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Evaluation of Biogenic Amines in Organic and Non-Organic Wines by HPLC OPA Derivatization

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Summary

Organic and non-organic wines, selected on the basis of consumers' preference towards healthy products, were produced from the grapes of *Vitis vinifera* varieties Semillon, Colombard, Cabernet Sauvignon, Merlot and Carignan and possible effects of different wine making techniques were considered. Concentrations of histamine, tyramine, putrescine, cadaverine, ethylamine, methylamine, tryptamine, agmatine and β-phenylethylamine were quantified by HPLC fluorescence detection of *o*-phthaldialdehyde (OPA) derivatives. The order of analyzed parameters in all wines from the highest to the lowest quantities was determined as follows: putrescine > histamine > ethylamine > methylamine > agmatine > tyramine > cadaverine > tryptamine. One of the analyzed compounds (β-phenylethylamine) was not detected. The highest average values for organic and non-organic wines were found as follows (in mg/L): putrescine 5.55, ethylamine 0.825 and histamine 0.628 in organic wines, and putrescine 3.68, histamine 1.14 and agmatine 0.662 in non-organic wines. Considering the wine type (organic/non-organic), an important difference was determined for putrescine. Putrescine content in organic wines was significantly greater than in non-organic ones ($p=0.008$). Evaluating colour of wines (white/red), a statistically significant difference was obtained for methylamine ($p=0.028$). Taking into account only grape varieties, statistically significant differences were found for histamine, methylamine, tyramine and cadaverine ($p<0.05$). The results of principal component analysis demonstrated close relations between the following biogenic amines and wines: agmatine and non-organic Colombard; tryptamine or cadaverine and both organic and non-organic Cabernet Sauvignon wines.

Key words: organic wine, biogenic amines, HPLC, grape varieties, OPA derivatization

Introduction

Biogenic amines are basic nitrogenous compounds formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones. They are endogenously synthesized from amino acid precursors by metabolic pathways in mammalian cells that usually involve decarboxylation of the parent amino acid. Similarly, they can be generated exogenously in the intestinal tract by bacteria-induced decarboxylation of amino acids released by the enzymatic hydrolysis of dietary proteins (*1,2*).

Biogenic amines in food and beverages are formed by the enzymes from raw material or are generated by microbial decarboxylation of amino acids (*3*). Prerequisites for a considerable biogenic amine formation are the availability of free amino acids, the presence of decarboxylase-positive microorganisms, conditions that allow bacterial growth, decarboxylase synthesis and decarboxylase activity (*4*). Biogenic amines are undesirable in all

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foods and beverages because if absorbed at too high concentrations, they may cause headaches, respiratory distress, heart palpitation, hypertension or hypotension, and several allergenic disorders (*3*). A legal upper limit of *m*(histamine)/*m*(food)=100 mg/kg and *m*(histamine)/*V*(alcoholic beverage)=2.0 mg/L has been suggested (*4*). The recommended upper limit of histamine in wines (in mg/L) is 2.0 in Germany, 5.0 to 6.0 in Belgium, 8.0 in France and 10.0 in Switzerland (*5*).

The identification of biogenic amines in wine samples has been carried out in several investigations. Soufleros *et al*. (*6*) demonstrated that the levels of histamine, tyramine and putrescine were lower after alcoholic fermentation and increased in most wines during malolactic fermentation. Biogenic amines such as methylamine, ethylamine and cadaverine, already present in grape must, were produced and degraded during vinification (*7*). More than 20 amines have been identified in wines and their total concentration has been reported to range from a few to about 50 mg/L, depending on many factors including wine making conditions, must fermentation and aging. Histamine, tyramine and putrescine are the most significant biogenic amines encountered in wines. Among these, histamine has been studied the most and several papers reported its presence in wine (*2,8–13*).

Organic viticulture has aroused great interest among ecologically aware consumers. In the European Union, organic agricultural production is regulated by Council Regulation (EEC) No 2092/91. Organizations of organic winegrowers all have their own rules and regulations (*14*). Although demands related to organic production vary, some rules are applied generally. For example, the use of herbicides, chemosynthetic insecticides and organic fungicides is uniformly prohibited.

