STUDY OF DIFFERENT BIOGENIC AMINES IN WINES FROM TWO PROTECTED DESIGNATIONS OF ORIGIN (PDOS) IN MURCIA (SPAIN) MULTIVARIATE CLASSIFICATION

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Abstract

Aims: Biogenic amines were determined in 109 wines from two wine-producing areas (Jumilla and Bullas PDOs) situated in the Murcia region (SE Spain).

Methods and Results: Biogenic amines (histamine, ethylamine, tyramine, phenylethylamine, putrescine, tryptamine and cadaverine) were analysed by RP-HPLC with o-phthaldialdehyde precolumn derivatization and fluorescence detection. Histamine and putrescine were the most prevalent amines in Jumilla wines (49 and 48%, respectively), whereas tryptamine and putrescine were the most prevalent ones in Bullas wines (19 and 38%, respectively). Multivariate analyses were used to attempt a preliminary classification of wines according to production area.

Conclusion: In general, wines from Jumilla PDO had a higher biogenic amine content than wines from Bullas PDO; however, all of them were safe from the health point of view.

Significance and impact of the study: This study demonstrates that the Jumilla and Bullas wines studied are safe to drink and that geographical classification based on biogenic amine profiles is not possible with the analysed samples.

Key words: biogenic amines, red wine, Jumilla PDO, Bullas PDO

Résumé

Objectifs: Les amines biogènes ont été dosées dans 109 vins issus de deux zones situées dans la région de Murcie (SE Espagne).

Méthodes et résultats: La teneur en amines biogènes (histamine, éthylamine, tyramine, phényléthylamine, putrescine, tryptamine et cadavérine) a été mesurée par RP-HPLC avec dérivation précolonne au o-phthaldialdéhyde et détection par fluorescence. L'histamine et la putrescine sont apparues comme les amines les plus répandues dans les vins de Jumilla (49 et 48 % respectivement), tandis que la tryptamine et la putrescine l'étaient dans les vins de Bullas (19 et 38 % respectivement). Des analyses multivariées ont été utilisées pour tenter une classification préliminaire des vins selon leur zone de production.

Conclusion: En général, les vins de l'AOC Jumilla montraient une teneur en amines biogènes plus élevée que ceux de l'AOC Bullas, sans pour autant présenter des risques d'un point de vue sanitaire.

Importance et impact de l'étude: Cette étude démontre que les vins de Jumilla et de Bullas étudiés sont propres à la consommation et que la classification en fonction de l'AOC n'est pas possible avec les échantillons analysés.

Mots clés: amines biogènes, vin rouge, AOC Jumilla, AOC Bullas

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INTRODUCTION

Wines are known to contain many biologically active compounds. The amount and composition of these compounds depend on the type of grapes, their degree of ripeness, the climate and soil of the viticultural area, as well as the winemaking techniques (Csomos *et al.*, 2002).

Biogenic amines (BAs) are organic nitrogenous compounds of low molecular weight, which are formed during metabolic processes in living organisms and are found especially in fermented food products (Ancín-Azpilicueta *et al.*, 2008). They are mainly produced as a consequence of the decarboxylation of the respective precursor amino acids by substrate-specific enzymes resulting from the microorganisms present in fermented food (Coton *et al.*, 1999) or by transamination of aldehydes by amino acid transaminases (Zotou *et al.*, 2003). They can be aliphatic (putrescine and cadaverine), aromatic (tyramine, phenylalanine) or heterocyclic (histamine). Aliphatic amines are associated with deficient sanitary conditions, aromatic and heterocyclic ones are suspected to have a toxic effect (Mafra *et al.*, 1999).

