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Occurrence of biogenic amines in wine: The role of grapes

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ABSTRACT

The evolution of biogenic amines from must to wine has been studied in seven different grape cultivars before and after malolactic fermentation. Alcoholic and malolactic fermentations have been carried out using selected yeasts and bacteria that, in a previous study, were unable to produce biogenic amines. The study has been performed under aseptic conditions to exclude possible interferences due to uncontrolled contaminating microorganisms present in grapes and/or in the environment. The goal of this work was to investigate the influence of grape on biogenic amines content of the wine. The results obtained showed that grape variety is related to the presence of some biogenic amines in wines and that, climatic conditions also affect the accumulation of these compounds in grapes.

1. Introduction

Biogenic amines (BA) occur in different kinds of food, such as cheese, fish products, beer and wine. They are undesirable in all foods and beverages because, if present at high concentrations, they may induce headaches, respiratory distress, hyper-hypotension and several allergenic disorders (Silla-Santos, 1996). The presence of BA in wines has been studied extensively for 30 years and particularly over the last 10 years, as a consequence of the increasing attention to consumer protection. In order to attempt to eliminate BA in wine, it is necessary to identify the source of these compounds. In spite of the many studies carried out, there is no agreement on this. Generally, malolactic fermentation (MLF) is considered a crucial factor for BA production, and studies have shown that, in this phase, the main BA generated are putrescine, histamine and tyramine (Lonvaud-Funel, 2001; Marcobal, Martin-Alvarez, Polo, Munoz, & Moreno-Arribas, 2005). However, published data are complex and sometimes contradictory. Several studies have found that BA are principally formed by *Oenococcus oeni*, the main agent of MLF, but other authors have found that only *Lactobacillus* spp. is able to produce them (Costantini, Cersosimo, Del Prete, & Garcia-Moruno, 2006; Guerrini, Mangani, Granchi, & Vincenzini, 2002; Landete, Ferrer, & Pardo, 2005; Lonvaud-Funel & Joyeux, 1994; Moreno-Arribas, Polo, Jorganes, & Muñoz, 2003). Others have found that BA are formed by yeasts during alcoholic fermentation (AF) (Bouteau, Duischaver, & Ashton, 1984; Caruso et al., 2002; Torrea & Ancìn, 2001). In several studies, BA have been suggested as indicators of a lack of hygiene during the winemaking process or associated (in the case of putrescine and cadaverine) with poor sanitary conditions of grapes (Leitao, Marques, & Romao, 2005). These contradictory data can, in part, be explained by the difficulties in analysis, in addition to winemaking or experimental conditions. Analytical determination of BA is not simple because of their structure and because wine is a complex matrix and BA are usually present at low levels.

High-performance liquid chromatography (HPLC) is the most used analytical method due to its high resolution and sensitivity. In the last few years, different HPLC methods have been proposed (Costantini et al., 2006; Mafra, Herbert, Santos, Barros, & Alves, 1999; Pereira Monteiro & Bertrand, 1994; Soufleros, Bouloumpasi, Zotou, & Loukou, 2007). In spite of the sensitivity of the different methods, the problem of real identification of very small chromatographic peaks in a complex chromatogram remains, based only on retention times.

The analytical problems also concerned the PCR-based methods for the detection of microorganisms, potential producers of BA, as demonstrated by the contradictory results obtained by different authors. These methods are based on the determination of the genes codifying for the aminoacid decarboxylase enzymes, the enzymes necessary for the decarboxylation of the aminoacid precursors of the BA. Recently, Lucas et al. (2005 and 2008), reported that *hdc* (histidine decarboxylase) gene is located on an unstable plasmid, and because of this instability, cells can lose the capacity to form histamine when they are grown on a synthetic laboratory medium. This could explain these contradictory data.

Some authors have reported the presence of some BA in grapes: Broquedis, Dumery, and Boucard (1989) reported the presence of

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polyamines, particularly putrescine and spermidine, whereas Vidal-Carou, Ambatlle-Espunyes, Ulla-Ulla, and Mariné-Font (1990) found small quantities of histamine.

The aim of this study was to determine the role of grapes in the BA content of wine. We studied seven different grape cultivars under sterile conditions and noted how the BA content changed in must, in wine at the end of AF and in wine after MLF using selected yeasts and bacteria. The content of BA has been analysed by HPLC. Yeast and bacteria have been previously tested for the production of BA by TLC and PCR methods, respectively.

2. Materials and methods

2.1. Cultivars

The study was carried out on a 12-year-old experimental vineyard of the "Unità di ricerca per le produzioni enologiche dell'Italia centrale-CRA", located in Velletri (Rome) in the Lazio region (Italy) (41°40.5′N latitude, 12°50.7′E longitude) at 355 m above sea level. The trials were made using *Vitis vinifera* cultivars Merlot, Syrah, Sangiovese, Cesanese d'Affile, Carmenere, Montepulciano and Cabernet Franc. Grapes were harvested at technological maturation. These cultivars all had the same cordon spur training system, i.e., cordon-trained and spur pruned. Seven kilograms of grapes have been used for each grape variety. To obtain sterile must, grapes were washed with a potassium metabisulfite solution (1500 mg/L) and then washed with sterile water, under aseptic conditions. The grapes were crushed and pressed using a basket press. Skins, seeds and stems were manually separated, weighed, divided into equal parts and then added to each must before fermentation.

2.2. Years

BA content has been examined during two vintages. Grapes belonging to the same varieties and the same vines have been studied in the years 2004 and 2005.

