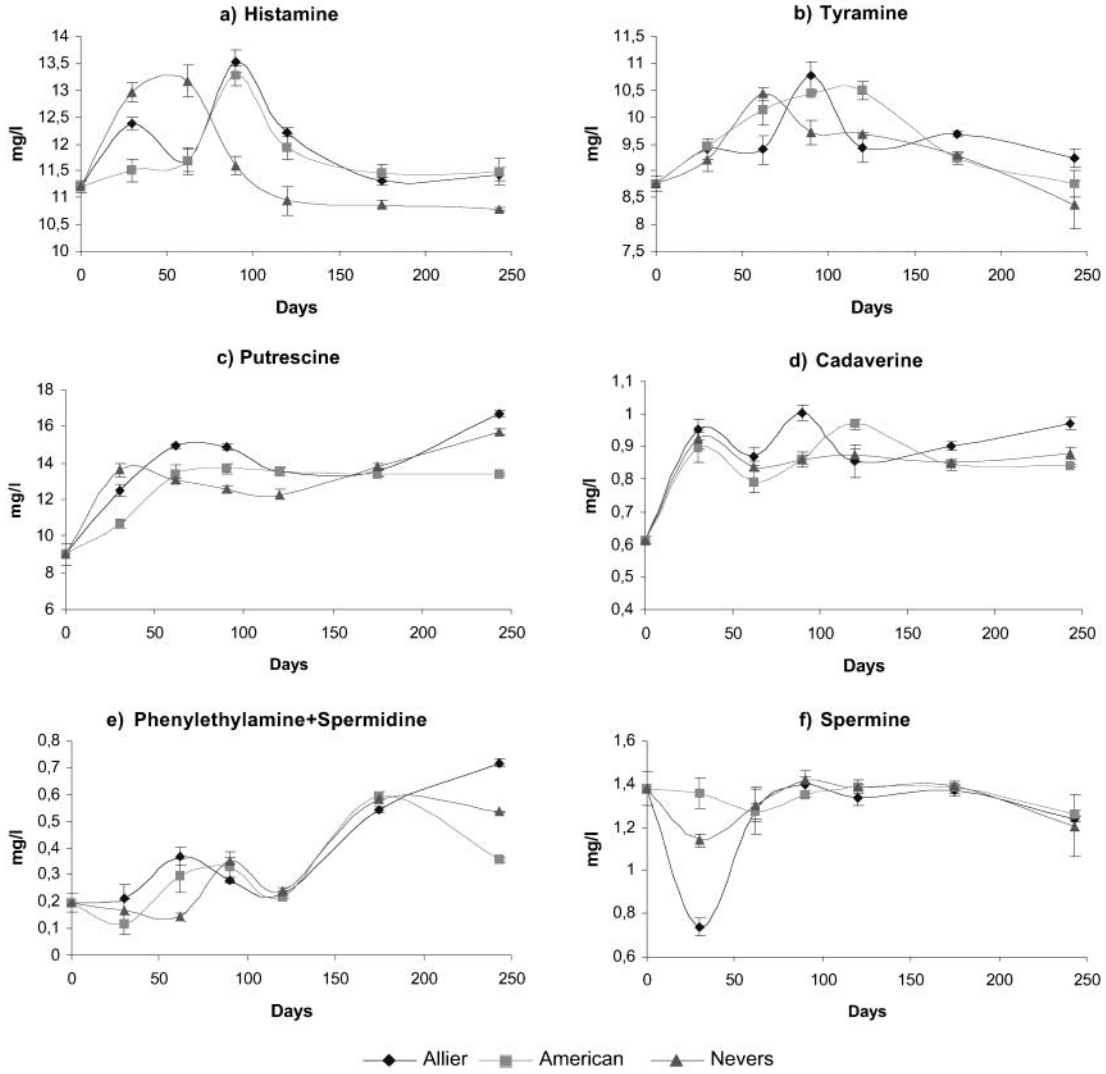

Figure 2. Evolution of biogenic amines in wine aged in French (Allier and Nevers) and American oak barrels. All results are given with their standard deviation ($n = 4$).