Organic agriculture in Turkey began with organically grown dried grapes and figs through the demands of European countries in 1984. The number and quantity of organic products have gradually increased. As a result of these developments, the Ministry of Agriculture and Rural Affairs published Turkish Legislation on Organic Agriculture in Official Newspaper in 1994. It conforms to the EU Regulation EU numbered 2092/91 and basic standards of IFOAM (International Federation of Organic Agriculture Movements).

The aim of the present study is to determine the biogenic amine levels in wines from five different organic and non-organic grapes of *Vitis vinifera* varieties (Merlot, Carignan, Cabernet Sauvignon, Semillon and Colombard) and to describe the relations between parameters.

Materials and Methods

Materials

Grapes of *Vitis vinifera* var. Carignan (red), Cabernet Sauvignon (red) Merlot (red), Colombard (white) and Semillon (white), were used. Organically and non-organically grown grapes were supplied from Yazgan Company, Turkey. Organic grapes were produced under the control of INAC Company (International Nutrition and Agriculture Certification) that has certification for organic grape production.

Wine processing

All grapes (25 kg of each grape variety) were transported to the Food Engineering Department and crushed within 24 hours of harvest.

Organically grown grapes were hand harvested from a vineyard at KemalPasa, Ulucak, Turkey. Maturity was evaluated according to sugar content and acidity of each grape variety. Non-mechanically crushed grapes were allowed to ferment with active yeasts in musts. Before fermentation, 5.0 % SO_2 solution was added into must until 25.0 mg/L of total SO_2 concentration was obtained. Fining and filtering were done without using any stabilizing and fining agents. All organic wine production steps were done according to standards for organic winemaking and Council Regulation (EEC) No 2092/91.

Non-organically grown white grapes were crushed, destemmed and pressed immediately in a hydraulic press. Portions of juices (3-litre glasses) were collected, sulphited (50.0 mg/L of SO_2) and kept for 6 hours. After that the juices were inoculated with Fermivin (2.0 %, *Saccharomyces cerevisiae* 7013 (INRA), Gist Brocades Co.). Pectolytic enzyme Rapidase Ex Color (2.0 g/hL) was added and allowed to ferment at 25 °C until it was dry. Non-organically grown red grapes were crushed, destemmed and prepared for skin fermentation treatments. The crushed grapes were sulphited (50.0 mg/L of SO_2), inoculated with Fermirouge (2.0 %, *Saccharomyces cerevisiae* 7000 (INRA), Gist-Brocades Co.), supplied with pectolytic enzyme Rapidase Ex Color (4.0 g/hL) and the skin was allowed to ferment for 5 days at 25 °C. The pomace was stirred and pushed down twice a day. After that alcohol fermentation in glass vessels was allowed until it was dry. Bentonite (150 g/hL) and gelatin (250 g/hL, Merck) were used as fining agents for white wines. For the red ones, only gelatin (250 g/hL) was used. After filtration and bottling, wines were stored at 15 °C. The concentrations of biogenic amines were determined after six months of aging. For each wine type (organic/non-organic) and each grape variety production was done in duplicate.

Reagents and standards

Biogenic amine standards used were supplied from the following companies: histamine, tyramine, cadaverine, tryptamine, agmatine and β-phenylethylamine from Sigma, methylamine from Merck, putrescine from Fluka and ethylamine from Acros Organics. Standard solutions of biogenic amines were prepared by dissolving each of them separately in 0.10 M HCl solution. These standard solutions contained 1.0 mg of base form of biogenic amine in 1 mL. To prepare a standard mixture of biogenic amines, suitable volumes (0.5 to 1 mL) of standard solutions were mixed, adjusted to pH=9.0 and diluted to 25.0 mL with distilled water. The other reagents: sodium hydroxide, ammonium chloride, *o*-phthaldialdehyde (OPA) and 2-mercaptoethanol were supplied from Merck, hydrochloric acid and tetrahydrofuran from J.T. Baker, methanol HPLC grade from Lab-Scan, boric acid and sodium acetate$\times 3H_2O$ from Riedel.