2.3. Alcoholic fermentation

Four independent replications were made for each must. Fermentations were conducted under aseptic conditions in 1.0 L conical flasks containing 500 mL of must. Musts were inoculated with pure cultures of *Saccharomyces cerevisiae*, pre-grown in the same sterile juice for 72 h at 28 °C, followed by 12 h temperature equilibration at 20 °C. *S. cerevisiae* has been previously tested in a synthetic medium by TLC and HPLC according to the methods described by Garcia-Moruno, Carrascosa, and Muñoz (2005) and Costantini et al. (2006).

Just before the inoculation, total and viable cell counts were performed. After inoculation, the final concentration of yeast cells in the musts was about 10^6 cells/mL. Fermentation of the inoculated musts was carried out at a controlled temperature of 20 °C and they were monitored by weight loss caused by CO_2 production.

2.4. Malolactic fermentation

At the end of the alcoholic fermentation (AF), yeasts were removed from wine by filtering through a 0.45 μm pore size filter under sterile conditions. The freeze-dried lactic acid bacteria *O. oeni* (Lalvin, Lallemand SA, France) were rehydrated, according to the manufacturer's instructions, in distilled water at 25 °C for 15 min and then added to each sample of filtered wine to a concentration of 5×10^6 cells/mL. The malolactic fermentations (MLF) were conducted at a controlled temperature of 25 °C. According to the methods described by Garcia-Moruno et al. (2005) and Costantini et al. (2006), *O. oeni* was previously tested by TLC and HPLC using a synthetic medium to test its ability to produce BA. It had also been tested by PCR to detect histidine decarboxylase, tyramine decarboxylase and ornithine decarboxylase genes (Costantini et al., 2006).

2.5. Media analysed

The following samples have been considered as medium: Must at the beginning of AF (Must), must (72 h) after the start of fermentation (Must 72 h), wine at the end of AF (Wine/AF) and wine at the end of MLF (Wine/MLF).

2.6. Chemical analysis of musts and wines

Chemical analysis of musts and wines are shown in Tables 1 and 2, respectively. Soluble solids (reducing sugars) were measured in degrees Brix, and titratable acidity and pH were determined according to standard methods (OIV, 1991). Malic acid and lactic acid were determined by Enochem autoanalyzer (Chem Italia Servizi-Chematech R&D) and using their relative enzymatic kits.

2.7. HPLC analysis of biogenic amines in must and wines

The determination of BA in wine was carried out by HPLC. Analysis was performed with a Hewlett–Packard I model 1100 high-pressure liquid chromatograph (Hewlett–Packard, Palo Alto, CA, USA) with a fluorimetric detector according to the method described by Costantini et al. (2006). Briefly, the samples were subjected to an automatic precolumn derivatization procedure using *o*-phthalaldehyde (OPA Reagent, Agilent Technologies, Palo Alto,

Table 1
Chemical analysis of the musts; 2004–2005

	Merlot	Syrah	Cabernet Franc.	Montepulciano	Sangiovese	Carmenere	Cesanese d'Affile
Cultivar Year 2004							
Reducing sugars (g/L)	193 ± 0.20	183 ± 0.22	190 ± 0.25	196 ± 0.28	193 ± 0.21	192 ± 0.19	197 ± 0.30
TA (g/L)	7.80 ± 0.04	8.60 ± 0.05	6.00 ± 0.04	8.00 ± 0.04	7.95 ± 0.30	7.20 ± 0.02	7.50 ± 0.02
pH	3.42 ± 0.02	3.24 ± 0.01	3.25 ± 0.01	3.16 ± 0.01	3.18 ± 0.02	3.50 ± 0.01	3.20 ± 0.01
Malic acid (g/L)	3.76 ± 0.12	3.70 ± 0.10	3.10 ± 0.09	3.70 ± 0.15	3.67 ± 0.30	4.10 ± 0.16	4.80 ± 0.12
Cultivar Year 2005							
Reducing sugars (g/L)	196 ± 0.21	194 ± 0.22	201 ± 0.20	200 ± 0.19	197 ± 0.21	195 ± 0.22	199 ± 0.23
TA (g/L)	7.30 ± 0.02	7.50 ± 0.03	5.85 ± 0.02	7.00 ± 0.03	7.50 ± 0.02	6.67 ± 0.01	6.90 ± 0.02
pH	3.31 ± 0.01	3.20 ± 0.01	3.35 ± 0.01	3.20 ± 0.02	3.21 ± 0.02	3.45 ± 0.01	3.25 ± 0.01
Malic acid (g/L)	2.80 ± 0.09	3.36 ± 0.09	2.79 ± 0.10	3.46 ± 0.12	3.10 ± 0.09	3.76 ± 0.15	3.10 ± 0.20

Values represent the average of duplicate replications ± standard deviation.
Reducing sugars measured as BRIX.
Titratable acidity (TA) expressed in g/L of tartaric acid.