concentration was reached after 90 days in the wine aged in American and Allier oak and after 62 days in the wine aged in Nevers oak (**Figure 2a**). Subsequently, the concentration of this amine decreased so that at the end of the period under study the concentration of histamine in the three samples of wine was similar to the concentration in the wine before it was introduced in the barrels. The values of this amine in the wine after aging were found to be in the range of 8−20 mg/L, where wines can present adverse effects if they are consumed in important amounts (*21*, *22*). Gerbaux and Monamy (*16*) also found an increase in the concentration of histamine between 4 and 8 months after malolactic fermentation in Pinot Noir and Chardonnay wines. The production of histamine during the first months of aging was probably due to the proliferation of microorganisms with decarboxylase activity and could be favored by the release of amino acids at the end of fermentation because of yeast autolysis (*23*, *24*) and alteration of yeast plasma membrane (*25*, *26*). Amino acid decarboxylations to obtain biogenic amines are an additional mechanism for energy generation for cells deprived of other substrates and also constituted part of the defense mechanisms against the acid environment (*17*). This would explain the decarboxylation of histidine and thus the increase in the concentration of histamine when the main sources of energy have been metabolized. Likewise, some authors have reported an increase of amines through cell lysis (*27*).

In the wines aged during 90 days in American and Allier oak barrels and during 62 days in Nevers oak barrels the concentration of histamine decreased due to a probable degradation. Some biogenic amines, such as histamine, tyramine, and, to a lesser extent, phenylethylamine and tryptamine are inactivated by amine oxidases present in higher organisms. These oxidases have also been described for bacteria (*28*). In some fermented foods it has been observed that the degradation of histamine could be due to the action of histamine oxidase, an enzyme that degrades histamine, thus preventing its accumulation (*29*, *30*). In wine, the action of these enzymes would be hindered by acid pH as the greatest activity of these enzymes takes place at neutral or basic pH. However, Umezu et al. (*31*) found oxidase activity below pH 4 in a synthetic medium. It is not likely the oxygen requirement of these enzymes would be a limiting factor for their activity, as the barrels permit the microoxygenation of the wine. Vidal-Carou et al. (*18*) found a decrease in the concentration of histamine in red wines stored in bottles for 80 days at temperatures between 20 and 24 °C, which showed clear signs of spoilage. In our case the three wines aged for 243 days in the barrels showed a higher volatile acidity than the young wine (**Table 1**). However, in all cases the values