Apparatus

Chromatographic experiments were performed using Hewlett-Packard 1050 liquid chromatograph equipped with

Waters 470 scanning fluorescence detector, a gradient elution pump and an injection loop of 20 μL. Excitation and emission wavelengths were 340 and 420 nm, respectively. The chromatographic column was Phenomenex, Bondclone C_{18} (particle size 10 μm, 300×3.9 mm i.d.).

Preparation of derivatization reagent

Method of Üren and Karababa (*15*) was used to prepare OPA reagent. A mass of 0.20 g of *o*-phthaldialdehyde was dissolved in 9.0 mL of methanol. To this solution 1.0 mL of 0.40 M (pH=9.0) borate buffer and 160 μL of 2-mercaptoethanol were added. The borate buffer was prepared as follows: 2.47 g of boric acid was dissolved in sufficient distilled water, pH was adjusted to 9.0 by 1.0 M NaOH solution and then diluted to 100 mL. The OPA reagent was stored at 4 °C.

Derivatization of standards and samples

Standard biogenic amines were derivatized prior to column injection as follows (*15*): to 400 μL of methanol 25.0 μL of standard mixture of biogenic amines and 475 μL of distilled water were added. Following the addition of 100 μL of OPA reagent, the mixture was filtered through a 0.50-μm pore size filter (Hamilton 81610 Gastight) and 20.0 μL of filtrate were immediately injected on the column. Samples were derivatized as follows: the pH of 25.0 mL of wine sample was adjusted to 9.0 with 0.10 M NaOH solution and diluted to 30.0 mL with distilled water. To 100 μL of wine sample 400 μL of methanol and 400 μL of distilled water were added. After the addition of 100 μL of OPA reagent, the mixture was filtered through a 0.50-μm pore size filter and 20.0 μL of the solution were immediately injected on the column. Derivatization temperature was 25 °C. Chromatograms were obtained for two aliquots of the same wine sample which underwent the whole analytical procedure. Quantifications were performed by the standard addition method (*7*,*10*,*15*). A volume of 10 μL of standard mixture of biogenic amines and 100 μL of wine sample were added into 390 μL of distilled water and derivatization was carried out by adding 100 μL of OPA reagent, following the addition of 400 μL of methanol. A volume of 20.0 μL of the solution was injected on the column. This standard addition procedure was performed in two replicates. According to Üren and Karababa (*15*) reproducibilities (RSD/%) of biogenic amine determinations were 8.8, 15.7, 11.2, 8.4, 11.1, 22.3, 14.6, 15.5 and 16.1 for agmatine, histamine, methylamine, ethylamine, tyramine, tryptamine, putrescine, cadaverine and β-phenylethylamine, respectively. Mafra *et al.* (*7*) found values between 3.8 and 22.8 for pre-column *o*-phthaldialdehyde derivatization of biogenic amines. Mafra *et al.* (*7*) also reported that detector response was linear up to 0.5 mg/L of individual biogenic amines on average in the injected solution of OPA derivatives.

Chromatographic conditions

Column temperature was 25 °C with the flow rate of 1 mL/min. Method of Üren and Karababa (*15*) was used to prepare the mobile phase as follows: solvent A: [0.050 M acetate buffer/tetrahydrofuran (96/4)]:methanol, 60:40; solvent B: methanol. The pH of solvent A was adjusted to 6 and filtered through Schleicher & Schuell 589/3 filter paper. Both solvents were degassed for 15 min before use. A binary gradient elution was used for the separation of OPA derivatives of biogenic amines. Solvent A (in %): 75.00 (0 min), 75.00 (8 min), 66.67 (12 min), 50.00 (25 min), 0 (30 min), 66.67 (35 min), 75.00 (40 min); solvent B (in %): 25.00 (0 min), 25.00 (8 min), 33.33 (12 min), 50.00 (25 min), 100 (30 min), 33.33 (35 min), 25.00 (40 min).