Table 2
Chemical analysis of wine at the end of alcoholic fermentation; 2004–2005

	Merlot	Syrah	Cabernet Franc.	Montepulciano	Sangiovese	Carmenere	Cesanese d'Affile
Cultivar Year 2004							
Ethyl alcohol% (v/v)	12.72 ± 0.028	12.07 ± 0.14	12.64 ± 0.058	12.65 ± 0.11	12.44 ± 0.08	12.51 ± 0.04	12.67 ± 0.03
TA (g/L)	6.80 ± 0.033	7.76 ± 0.033	6.89 ± 0.05	7.81 ± 0.02	7.33 ± 0.023	6.68 ± 0.11	8.24 ± 0.14
pH	3.38 ± 0.04	3.21 ± 0.010	3.41 ± 0.005	3.12 ± 0.01	3.30 ± 0.005	3.52 ± 0.008	3.18 ± 0.01
Malic acid (g/L)	2.93 ± 0.062	3.31 ± 0.010	2.89 ± 0.09	2.96 ± 0.03	2.79 ± 0.037	3.71 ± 0.072	4.09 ± 0.11
Lactic acid (g/L)	nd	nd	nd	nd	nd	nd	nd
Cultivar Year 2005							
Ethyl alcohol % (v/v)	12.28 ± 0.09	11.74 ± 0.09	12.33 ± 0.11	12.00 ± 0.12	11.80 ± 0.06	11.77 ± 0.11	12.23 ± 0.11
TA (g/L)	7.25 ± 0.05	8.28 ± 0.02	6.03 ± 0.10	7.47 ± 0.13	7.58 ± 0.17	6.11 ± 0.03	7.50 ± 0.20
pH	3.38 ± 0.01	3.25 ± 0.005	3.54 ± 0.02	3.32 ± 0.006	3.29 ± 0.025	3.61 ± 0.008	3.39 ± 0.01
Malic acid (g/L)	2.50 ± 0.09	3.41 ± 0.30	2.53 ± 0.29	3.03 ± 0.035	2.72 ± 0.09	3.23 ± 0.075	3.75 ± 0.05
Lactic acid (g/L)	nd	nd	nd	nd	nd	nd	nd

Values represent the average of four replications ± standard deviation.
Nd, not detected.
Mean value were calculated by using zero for nd.
Titratable acidity (TA) expressed in g/L of tartaric acid.

CA, USA). All separations were performed on an Alltima C18 column, with a 5-mm-thick film, measuring 200 by 4.6 mm (Alltech, Deerfield, IL, USA), with a matching guard cartridge of the same type (7.5 by 4.6 mm). Samples were injected in duplicate onto the column after being filtered through a 0.2 mm filter (Schleicher and Schuell, Keen, NH, USA). As mobile phases, two eluents were used: Eluent A (1.224 g of sodium acetate trihydrate, 500 mL of water, 0.09 mL of triethylamine, and 1.5 mL of tetrahydrofuran) and eluent B (1.088 g of sodium acetate trihydrate, 100 mL of water, 200 mL of acetonitrile, and 200 mL of methanol). A 65 min gradient programme commenced with an initial concentration of 10% of eluent B at a flowrate of 0.450 mL/min and terminated with 100% of eluent B at a flowrate of 0.700 mL/min; at the end of the analysis, there is a post-run of 15 min where the flowrate and % of eluent B come back to the initial condition. Fluorescence wavelengths for excitation and emission were 340 and 450 nm, respectively. Quantification of the BA was performed with an internal standard of 15 mg/L of norvaline solution.

BA standard: A 20 mg/l (in methanol 75%) solution containing agmatine, cadaverine, ethylamine, ethanolamine, phenylethylamine, histamine, putrescine, triptamine and tyramine was injected as standard solution, all purchased by Sigma.

2.8. Climatic data

Climatic data were supplied by the weather station belonging to the Regional Institute of Meteorology. This weather station is located at "Unità di Ricerca di Velletri" (Rome) and it is representative of the weather in the area.

2.9. Statistical analysis

The statistic study of the BA content determined in wines was carried out with analysis of variance (ANOVA), and the means were compared with the least significant difference (LSD) test. For data analysis, the Statistica package (Version 7.1, StatSoft Inc., Tulsa, OK, USA) was used.

2.10. List of abbreviations used in the study

AGM agmatine
ETHA ethanolamine
ETHYL ethylamine
PUT putrescine
TYM tyramine
ANOVA analysis of variance
LSD test least significant difference test
AF alcoholic fermentation
MLF malolactic fermentation

3. Results

3.1. Oenological parameters

The data reported in Table 1 show that the oenological parameters in 2004 and 2005 year were similar for all the cultivars, even though, in 2004, the harvest was about 15 days before that in 2005 as a result of climatic conditions.

In the 2003–2004 cycle, the annual rainfall was very favourable for fruit ripening. Rainfall was heavy in autumn (accumulated rain about 350 mm) and at the beginning of winter (accumulated rain, in December, about 120 mm), but the winter itself was dry (accumulated rain from January to March about 60 mm). In the spring, rainfall was plentiful (accumulated rain in April, May and June was about 260 mm), but the ripening period was dry (accumulated rain from July to September about 65 mm). The temperature was mild with few fluctuations and average temperatures were not extreme (the average minimum temperature was 8 °C in January and the average maximum temperature was 28 °C in August), but about 2 °C higher than in 2005. These climatic conditions are generally associated with high wine quality.

In the 2004–2005 cycle, there was a little rain in the autumn (accumulated rain about 150 mm), with no rainfall after harvest and very little rain in the winter (accumulated rain from January to March was about 70 mm). In the spring and summer, there was considerable rain, especially during the ripening period (accumulated rain from July to September was about 400 mm). The average temperature was very low during the ripening period (the average minimum temperature was 12 °C in September and average maximum temperature of 19 °C in August) and mild in summer. These climatic conditions are generally associated with low wine quality.

At the end of AF, wines reached dryness and lactic acid was not found (Table 2). In all wines, the end of the MLF was indicated both by the complete degradation of malic acid and by the parallel increase in concentration of lactic acid and pH value.