High concentrations of BAs can cause undesirable physiological effects in sensitive individuals, especially when alcohol and acetaldehyde are present (Bauza *et al.*, 1995). In wines, doses of 3 mg can be enough to produce such effects (Gloria *et al.*, 1998). Most food-borne intoxications caused by BAs are related to histamine (headaches, hypotension and digestive problems) or aromatic amines such as tyramine and phenylethylamine (migraines and hypertension) (Marcobal *et al.*, 2005). The complexity of the interactions between amines and with other compounds is the principal reason for the difficulty in defining the maximum tolerated limits (García-Marino *et al.*, 2010). The total BA content in wines varies from trace amounts up to 130 mg/l (Soufleros *et al.*, 1998). The toxicity levels for histamine are as follows: 8-40 mg, mild poisoning; 40-100 mg, moderate poisoning; over 100 mg, severe poisoning. In comparison, the consumption of over 100 mg of tyramine can cause migraines (Aygün *et al.*, 1999). The lack of legislation on the tolerated BA content in wine makes it difficult to import and export this product (Anli *et al.*, 2004). Some countries have established recommendations for histamine (2-10 mg/l), levels of Bas below 10 mg/ml seem to be acceptable.

The processes that generate BAs, together with the factors that influence their quantitative and qualitative presence are in some cases not well defined yet, although some authors have contributed their own conclusions. After some years of controversy about the origin of BAS in wines, many researchers have presented evidence supporting that, in winemaking, amines are mainly formed during malolactic fermentation (Bauza *et al.*, 1995; Soufleros *et al.*, 1998) by the action of lactic acid bacteria causing decarboxylation of free amino acids (Moreno-Arribas *et al.*, 2000) through substrate-specific decarboxylase enzymes. This property is not linked to a specific bacterial species, but it is usually strain-dependent (Leitao *et al.*, 2000; Moreno-Arribas *et al.*, 2003). This could, at least, partially explain why BAs are randomly produced: they are found in some wines and not in others. The main histamine and putrescine producers have been determined to belong to the Oenococcus oeni species (Lucas *et al.*, 2008).

One factor that determines the amino acid decarboxylase activity of bacteria in wines is pH. The trend of harvesting grapes with maximum ripeness results in wines with lower acidity and higher pH. Consequently, the diversity of the bacterial microflora increases, and with it the incidence of BA formation.

Another factor to to keep in mind is the possible formation of BAs during the maturation and aging of the wine. In recent studies, a progressive increase in the content of certain amines has been demonstrated during aging, particularly histamine, tyramine, putrescine and diaminobutane (García-Marino *et al.*, 2010).

In general, it has been observed that red wines contain more amines than either white or rosé wines (Ancín-Azpilicueta *et al.*, 2010), mainly because of winemaking practices and grape ripeness (Bauza *et al.*, 1995). Indeed, amines can already be found in red grapes and must (Daeschel, 1996).

The main purpose of the present work was to provide information about the occurrence of these compounds in wines from two Protected Designations of Origin (PDO, Denominación de Origen in Spanish) in Murcia (SE Spain) called D.O. Jumilla and D.O. Bullas. We also attempted to distinguish these different areas by using multivariate analysis on the data obtained from BA measurements.

MATERIALS AND METHODS

1. Wine samples

We studied a set of 109 randomly purchased red wines from two PDOs of the Murcia region (SE Spain), Jumilla (n = 74) and Bullas (n = 35), from the 2008 vintage. All analyses were done in triplicate.

2. Determination of biogenic amines in wines by HPLC

a) Reagents and standards

HPLC-grade acetonitrile, tetrahydrofuran and methanol supplied by J.T. Baker (Deventer, Holland) and ultrapure water generated by the Millipore Milli-Q system

(Bedford, MA) were used. Sodium acetate, o-phthaldialdehyde (OPA) and mercaptoethanol (MCE) were supplied by Merck (Darmstadt, Germany).

Biogenic amine (BA) standards including histamine, tyramine, putrescine, cadaverine, phenylethylamine, ethylamine and tryptamine were supplied by Sigma-Aldrich. Calibration solutions (100 ml each) were prepared by dissolving an accurately weighed amount of each amine in 0.1 N HCl and were stored at 4 °C for a maximum of one month. The calibration curve for each amine was obtained by analysing the standard solutions at different concentrations (1, 2, 10 and 20 ppm).

b) Chromatographic system

A liquid chromatography system consisting of a 1525 binary HPLC pump, a 717 Plus autosampler and a 2475 fluorescence detector (all provided by Waters, Milford, MA) was used. Chromatographic data were collected and analysed with Empower software (Waters, Milford, MA). The separations were performed on a Phenomenex (Kromasil C18 250 x 4.6 mm i.d.; 100 Å, 5 µm) column with a matching guard cartridge.