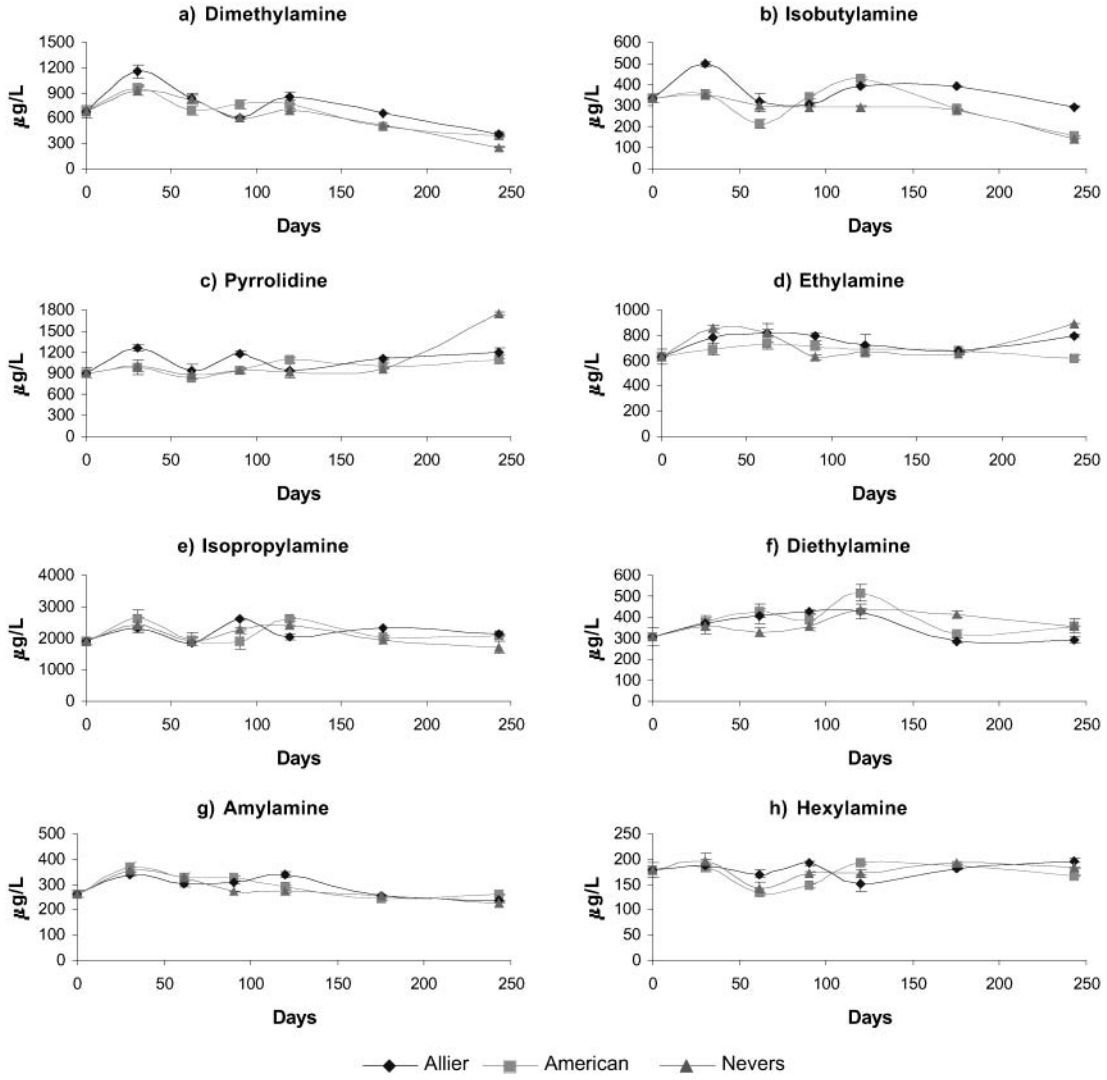

Figure 3. Evolution of volatile amines in wine aged in French (Allier and Nevers) and American oak barrels. All results are given with their standard deviation ($n = 4$).

were below 0.7 g of HAc/L, a limiting value to obtain a wine with good organoleptic characteristics and sound conservation.

Tyramine varied in a similar way to histamine in the three samples (**Figure 2b**). This amine, like histamine, was produced at the beginning of the aging period in the three wine samples and showed a maximum concentration somewhat earlier in Nevers oak than in American and Allier oak. Tyramine was subsequently degraded, and at the end of the studied period its concentration, in the three samples, was similar to the concentration in the wine before it was introduced in the barrels. This amine did not reach concentrations between 25 and 40 mg/L in any of the wines, a range at which wine should be consumed with caution (*22*). The production of tyramine at the beginning of wine aging was probably due to the action of microorganisms, which, through decarboxylation of the precursor amino acid, obtained energy to survive in a medium poor in nutrients. With regard to the degradation of tyramine the action of tyramine oxidase enzyme has been observed in some fermented food. This enzyme catalyzes the oxidation of tyramine, thus preventing its accumulation in food (*28*−*31*). However, the tyramine oxidase activity would be hindered by the pH acid of the wine as happened in the case of the degradation of histamine. Vidal-Carou et al. (*18*) observed the degradation of this amine in red wines stored in bottles at 4 °C for 80 days without any sign of spoilage.

Putrescine was the most abundant amine in all of the wines after 243 days of storage in barrels (**Figure 2c**). The concentration of this amine at the end of the period under study was higher than the concentration in the wine after malolactic fermentation. An increase of 48% was observed for the wine aged in American oak barrels, 85% for the wine aged in Allier oak barrels, and 74% for the wine aged in Nevers oak barrels. These results agree with those of Gerbaux and Monamy (*16*), who found that putrescine along with histamine and tyramine accumulated in Chardonnay and Pinot Noir wines during storage in bottles. The concentration of cadaverine increased slightly over the first month of aging in all of the samples, and subsequently it hardly changed during the remaining period under study (**Figure 2d**). Putrescine and cadaverine, unlike histamine or tyramine, were not degraded in the second half of aging, so that it seems there was no oxidation of these amines by oxidase enzymes in the wines.

The concentration of phenylethylamine plus spermidine fluctuated during aging in all of the wines (**Figure 2e**). In the wines aged in Allier and Nevers oak the maximum concentration was reached at the end of the studied period. In the wine stored in American oak a decrease in the levels of these amines was observed in the final stage of aging. Consumption of phenylethylamine through the action of oxidase enzymes has been reported; Leuschner et al. (*28*) observed oxidation of this amine

in a synthetic medium, although to a lesser extent than in the cases of histamine and tyramine. The concentration of spermine hardly changed in the wines aged in American and Nevers oak. In the wine aged in Allier oak this amine showed a slight decrease at the beginning of the storage process (**Figure 2f**). Consequently, it seems that spermine was not relevant for the bacteria to obtain energy, nor was it degraded to any considerable extent.