3.2. Detection on ability to produce BA by microorganisms

The *S. cerevisiae* strain used in this study was previously tested in a synthetic medium by TLC and HPLC as described in Section 2 and it was concluded that it did not produce the amines histamine, putrescine, cadaverine, tyramine and agmatine.

The *O. oeni* strain used in this study was analysed by TLC and HPLC using a synthetic medium as described in Section 2 and it was concluded that it was not a producer of the BA histamine, putrescine, cadaverine, tyramine and agmatine. It had also been tested by PCR to detect histidine decarboxylase, tyramine decarboxylase and ornithine decarboxylase genes and these were not found.

3.3. BA content in the media

Results for BA content in musts are shown in Table 3. In all of the seven musts analysed, only ethanolamine (ETHA), ethylamine (ETHYL) and putrescine (PUT) were found. Histamine (HISTA), tyramine (TYM), cadaverine (CAD) and agmatine (AGM) were not found.

Table 4 lists BA content in the must 72 h after the start of AF, and shows that PUT was immediately reduced after the first 72 h. This result was not surprising because PUT is an intermediate in the biosynthesis of polyamines (Tabor, Rosenthal, & Tabor, 1958) and can be metabolised by the yeast.

Results for BA content at the end of the AF are shown in Table 5. AGM and TYM appeared during the AF for some of the cultivars studied: TYM was present only in Montepulciano, Sangiovese and Cesanese d'Affile cultivars in 2004 and AGM was present in all of the cultivars but only in 2005.

Table 6 shows the amine content in wines at the end of the MLF. It can be observed that, comparing the data in Tables 5 and 6, TYM and AGM appeared in low quantities during the AF and later AGM increased during the MLF while TYM disappeared.

Compared with other published works, our data refer to substratum grapes where washing with sulfurous anhydride had effectively destroyed yeasts and indigenous bacteria, therefore fermentations were similar to sterile musts unlike many studies which analysed commercial wines, where the presence of indigenous microorganisms can affect the profile of amines in the wine. The addition of sodium azide to the samples eliminated further microbial development before the analysis.

3.4. Statistical analysis of the results

The analysis of variance (ANOVA) showed significant differences ($p \leqslant 0.05$) among individual and total amine values and years, cultivar, and medium of fermentation.

The total BA concentration in two years is reported in Fig. 1. The data showed a significant difference ($p \leqslant 0.05$) between the total amine content and the year.

Table 3
Biogenic amines (mg/L) in musts; 2004–2005

Biogenic amines (mg/L)	Merlot	Syrah	Cabernet Franc.	Montepulciano	Sangiovese	Carmenere	Cesanese d'Affile
Must Year 2004							
ETHA	4.43 ± 0.15	3.98 ± 0.12	3.02 ± 0.10	3.76 ± 0.15	4.44 ± 0.13	4.84 ± 0.10	5.03 ± 0.09
AGM	nd	nd	nd	nd	nd	nd	nd
ETHYL	5.28 ± 0.18	5.12 ± 0.16	5.78 ± 0.15	4.65 ± 0.12	5.37 ± 0.16	5.86 ± 0.20	6.0 ± 0.09
TYM	nd	nd	nd	nd	nd	nd	nd
PUT	4.75 ± 0.09	5.47 ± 0.08	3.06 ± 0.06	nd	nd	15.41 ± 0.10	7.99 ± 0.07
Total amines	14.46 ± 0.59	14.57 ± 0.51	11.86 ± 0.44	8.41 ± 0.38	9.81 ± 0.41	26.11 ± 0.42	19.02 ± 0.35
Must Year 2005							
ETHA	12.22 ± 0.09	11.65 ± 0.08	9.83 ± 0.07	10.73 ± 0.06	13.70 ± 0.08	11.52 ± 0.07	11.65 ± 0.06
AGM	nd	nd	nd	nd	nd	nd	nd
ETHYL	14.46 ± 0.10	14.07 ± 0.09	13.18 ± 0.10	13.96 ± 0.08	15.39 ± 0.05	14.47 ± 0.10	14.09 ± 0.08
TYM	nd	nd	nd	nd	nd	nd	nd
PUT	11.0 ± 0.12	14.47 ± 0.10	13.52 ± 0.10	13.67 ± 0.09	14.52 ± 0.09	27.62 ± 0.08	26.17 ± 0.09
Total amines	37.60 ± 0.44	40.19 ± 0.38	36.53 ± 0.38	38.36 ± 0.33	43.31 ± 0.31	53.61 ± 0.35	51.91 ± 0.33

Values represent the average of duplicate replications ± standard deviation.
Nd, not detected.
Mean values were calculated by using zero for nd.
AGM: agmatine; ETHA: ethanolamine; ETHYL: ethylamine; PUT: putrescine; TYM: tyramine.