c) Chromatographic conditions

Eluent A contained sodium acetate 0.05 M at pH 6.6 and tetrahydrofuran (99:1 v/v). Eluent B contained methanol and acetonitrile (50:50 v/v). The gradient elution program was as follows: 0-12 min (57% A, 1 ml/min), 12-20 min (15% A, 1 ml/min), 20-53 min (0% A, 0.60 ml/min), 53-70 min (60% A, 1 ml/min). OPA-derivatized amines were detected using a fluorescence detector (excitation wavelength of 340 nm and emission wavelength of 420 nm). A standard mixture of BAs (final volume = 100 ml) was prepared by adding 1 ml of each amine stock solution to 0.1 N HCl. Its chromatographic profile is shown in Figure 1.

The HPLC coupled to OPA derivatization method was used for the separation of BA precursors as it allows high recovery and meet the necessary requirements with respect to accuracy, repeatability, cost and sensitivity (Soufleros *et al.*, 1998).

d) Derivatization procedure

Precolumn derivatization was used. The formation of the *o*-phthaldialdehyde (OPA) derivatives was performed automatically. The reagent solution consisted of 250 mg of OPA and 250 µl of 2-mercaptoethanol (MCE) in 5 ml of methanol and 50 ml of borate buffer (0.4 M, pH 10.5). An autosampler was used for the derivatization and 5 µl of wine sample were automatically mixed with 5 µl of reagent solution. Wine samples were filtered through Millipore filters (0.45 µm) and then directly injected into the HPLC system.

The identification of the compounds was carried out by injecting individual standards and comparing the UV spectra recorded with the diode array detector with those reported in the literature (Figure 1).

3. Statistical data treatment

All analyses were performed with the statistical package Statgraphics 5.1 Plus. The statistical methods used were as follows: regression analysis for the calibration curves; correlation analysis (Pearson) among all studied variables; cluster analysis (CA); linear discriminant analysis (LDA); and principal component analysis (PCA).

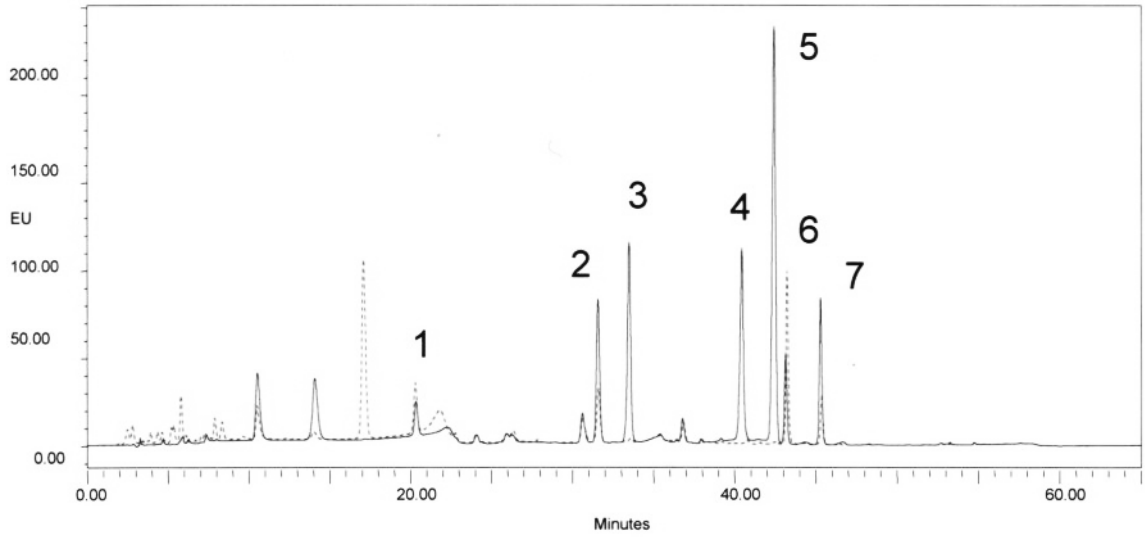


Figure 1 - RP-HPLC chromatographic profiles of the OPA-derivates of a standard solution (-) of biogenic amines and wine (…).
Peak identities: histamine (1), ethylamine (2), tyramine (3), tryptamine (4), phenylethylamine (5), putrescine (6), cadaverine (7).