Evolution of Volatile Amines during Aging of Wine in Oak Barrels. The concentration of dimethylamine (**Figure 3a**) increased during the first month of wine storage in the barrels; subsequently, it decreased so that after 243 days, the concentration of this amine in the aged wines was less than the concentration at the end of malolactic fermentation. Dimethylamine in all cases showed concentrations >50 μg/L, a level that could have a negative effect on beer aroma according to Palamand et al. (*32*). However, the threshold levels of organoleptic impact for dimethylamine are probably higher in wine than in beer, so we cannot affirm that the concentrations found in this study have an impact on the aroma. The concentration of isobutylamine at the end of the aging period (**Figure 3b**) decreased in the wine stored in American oak (49.6%) and in the wine stored in Nevers oak (53%); in the wine stored in Allier oak the concentrations after aging and after malolactic fermentation were similar.

The concentrations of pyrrolidine and ethylamine oak (**Figure 3c**,**d**) increased in the latter phase of aging in the barrels of Nevers. The concentrations of these amines in the other barrels did not show significant changes. The concentration of ethylamine was <2000 μg/L in all of the wines, a value reported by Palamand et al. (*32*) as negative for beer aroma. The concentrations of isopropylamine (**Figure 3e**), diethylamine (**Figure 3f**), amylamine (**Figure 3g**), and hexylamine (**Figure 3h**) remained almost constant over the whole period of aging in the three samples.

Ough and Daudt (*33*) studied the evolution of volatile amines during the storage of Pinot Noir and Riesling wines. These authors found that some amines were consumed while others were produced during aging, although the data were different depending on the type of wine and fermentation temperature. In our study the concentrations of most of the volatile amines did not change during aging of wine in the barrels, although a decrease for dimethylamine and isobutylamine was observed. These two amines could have been consumed by residual bacteria present in the wine for the production of carbon skeletons or amino groups.

The increase in the concentrations of ethylamine and pyrrolidine in Nevers oak barrels could be due to yeast autolysis and/or reductive amination of the corresponding aldehyde or the transamination of the aldehyde from an amino acid (*12*). These reactions would have been possible due to the presence of amino groups in the medium resulting from amino acids or from other amines.

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LITERATURE CITED

(1) Bauza, T.; Blaise, A.; Teissedre, P. L.; Cabanis, J. C.; Kanny, G.; Moneret-Vautrin, D. A. Les amines biogènes du vin. Métabolisme et toxicité. *Bull. O. I. V.* **1995**, *767*−*768*, 42−67.

(2) Lehtonen, P.; Saarinen, M.; Vesanto, M.; Riekkola, M. L. Determination of wine amines by HPLC using automated precolumn derivatisation with *o*-phthalaldehyde and fluorescence detection. *Z. Lebensm. Unters. Forsch.* **1992**, *194*, 434−437.

(3) Mayer, K.; Vetsch, U. pH and biological degradation in wine. *Schweiz. Z. Obst- Weinbau* **1973**, *109*, 635−639.

(4) Chu, C. H.; Bejdanes, L. F. Effect of diamines, polyamines and tuna fish extracts on the binding of histamine to mucin in vitro. *J. Food Sci*. **1981**, *47*, 79.

(5) Forsythe, W. I.; Redmond, A. Two controlled trials of tyramine in children with migraine. *Dev. Med. Child Neurol*. **1974**, *16*, 794−799.

(6) Glória, M. B. A.; Watson, B. T.; Simon-Sarkadi, L.; Daeschel, M. A. A survey of biogenic amines in Oregon Pinot noir and Cabernet sauvignon wines. *Am. J. Enol. Vitic.* **1998**, *49*, 279−282.

(7) ten Brink, B.; Damink, C.; Joosten, H. M. L. J.; Huis in't Veld, J. H. J. Occurrence and formation of biologically active amines in foods. *Int. J. Food Microbiol.* **1990**, *11*, 73−84.

(8) Straub, B. W.; Kicherer, M.; Schilcher, S. M.; Hammes, W. P. The formation of biogenic amines by fermentation organisms. *Z. Lebensm. Unters. Forsch.* **1995**, *201*, 79−82.

(9) Lehtonen, P. Determination of amines and amino acids in wine. A review. *Am. J. Enol. Vitic.* **1996**, *47*, 127−132.

(10) Halász, A.; Baráth, A.; Simon-Sarkadi, L.; Holzapfel, W. Biogenic amines and their production by microorganisms in food. *Trends Food Sci. Technol.* **1994**, *5*, 42−49.

(11) Ough, C. S.; Daudt, C. E.; Crowell, E. A. Identification of new volatile amines in grapes and wines. *J. Agric. Food Chem*. **1981**, *29*, 938−941.