Table 4
Biogenic amines (mg/L) in musts after 72 h; 2004–2005

Biogenic amines (mg/L)	Merlot	Syrah	Cabernet Franc.	Montepulciano	Sangiovese	Carmenere	Cesanese d'Affile
Wine Year 2004							
ETHA	4.47 ± 0.10	4.10 ± 0.81	3.79 ± 0.08	4.94 ± 0.07	4.26 ± 0.05	5.51 ± 0.07	6.09 ± 0.07
AGM	nd	nd	nd	nd	nd	nd	nd
ETHYL	5.06 ± 0.11	3.18 ± 0.10	3.74 ± 0.07	3.79 ± 0.03	4.16 ± 0.06	5.56 ± 0.09	4.83 ± 0.04
TYM	nd	nd	nd	nd	nd	nd	nd
PUT	2.07 ± 0.03	5.88 ± 0.08	nd	nd	5.64 ± 0.05	2.05 ± 0.04	nd
Total amines	11.60 ± 0.25	13.16 ± 0.25	7.53 ± 0.29	8.73 ± 0.07	14.06 ± 0.21	13.12 ± 0.22	10.92 ± 0.28
Wine Year 2005							
ETHA	11.46 ± 0.08	8.49 ± 0.08	10.99 ± 0.08	12.81 ± 0.07	15.73 ± 0.07	14.48 ± 0.07	15.15 ± 0.07
AGM	nd	nd	nd	nd	nd	nd	nd
ETHYL	14.35 ± 0.09	9.88 ± 0.08	15.23 ± 0.07	16.76 ± 0.09	18.42 ± 0.06	20.29 ± 0.09	15.74 ± 0.08
TYM	nd	nd	nd	nd	nd	nd	nd
PUT	8.17 ± 0.09	2.72 ± 0.03	2.76 ± 0.03	6.75 ± 0.05	5.23 ± 0.05	6.54 ± 0.04	4.88 ± 0.03
Total amines	33.98 ± 0.42	21.09 ± 0.25	28.98 ± 0.29	36.32 ± 0.32	39.38 ± 0.27	41.43 ± 0.28	35.77 ± 0.28

Values represent the average of four replications ± standard deviation.
Nd, not detected.
Mean values were calculated by using zero for nd.
AGM: agmatine; ETHA: ethanolamine; ETHYL: ethylamine; PUT: putrescine; TYM: tyramine.

Table 5
Biogenic amines (mg/L) in wine at the end of alcoholic fermentation; 2004–2005

Biogenic amines (mg/L)	Merlot	Syrah	Cabernet Franc.	Montepulciano	Sangiovese	Carmenere	Cesanese d'Affile
Wine Year 2004							
ETHA	4.46 ± 0.44	6.01 ± 0.41	5.44 ± 0.28	6.62 ± 0.34	5.37 ± 0.31	6.64 ± 0.78	9.64 ± 0.28
AGM	nd	nd	nd	nd	nd	nd	nd
ETHYL	1.33 ± 0.11	2.74 ± 0.08	2.39 ± 0.27	2.58 ± 0.56	2.71 ± 0.10	0.56 ± 0.10	2.48 ± 0.11
TYM	nd	nd	nd	1.70 ± 0.43	1.78 ± 0.25	nd	1.56 ± 0.51
PUT	0.90 ± 0.10	0.95 ± 0.12	1.82 ± 0.26	1.11 ± 0.15	1.10 ± 0.30	1.74 ± 0.30	1.94 ± 0.15
Total amines	6.69 ± 0.50	9.70 ± 1.05	9.65 ± 0.43	12.01 ± 1.50	10.96 ± 0.80	8.94 ± 1.05	15.62 ± 0.28
Wine Year 2005							
ETHA	19.86 ± 1.06	17.91 ± 0.81	11.31 ± 0.44	15.49 ± 0.35	14.68 ± 0.42	16.43 ± 1.33	21.80 ± 1.52
AGM	0.61 ± 0.08	0.42 ± 0.08	0.59 ± 0.08	2.90 ± 0.50	0.33 ± 0.07	0.61 ± 0.10	1.72 ± 0.30
ETHYL	1.91 ± 0.20	1.78 ± 0.25	0.97 ± 0.10	1.26 ± 0.15	1.77 ± 0.20	1.72 ± 0.15	2.00 ± 0.20
TYM	nd	nd	nd	nd	nd	nd	nd
PUT	3.49 ± 0.09	3.56 ± 0.15	1.15 ± 0.30	1.66 ± 0.11	1.33 ± 0.48	1.83 ± 0.20	2.01 ± 0.09
Total amines	25.87 ± 1.02	23.67 ± 1.15	14.02 ± 0.90	21.31 ± 0.90	18.11 ± 0.95	20.59 ± 1.31	27.53 ± 2.01

Values represent the average of four replications ± standard deviation.
Nd, not detected.
Mean values were calculated by using zero for nd.
AGM: agmatine; ETHA: ethanolamine; ETHYL: ethylamine; PUT: putrescine; TYM: tyramine.

Table 6
Biogenic amines (mg/L) in wine at the end of malolactic fermentation; 2004–2005