The purposes of these statistical methods are different. The sign of the Pearson's correlation coefficient indicates the direction of the relationship and its absolute value indicates the strength of the correlation, with larger absolute values indicating stronger relationships. Cluster analysis (CA) is used to classify objects into groups. LDA is a method to discriminate between two or more groups of samples. It is a supervised pattern recognition technique, which means that the class membership has to be known prior to analysis. Finally, PCA has proved to be a powerful tool for pattern recognition, classification, modelling and other aspects of data evaluation.

RESULTS AND DISCUSSION

1. Study of amine contents in wine

The physicochemical properties (alcohol, total sulfur dioxide, total acidity, pH, methanol, anthocyanins and total phenols) of the analysed Jumilla and Bullas wines are reported in Table 1. The concentration of biogenic amines (BAs) detected by HPLC in these wines varied over a wide range, probably reflecting large compositional difference among the wine samples (data shown in Table 2). In our case, the wine samples from Jumilla PDO had higher pH values (3.68-3.71) that the ones from Bullas (3.57-3.62), and they had the highest concentrations of BAs. In addition, the concentration of sulfur dioxide was lower in the wines from this PDO, suggesting a lower antimicrobial protection that could explain the greater production of BAs. Silla-Santos (1996) postulated that the concentration of histamine and tryptamine in wine may be influenced by pH. The wide range of BA concentrations among the wine samples could also be explained by variations in the bacterial strains involved in malolactic fermentation and by some winemaking factors such as grape variety, vintage, aging of wines and grape skin maceration (Rosi *et al.*, 2009).

The concentrations of BAs in wines (range and mean ± standard deviation) together with the number of wines in which each of the amines were measured as it is indicated in Table 2. In general, putrescine was the most abundant compound with a concentration ranging from 0 to 35.13 mg/l. High concentrations of histamine (0 to 7.49 mg/l) and tyramine (0 to 9.89 mg/l) were also found. Regarding histamine, no sample had a concentration over 18 mg/l. These findings are in accordance with other results reported in the literature for wines from different areas of Spain (García-Villar *et al.*, 2007; Marcobal *et al.*,

Table 1 - Physicochemical properties from wines of Jumilla and Bullas PDOs.

	Jumilla (n=74)		Bullas (n=35)	
	Range	Mean ± SD	Range	Mean ± SD
Alcohol (% v/v)	13.81-14.07	13.94±0.13	13.84-14.03	13.94±0.10
Total sulfur dioxide (mg/L)	39.04-44.01	41.52±2.48	47.64-54.58	51.11±3.47
Total acidity (g/L)	5.17-5.32	5.24±0.07	5.10-5.24	5.17±0.07
pH	3.68-3.71	3.69±0.01	3.57-3.62	3.59±0.03
Methanol (mg/L)	184.1-198.1	191.1±6.69	159.9-185.3	172.6±12.70
Anthocyanins (mg/L)	419.9-449.1	434.5±4.58	381.3-445.0	413.2±3.18
Total Phenol Index	56.73-59.76	58.24±1.51	48.75-55.13	51.95±3.18

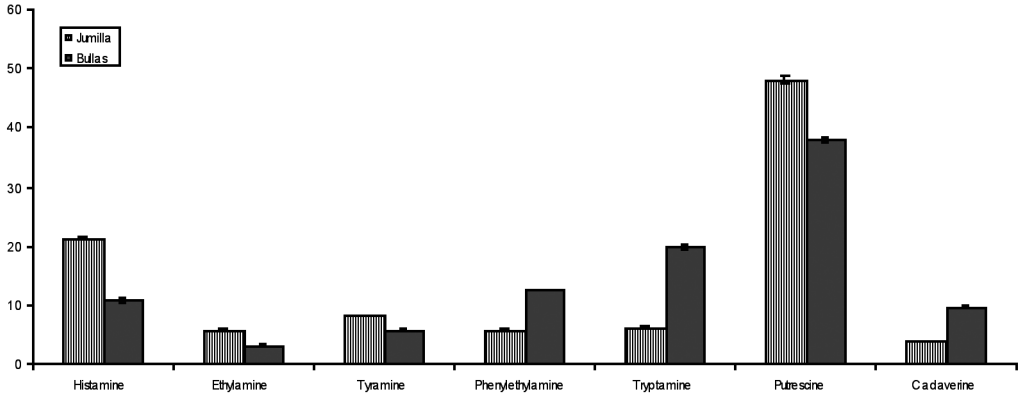


Figure 2 - Concentration (%) of selected amines in wines as function of the production area.