(12) Buteau, C.; Duitschaever, C. L.; Ashton, G. C. High-performance liquid chromatographic detection and quantitation of amines in must and wine. *J. Chromatogr.* **1984**, *284*, 201−210.

(13) Vidal-Carou, M. C.; Mariné-Font, A. Histamine in wines. *Rev. Agroquim. Tecnol. Aliment*. **1985**, *25*, 58−74.

(14) Torrea, D.; Ancin, C. Content of biogenic amines in a Chardonnay wine obtained through spontaneous and inoculated fermentations. *J. Agric. Food. Chem*. **2002**, *50*, 4895−4899.

(15) Aerny, J. Origin of histamine in wines. Updated knowledge. *Bull. O. I. V.* **1985**, *656*−*657*, 1016−1019.

(16) Gerbaux, V.; Monamy, C. Biogenic amines in Burgundy wines. Contents and origin in wines. *Rev. Fr. Oenol*. **2000**, *183*, 25−28.

(17) Lonvaud-Funel, A. Biogenic amines in wine: role of lactic acid bacteria. *FEMS Microbiol. Lett*. **2001**, *199*, 9−13.

(18) Vidal-Carou, M. C.; Codony-Salcedo, R.; Mariné-Font, A. Changes in the concentration of histamine and tyramine during wine spoilage at various temperatures. *Am. J. Enol. Vitic.* **1991**, *42*, 145−149.

(19) Torrea, D.; Ancin, C. Influence of yeast strain on biogenic amines content in wines: relationship with the utilization of amino acids during fermentation. *Am. J. Enol. Vitic*. **2001**, *52*, 185−190.

(20) Office International de la Vigne et du Vin. *Recueil des Méthodes Internationales d'Analyse des Vins et des Moûts*; Paris, France, 1990.

(21) Daeschel, M. Headache and wine. In *Proceedings of the Symposium on Wine and Health*; Rantz, J., Ed.; American Society for Enology and Viticulture: Davis, CA, 1996; pp 29−34.

(22) Soufleros, E.; Barrios, M.; Bertrand, A. Correlation between the content of biogenic amines and other wine compounds. *Am. J. Enol. Vitic.* **1998**, *49*, 266−278.

(23) Slaughter, J. C.; Flint, P. W. N.; Kular, K. S. The effect of CO_2 on the absorption of amino acids from a malt extract medium by *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett*. **1987**, *40*, 239−243.

(24) Kruger, L.; Pickerell, A. T. W.; Axcell, B. The sensitivity of different brewing yeast strains to carbon dioxide inhibition: Fermentation and production of flavour-active volatile compounds. *J. Inst. Brew*. **1992**, *98*, 133−138.

(25) Bidan, P.; Feuillat, M.; Moulin, J. P. Sparkling wines. French report. 65th Assemblée Générale de l'O. I. V. (General Confederation of OIV). *Bull. O. I. V.* **1986**, *663*−*64*, 563−626.
(26) Monteiro, F.; Bisson, L. F. Amino acid utilization and urea formation during vinification fermentations. *Am. J. Enol. Vitic*. **1991**, *42*, 199−208.
(27) Blackwell, B.; Mabbitt, L. A.; Marley, E. Histamine and tyramine content of yeast products. *J. Food Sci.* **1969**, *34*, 47−51.
(28) Leuschner, R. G.; Heidel, M.; Hammes, W. P. Histamine and tyramine degradation by food fermenting microorganisms. *Int. J. Food Microbiol*. **1998**, *39*, 1−10.
(29) Voigt, M. N.; Eitenmiller, R. R. Role of histidine and tyrosine decarboxylases and mono- and diamine oxidases in amine build-up in cheese. *J. Food Prot*. **1978**, *41*, 182−186.
(30) Enes Dapkevicius, M. L. N.; Nout, M. J. R.; Rombouts, F. M.; Houben, J. H.; Wymenga, W. Biogenic amine formation and degredation by potential fish silage starter microorganisms. *Int. J. Food Microbiol*. **2000**, *57*, 107−114.
(31) Umezu, M.; Shibata, A.; Umegaki, M. Production and oxidation of amines by *hiochi* bacteria. *J. Ferment. Technol*. **1979**, *57*, 505−511.
(32) Palamand, S. R.; Hardwick, W. A.; Markl, K. S. Volatile amines in beer and their influence on beer flavor. *Proc. Am. Soc. Brew. Chem*. **1969**, 54−58.
(33) Ough, C. S.; Daudt, C. E. Quantitative determination of volatile amines in grapes and wines. I. Effect of fermentation and storage temperature on amine concentrations. *Am. J. Enol. Vitic*. **1981**, *32*, 185−188.

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