Biogenic amines (mg/L)	Merlot	Syrah	Cabernet Franc.	Montepulciano	Sangiovese	Carmenere	Cesanese d'Affile
Wine Year 2004							
ETHA	4.64 ± 0.10	7.34 ± 0.30	5.12 ± 0.10	7.24 ± 0.50	6.47 ± 0.41	6.06 ± 0.56	10.27 ± 0.67
AGM	nd	nd	nd	nd	nd	nd	nd
ETHYL	0.87 ± 0.09	0.66 ± 0.06	0.76 ± 0.11	0.46 ± 0.05	1.15 ± 0.12	0.85 ± 0.09	0.58 ± 0.05
TYM	nd	nd	nd	nd	nd	nd	0.97 ± 0.15
PUT	1.08 ± 0.10	0.73 ± 0.11	1.00 ± 0.04	0.73 ± 0.12	0.54 ± 0.08	1.72 ± 0.37	1.82 ± 0.31
Total amines	6.59 ± 0.29	8.73 ± 0.38	6.88 ± 0.05	8.43 ± 0.40	8.16 ± 0.49	8.63 ± 0.30	13.64 ± 0.80
Wine Year 2005							
ETHA	16.90 ± 0.70	20.96 ± 1.50	13.38 ± 0.52	18.02 ± 1.02	15.62 ± 0.24	10.50 ± 0.08	20.62 ± 0.97
AGM	13.66 ± 1.24	16.87 ± 1.80	12.62 ± 1.73	10.28 ± 1.04	11.61 ± 1.05	12.27 ± 1.10	12.38 ± 1.87
ETHYL	2.52 ± 0.09	5.09 ± 0.92	2.28 ± 0.20	2.87 ± 0.05	4.16 ± 0.51	2.92 ± 0.32	2.29 ± 0.28
TYM	nd	nd	nd	nd	nd	nd	nd
PUT	4.47 ± 0.15	4.67 ± 0.52	2.98 ± 0.08	2.68 ± 0.44	2.70 ± 0.14	4.02 ± 0.33	2.51 ± 0.36
Total amines	37.55 ± 0.58	47.59 ± 2.05	31.26 ± 1.79	33.85 ± 1.05	34.09 ± 1.23	29.71 ± 1.52	37.80 ± 1.52

Values represent the average of four replications ± standard deviation.
Nd, not detected.
Mean values were calculated by using zero for nd.
AGM: agmatine; ETHA: ethanolamine; ETHYL: ethylamine; PUT: putrescine; TYM: tyramine.

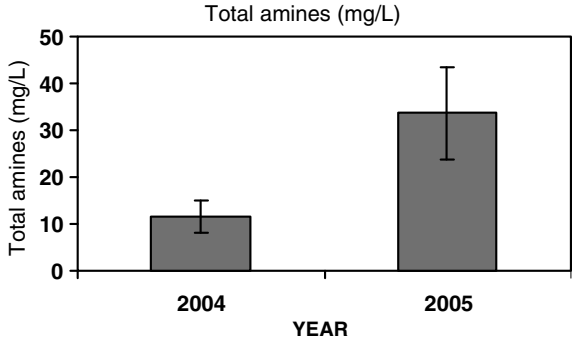

Fig. 1. Biogenic amine content in 2004 and 2005. Values are the mean of four repetitions. Bars indicate standard deviation.

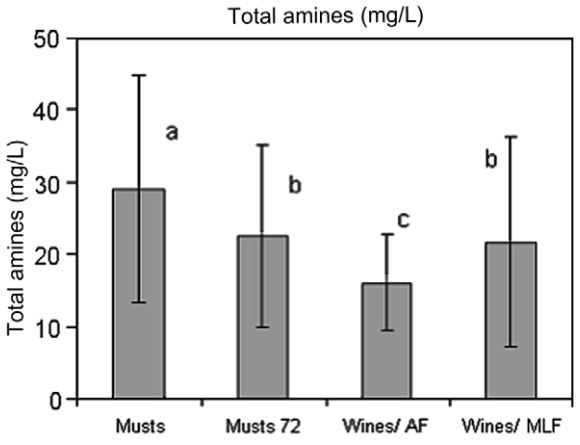

Fig. 2. Biogenic amine content in different media. Values are the mean of four repetitions. Bars indicate standard deviation. Mean values with the same letters are not significantly different according to the LSD test.

In 2004, the total amine content (independently from the medium and from the cultivar) was lower than in 2005; agmatine was always absent in 2004, and this could be related to the low level of general amine content.

A significant difference ($p \leqslant 0.05$) was also found between the total content of BA (calculated as addition of all amines found in the cultivars in two years) and the medium (Must, Must 72 h, Wine/AF, Wine/MLF) (Fig. 2), and between the total content of

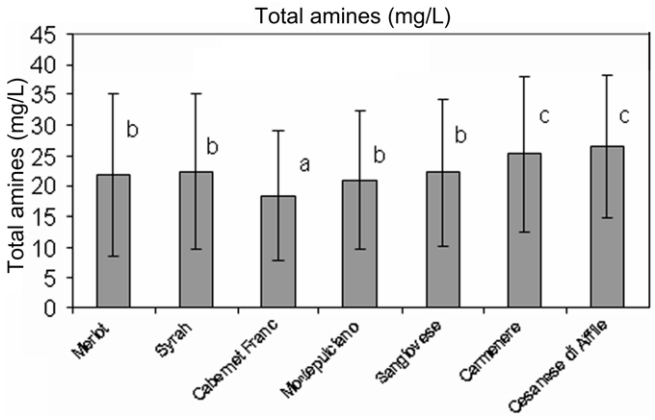

Fig. 3. Biogenic amine content in different cultivars. Values are the mean of four repetitions. Bars indicate standard deviation. Mean values with the same letters are not significantly different according to the LSD test.

BA (calculated as addition of all amines found in the media in two years) and cultivar (Fig. 3).

The wide range of values for standard deviation (Figs. 2 and 3) depend on the fact that the BA contents in 2004 and 2005 were very different.

3.4.1. Statistical analysis of individual biogenic amines

Each individual BA was examined by ANOVA to establish if it varied significantly in relation to year, cultivar and medium.

3.4.1.1. Individual biogenic amines and year effect. ANOVA point out that the content of the individual BA for all the examined cultivars differed significantly ($p \leqslant 0.05$) between the two years (data not shown). The total biogenic amines (TBA) content always was higher in 2005 than that in 2004 (Table 3). This suggests that the climatic conditions influence the content of BA.