2006; Martín-Álvarez *et al.*, 2006; Landete *et al.*, 2005) and Portugal (Ferreira and Pinho, 2006).

In the wines studied, the average content of total BAs was 5.72 mg/l for Jumilla wines and 5.38 mg/l for Bullas wines. The highest amount recorded was 50.20 mg/l and 18.01 mg/l and the lowest one was 0 mg/ml and 0.09 mg/l for Jumilla and Bullas wines, respectively. Wines from Jumilla had higher mean amounts of histamine (1.23 ± 0.29 mg/l), ethylamine (0.34 ± 0.07 mg/l), tyramine (0.47 ± 0.16 mg/l), phenylethylamine (0.34 ± 0.07 mg/l) and putrescine (2.75±0.64 mg/l). Marques *et al.* (2008) found that tyramine was the most prevalent BA in Portuguese wines from the 2003 harvest, while in 2004 putrescine was the most important one. Other authors reported that histamine, tyramine and putrescine were the BAs found at highest concentrations in wine, but cadaverine, phenylethylamine and isoamylamine could also be found (Bauza *et al.*, 1995; Silla-Santos *et al.*, 1996).

The wines from Bullas PDO had higher mean amounts of tryptamine (1.07 ± 0.29 mg/l) and cadaverine (0.53 ± 0.10 mg/l) than Jumill a wines. Zhijun *et al.* (2007) did not find tryptamine in any of the 38 tested red wines from different areas of China. The amounts of ethylamine and phenylethylamine never exceeded 1 mg/l in Bullas wines, and individual amine concentrations were similar to those reported in the literature (Soufleros *et al.*, 1998; Vázquez-Lasa *et al.*, 1998).

On the other hand, different results were observed when comparing individual BA content in percentage (%) rather than individual concentration (mg/ml) (Figure 2). This histogram shows that lower amounts of histamine, ethylamine, tyramine and putrescine were found in wines from Bullas PDO. Histamine, putrescine and tryptamine accounted for the largest percentages of total BAs and were detected in 25 and 16%, 31 and 30%, and 13 and 17 % of Jumilla and Bullas wines, respectively (data not shown). Similar findings were reported for Austrian (Lehtonen *et al.*, 1992), French (Bauza *et al.*, 1995; Soufleros *et al.*, 1998), Oregon (Gloria *et al.*, 1998) and other Spanish wines (Vázquez-Lasa *et al.*, 1998).

The maximum level of BAs allowed in wines by some countries, according to Lehtonen (1996), Bauza *et al.* (1995) and Loret *et al.* (2005), is 10 mg/l. Among the wines analysed in this study, only one from Jumilla PDO contained more than 10 mg/l BAs. Phenylethylamine and tyramine contents were found in all cases to be below the levels considered as toxic to human health: 3 and 25-40 mg/l, respectively (Bauza *et al.*, 1995). The recommended maximum limits for histamine in wine are 2 mg/l in Germany, 5-6 mg/l in Belgium, 10 mg/l in Switzerland and Austria, 8 mg/l in France and 3 mg/l in the Netherlands (Busto *et al.*, 1996).

2. Correlations

Pearson's correlation coefficient was calculated to evaluate the relationship between individual and total BA content in all analysed wines from Jumilla and Bullas PDOs, in order to obtain some data about its global characterization.

Significant correlations (n = 109; $p \leq 0.05$) was found as shown in Table 3. Most significant correlations showed a high confidence level ($p \leq 0.01$). Subsequent studies were focused on evaluating correlations between amines. These correlations were checked statistically, and analysis among individual amines showed a significant positive correlation ($p \leq 0.01$) between some of them, especially ethylamine and putrescine (r = 0.837), ethylamine and tyramine (r = 0.736), tyramine and putrescine (r = 0.703) and phenylethylamine and cadaverine (r = 0.611). Marcobal *et al.* (2005) reported a high correlation coefficient between some of the amines analysed here, especially histamine and putrescine (r = 0.630), phenylethylamine and cadaverine (r = 0.618), histamine

Table 2 - Biogenic amine contents (mg/l) of the 109 wines analysed.

	Jumilla (n=74)		Bullas (n=35)	
	Range	Mean ± SD	Range	Mean ± SD
Histamine	nd-17.14	1.23±0.29	nd-7.49	0.58±0.23
Ethylamine	nd-3.93	0.34±0.07	nd-0.80	0.18±0.04
Tyramine	nd-9.89	0.47±0.16	nd-1.29	0.32±0.06
Phenylethylamine	nd-3.93	0.34±0.07	nd-0.80	0.18±0.04
Tryptamine	nd-2.84	0.36±0.07	nd-8.47	1.07±0.29
Putrescine	nd-35.13	2.75±0.64	nd-14.97	2.05±0.46
Cadaverine	nd-1.83	0.22±0.05	nd-2.32	0.53±0.10
Total Ba[a]	nd-50.20	5.72±1.04	0.09-18.01	5.38±0.71

and: non detectable ; [a] Total biogenic amines (sum of histamine, ethylamine, tyramine, phenylethylamine, tryptamine, putrescine and cadaverine)

and tyramine (r = 0.571) and, to a lesser extent, histamine and methylamine (r = 0.441). These findings suggest that the formation of these amines may be affected by the same lactic acid bacteria responsible for the decarboxylation of the amino acids with different enzymatic activities. Soufleros *et al.* (1998) reported a high correlation coefficient between histamine and tyramine in French wines (r = 0.748). We also found a significant correlation between both of these BAs, although the correlation coefficient was lower (r = 0.251).

With the physicochemical parameters considered, we only found significant correlation between cadaverine and sulfur dioxide and between tyramine and total acidity, although the level of significance was lower ($p < 0.05$) and the correlation coefficients were low (r = -0.287 and r = -0.229, respectively).

3. Cluster analysis

Cluster analysis is used to classify objects into groups. It can be considered to be an alternative to principal component analysis (PCA). To be able to cluster objects, one must measure their similarity. The dissimilarity between two objects is a distance measure. The distance between two points is well defined; the simplest is the Euclidean distance. Ward's method is perhaps the most popular, as it basically looks at cluster analysis as an analysis of variance problem, instead of using distance metrics or measures of association.

In order to obtain a preliminary view of the main causes of variation in BAs, cluster analysis was carried out on the data obtained from the quantification of amines in the 109 wines studied. Figure 3 shows the resultant dendrogram. The squared Euclidean distance was taken as a measure of the proximity between two samples and the Ward's method was used as a linkage rule. The variables were previously standardized. Cluster analysis showed that grouping of wines based on BA concentration did not correlate with geographical origin. This result had been also found in 61 wines from different Spanish areas (Marcobal *et al.*, 2005). In our study, clustering by Ward's method was not able to clearly distinguish between Jumilla and Bullas wines. Therefore, the results from Figure 3 suggest that there were no differences in the levels of BAs according to their area of origin.

Table 3 - Pearson´s correlation coeficient valuesbetween individual and total biogenic amines.

	Coeficient
Histamine x Ethylamine	0.542**
Histamine x Tyramine	0.251**
Histamine x Putrescine	0.440**
Ethylamine x Tyramine	0.736**
Ethylamine x Putrescine	0.837**
Phenylethylamine x Cadaverine	0.611**
Phenylethylamine x Tryptamine	0.460**
Cadaverine x Tryptamine	0.421**
Tyramine x Putrescine	0.703**
Tyramine x Total acidity	-0.229*
Cadaverine x Total sulfur dioxide	-0.287*

Level of significance: p< 0,05*; p<0,01

4. Discriminant analysis

Linear discriminant analysis (LDA) was used to attempt to differentiate between Jumilla and Bullas wines. The discriminant functions are shown in Figure 4. The percentage of correctly classified wines, using all the variables analysed, was 73.42%. More specifically, 71.43% of Jumilla wines and 84.4% of Bullas wines were correctly classified. A good classification of wine samples