3.4.1.2. Individual biogenic amines and cultivar effect. The ANOVA showed a significant difference ($p \leqslant 0.05$) between the content of individual BA and the cultivar with only exception of AGM (Table 7). Considering that the cultivars were grown in the same pedoclimatic conditions and with the same training system, the differences observed, with exception the AGM, can be attributed exclusively to a genetic variety characteristic. While the no significant difference observed between the cultivars and AGM content, could mean that the content of AGM in the grape is more influenced by pedoclimatic conditions than by the type of cultivar.

3.4.1.3. Individual biogenic amines and medium effect. ANOVA showed a significant difference ($p \leqslant 0.05$) between the individual BA content and the fermentation medium (Table 8). Analysis of the means according to the LSD test point out that, during the alcoholic fermentation, ETHA and TYM content increased, while the content of ETHYL and PUT decreased. During malolactic fermentation is evident the ability of the lactic acid bacteria to increase the AGM content and to decrease the TYM content again to values near to zero. The lactic acid bacteria had no effect on the content of ETHYL since there was no significant difference between the ETHYL content in Wine/AF and Wine/MLF.

Table 8
Individual biogenic amines (mg/L) in the different media

Medium				
Biogenic amines (mg/L)	Must	Must (72 h)	Wine/AF	Wine/MLF
ETHA	7.91^{a}	8.73^{a}	11.54^{b}	11.65^{b}
AGM	nd^{a}	nd^{a}	0.51^{a}	6.40^{b}
ETHYL	9.83^{b}	10.07^{b}	1.87^{a}	1.96^{a}
TYM	nd^{a}	nd^{a}	0.36^{b}	0.069^{a}
PUT	11.23^{d}	3.76^{c}	1.76^{a}	2.26^{ab}

Mean concentration of individual biogenic amines in all cultivars in 2004–2005 years.
Mean values with the same superscript letters in the same line do not differ significantly ($p \leqslant 0.05$; LSD test).
Nd, not detected.
Mean value were calculated by using zero for nd.
AGM: agmatine; ETHA: ethanolamine; ETHYL: ethylamine; PUT: putrescine; TYM: tyramine.

3.5. Correlations

Pearson's correlation coefficient, calculated to evaluate the relationships between individual and total BA contents, analysed in all media (Must, Must 72, Wine/AF, and Wine/MLF) in the two years, and between these and oenological parameters, showed significant correlation ($n = 77$; $p \leqslant 0.05$) as indicated in Table 9. Negative values indicate an inversely proportional correlation. Most significant correlations show a high confidence level ($p \leqslant 0.01$). A marked correlation was found between ETHYL and total biogenic amines (TBA) ($n = 77$, $r = 0.77$, with $p \leqslant 0.01$); between PUT and TBA ($n = 77$, $r = 0.79$, with $p \leqslant 0.01$); between ETHYL and PUT ($n = 77$, $r = 0.67$ with $p \leqslant 0.01$). ETHYL and PUT were the only individual amines to be inversely correlated to ethanol ($n = 77$, $r = -0.77$, $p \leqslant 0.01$; and $n = 77$, $r = -0.69$, $p \leqslant 0.019$, respectively). In fact, they decreased during AF. Significant correlations were found between acid parameters and ETHYL, with higher values of this amine with increasing TBA content and decreasing pH. The correlation between ETHA and malic and lactic acid ($n = 77$, $r = -0.27$, $p \leqslant 0.01$ and $n = 77$, $r = 0.34$, $p \leqslant 0.01$, respectively) was also good.

4. Discussion

In the discussion of the results, it should be remembered that the determination of BA, from must to wine after the MLF, has been

Table 7
Individual biogenic amines (mg/L) in different cultivars

Cultivar							
Biogenic amines (mg/L)	Merlot	Syrah	Cabernet Franc.	Montepulciano	Sangiovese	Carmenere	Cesanese d'Affile
ETHA	9.80^{b}	10.02^{b}	7.86^{a}	9.95^{b}	10.03^{b}	9.49^{b}	12.53^{c}
AGM	1.78	2.16	1.65	1.64	1.49	1.61	1.76
ETHYL	5.72^{b}	5.15^{a}	5.54^{ab}	5.79^{b}	6.64^{d}	6.52^{cd}	6.00^{bc}
TYM	nd^{a}	nd^{a}	nd^{a}	0.21^{b}	0.22^{b}	nd^{a}	0.32^{c}
PUT	4.49^{c}	4.80^{d}	3.28^{a}	3.32^{a}	3.84^{b}	7.62^{f}	5.91^{e}

Mean concentration of individual biogenic amines in all media in 2004–2005 years.
Mean values with the same superscript letters in the same line do not differ significantly ($p \leqslant 0.05$; LSD test).
Nd, not detected.
Mean values were calculated by using zero for nd.
AGM: agmatine; ETHA: ethanolamine; ETHYL: ethylamine; PUT: putrescine; TYM: tyramine.

Table 9
Correlation coefficient values between individual and total biogenic amines and between these and oenological parameters

	Correlation coefficient values
ETHA × AGM	0.56*
ETHA × TYM	−0.29*
ETHA × TBA	0.57*
ETHA × Malic acid	−0.27*
ETHA × Lactic acid	0.34*
AGM × ETHYL	−0.29**
AGM × Ethanol	0.28*
ETHYL × PUT	0.67*
ETHYL × TBA	0.77*
ETHYL × Ethanol	−0.77*
ETHYL × TA	0.25**
ETHYL × pH	−0.25**
TYM × TBA	−0.27**
TYM × Ethanol	0.27**
TYM × PH	−0.39*
PUT × TBA	0.79*
PUT × Ethanol	−0.69*
PUT × TA	0.29**
TBA × Ethanol	−0.56*

AGM: agmatine; ETHA: ethanolamine; ETHYL: ethylamine; PUT: putrescine; TYM: tyramine; TA: titratable acidity; TBA: total biogenic amines. (−) Negative correlation.
* $p \leqslant 0.01$.
** $p \leqslant 0.05$.

realized using sterile musts and with selected yeast and bacteria. The study has been planned to exclude possible interferences due to uncontrolled contaminating microorganisms.

After these preliminary remarks, the results show that the amines ETHA, ETHYL and PUT are all present in the grape. This agrees with the results of other authors: Ethanolamine and ethylamine were found in grape by Ough, Daudt, and Crowell (1981);Broquedis et al. (1989) found putrescine. Of these amines, only ethanolamine increases during AF: Ethanolamine is a precursor of phosphatidylcholine, the most abundant phospholipid in the membranes of eukaryotic cells (Choi, Martin, Murphy, & Voelker, 2004) and because of regulation phenomena in the metabolism of phospholipids, it is probably surrendered outside in the medium. This explains its increment.

Putrescine (PUT), initially present in must, strongly decreases during AF. This could be due to the fact that PUT is a polyamine and yeasts incorporate it in their metabolism, for example, as spermidine and spermine precursor. Uptake of putrescine is dependent on membrane potential, whereas excretion involves an exchange reaction between putrescine and ornithine (Igarashi & Kashiwagi, 1999). In *S. cerevisiae*, the gene for a polyamine transport protein (TPO1) was identified (Igarashi & Kashiwagi, 1999). During MLF, the concentration of PUT was almost unchanged.

Also ethylamine decreases during AF. This may be because it is employed as a carbon and nitrogen source by yeasts. At the end of AF, the remaining quantities of this amine were about 1–2 mg/L and that did not change significantly during MLF.

Agmatine (AGM), that was not present in musts, appears in low quantities during AF and it increases during MLF. Since we know that *S. cerevisiae* and *O. oeni* do not produce it, this increase could be due to hydrolysis of AGM–hydroxycinnamic acid complexes, coming from the grapes, as a result of the action of yeast and lactic acid bacteria. Even if there are no specific studies on grape, it is known that these hydroxycinnamic acid amides can be accumulated in plants in response to stress (Jin, Yoshida, Nakajima, & Murai, 2003; Newman, Von Röepenack-Lahaye, Parr, Daniels, & Dow, 2001; von Röepenack, Parr, & Schulze-Lefert, 1998). Recent studies indicate that synthesis of hydroxycinnamoylagmatine derivatives is induced in response to fungal infection of leaves (Peipp, Maier, Schmidt, Wray, & Strack, 1997; von Röepenack et al., 1998). Additionally, hydroxycinnamoylagmatine derivatives have been found in wheat (Jin, & Yoshida, 2000), and histochemical staining of epidermal leaf tissue indicates that these compounds might accumulate in cereals in general as a response to fungal infection (Wei, De Neergaard, Thordahl-Christensen, Colline, & Smedegaard-Petersen, 1994). These compounds and their derivatives may be implicated in cell wall fortification, restricting pathogen ingress, as well as being cytotoxic to the invading pathogens (Stoessl & Unwin, 1970; von Röepenack et al., 1998; Wei et al., 1994).

Tyramine (TYM) was absent in the musts analysed in this study, but in some studies, small quantities of this amine have been determined in must (Herbert, Cabrita, Ratola, Laureano, & Alves, 2005; Hernández-Orte, Peña-Gallego, Ibarz, Cacho, & Ferreira, 2006). The results show that small quantities of TYM appeared during AF, and since we know that yeasts do not produce it, in this case, the increase could also be caused by the hydrolysis of TYM–hydroxycinnamic acid complexes of grapes. Even if there are no specific studies on grapes, it is known that hydroxycinnamic amides are widely distributed in plants (Facchini, Hagel, & Zulak, 2002). These compounds are synthesized by enzymatic condensation of a coenzyme A-activated phenylpropanoid with an amine, either an aliphatic amine derived from polyamine biosynthesis or an arylamine, predominantly tyramine or anthranilate. The hydroxycinnamic acid amides are involved in plant defence responses, and some were found to be phytoanticipins, others phytoalexins (Kristensen, Burhenne, & Rasmussen, 2004). Pearce, Marchand, Griswold, Lewis, and Ryan (1998) demonstrated the synthesis of feruloyltyramine and *p*-coumaroyltyramine in response to wounding in tomato leaves.

Summarizing, data show that the amines ETHA, ETHYL and PUT are present in grapes and, also without external microbial contamination, some amines can appear in wine at the end of the AF or MLF as a consequence of the normal metabolic processes of yeast and bacteria. These amines are: ethanolamine, an intermediate in phospholipid synthesis that is surrendered in wine by *S. cerevisiae*; agmatine and tyramine, as probable consequences of hydrolysis of hydroxycinnamic amide compounds in grapes by the action of yeast and bacteria.

In conclusion, from these results, it is evident that grape variety is related to the presence of BA in wines. Significant differences were also found between the amine content in 2004 and 2005, indicating that climatic conditions also affect the accumulation of these compounds in grapes.

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