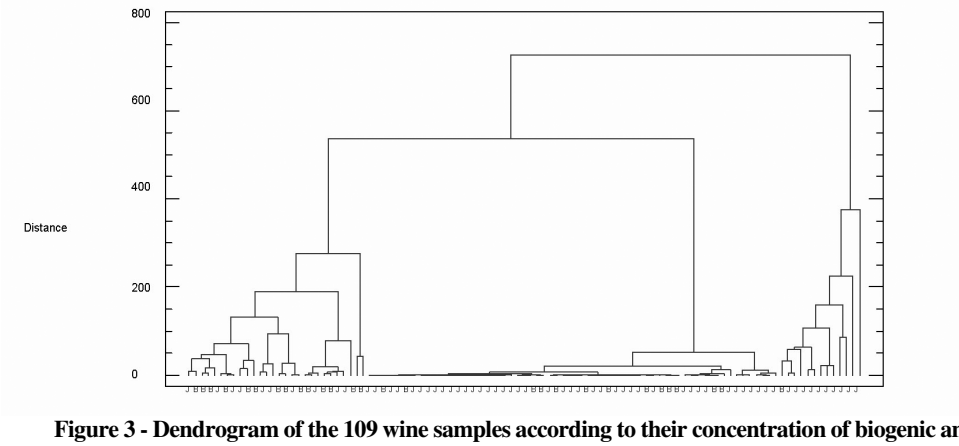


Figure 3 - Dendrogram of the 109 wine samples according to their concentration of biogenic amines.
The samples' labels correspond to the production areas: Jumilla (J) and Bullas (B) PDOs.

was achieved using tyramine, histamine and phenylethylamine. Discriminant analysis was able to identify the production area of these wines according to their BA content. Nevertheless, this statistical tool could not completely differentiate between them.

5. PCA analysis

Principal component analysis (PCA) is a powerful visualisation tool for data evaluation, which can graphically represent intersample and intervariable relationships. Moreover it provides a way to reduce the dimensionality of the data. PCA is an unsupervised method of pattern recognition in the sense that no grouping of the data has to be known before the analysis. Using PCA, class membership is easy to indicate on a score plot.

The percentage of captured variance was 25%, 15% and 12% for PC1, PC2 and PC3, respectively. The interpretation of the amine pattern of wines was mainly based on the representation of information contained in PC2 and PC3 (Figure 5A), since all the samples appeared together in the plot of PC1 versus PC2 (data not shown). In spite of that, the interpretation should be cautious as the percentage of variance retained with components 2 and 3 was quite low. The sample distribution on PC2 and PC3 reflected the influence of the production area: wines from Bullas were mainly situated in the top right region of the graph, while wines from Jumilla appeared in the left bottom region. Obviously, this distribution should not be rigidly interpreted, as some samples appeared in the intermediate region and were intermingled to some degree. Similarly, García-Villar *et al.* (2007) observed that wines from certain Spanish regions (Valdepeñas, Penedés and La Mancha) were found in compact areas in a PCA plot, whereas others (Navarra and Rioja wines) were widely distributed without the presence of distinct areas.

Regarding the loading plots, the distribution of variables on PC2 and PC3 (Figure 5B) showed that tryptamine, phenylethylamine and cadaverine were clustered to the right. This finding confirmed that, as mentioned above, these three amines are reasonably correlated and their behaviour is similar. Moreover, they were present in higher amounts in wines from Bullas. The same occurred with ethylamine, tyramine, histamine and putrescine, which were clustered to the left and were present in higher amounts in wines from Jumilla.

CONCLUSIONS

Wines from Jumilla PDO had a higher content of BAs than wines from Bullas PDO. Nonetheless, the levels of BAs found in the wines studied were generally low, especially when compared with other foodstuffs (Soufleros *et al.*, 2007), where BAs can be present in much higher concentrations.

From this work, it can be concluded that most of the wines from Jumilla PDO have low concentrations of BAs, because only 3% had concentrations of tyramine and histamine greater than 10 mg/l. Regarding the wines from

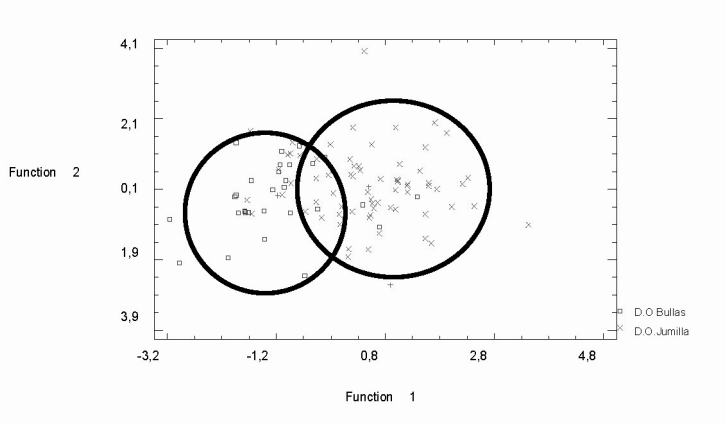
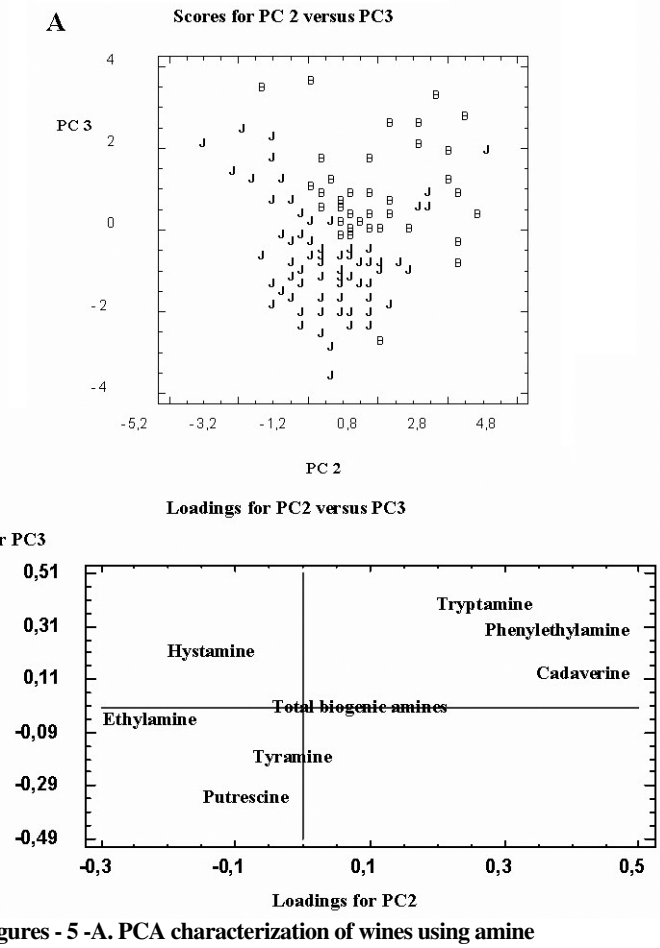


Figure 4 - Distribution of wines in the two-dimensional coordinate system defined by the two first discriminant functions used to differentiate between their production area (Bullas or Jumilla PDO).



Figures - 5 -A. PCA characterization of wines using amine contents and physicochemical parameters as analytical data. 5.B. Loadings for PC2 versus PC3.

Bullas PDO, none of them had concentrations of tyramine and histamine higher than 8 mg/l and only 3% contained more than 10 mg/l of putrescine.

The multivariate analysis methods were not equally useful for classifying wines with respect to their production area (Jumilla or Bullas). Cluster analysis was not enough to classify all the wines studied since the groups formed on the basis of BA concentration did not correlate with any grouping based on their area of origin. PCA (PC2 versus PC3) allowed for a better classification, although the interpretation should be cautious as the percentage of variance retained with components 2 and 3 was quite low. Only LDA correctly classified 71% of wines from Jumilla PDO and 84% of wines from Bullas PDO; so it was the best multivariate analysis method to classify the wines by area of origin.

From these results, it is evident that other factors (precursors, types of bacteria, etc.) are related to the presence of BAs in wines.

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