Data evaluation

Significant differences between averages were obtained at 95 % significance level. The least significant differences (LSD) test and all other tests were performed by using SPSS 10 program. Statistica was used for matrix evaluation. The cluster analysis was performed as joining type (tree cluster) by using raw data. Furthest neighbour shape was selected as linkage and 1-Pearson r as a distance measure. Missing data were case wise deleted. Scale plot was demonstrated as (Dlink/Dmax) ×100.

Principal component analysis (PCA) was performed using multivariate exploratory techniques. PCA permits visualization of the original arrangement of wines in an *n*-dimensional space by identifying the directions in which most of the information is retained. It is therefore possible to explain differences among various wines by means of these factors obtained from the generalized correlation matrix of the data sets and at the same time determine which variables contribute most to such differentiation.

Results and Discussion

The mean values and standard deviations of biogenic amines in all wines (Table 1) were found as follows (in mg/L): agmatine (0.331±1.0), histamine (0.886±1.1), methylamine (0.428±0.36), ethylamine (0.607±0.45), tyramine (0.191±0.46), tryptamine (0.016±0.052), putrescine (4.62±1.3) and cadaverine (0.070±0.16). The highest concentrations of agmatine, histamine, methylamine, ethylamine, tyramine, tryptamine, putrescine and cadaverine were determined (in mg/L) in: Colombard (non-organic) 3.31, Merlot (organic) 3.14, Merlot (organic) 1.15, Merlot (organic) 1.40, Merlot (non-organic) 1.42, Cabernet Sauvignon (organic) 0.165, Merlot (organic) 6.28, Cabernet Sauvignon (non-organic) 0.490, respectively. Chromatogram of standard mixture of biogenic amines under the conditions specified above can be seen in Fig. 1. The order of analyzed parameters from the highest to the lowest quantities for all wines was determined as follows: putrescine > histamine > ethylamine > methylamine > agmatine > tyramine > cadaverine > tryptamine.

In the review written by Lehtonen (*5*), the mean values of histamine were reported as 0.26 and 3.4 mg/L, of tyramine 0.6 and 3.1 mg/L, of putrescine 1.1 and 14.3 mg/L and cadaverine 0.3 and 0.5 mg/L in white and red wines respectively. Loukou and Zotou (*16*) evaluated the biogenic amine content in Greek alcoholic beverages and determined the levels of methylamine of 0.513–0.903 and 0.588–1.503 mg/L, ethylamine of 0.537–2.162 and 0.544–2.639 mg/L, putrescine of 0.528–2.539 and 0.900––3.148 mg/L, cadaverine of 0.118–0.208 and 0.037–0.528

Table 1. Concentrations of biogenic amines in organic or non-organic wines (in mg/L)

Wine type	1	2	3	4	5	6	7	8
Semillon non-organic	n.d.	n.d.	0.180	0.241	n.d.	n.d.	3.22	n.d.
Semillon organic	n.d.	n.d.	0.286	1.250	n.d.	n.d.	5.99	n.d.
Colombard non-organic	3.31	1.96	n.d.	0.254	n.d.	n.d.	2.39	n.d.
Colombard organic	n.d.	n.d.	0.108	0.222	n.d.	n.d.	6.17	n.d.
Cabernet Sauvignon non-organic	n.d.	1.25	0.621	0.329	n.d.	n.d.	4.38	0.490
Cabernet Sauvignon organic	n.d.	n.d.	0.231	0.227	n.d.	0.165	4.51	0.210
Merlot non-organic	n.d.	1.78	0.849	0.505	1.420	n.d.	4.54	n.d.
Merlot organic	n.d.	3.14	1.150	1.400	0.492	n.d.	6.28	n.d.
Carignan non-organic	n.d.	0.735	0.478	0.610	n.d.	n.d.	3.87	n.d.
Carignan organic	n.d.	n.d.	0.377	1.030	n.d.	n.d.	4.81	n.d.
Mean±s.d.	0.331±1.0	0.886±1.1	0.428±0.36	0.607±0.45	0.191±0.46	0.016±0.052	4.62±1.3	0.070±0.16

n.d.: not detected; 1 agmatine, 2 histamine, 3 methylamine, 4 ethylamine, 5 tyramine, 6 tryptamine, 7 putrescine, 8 cadaverine

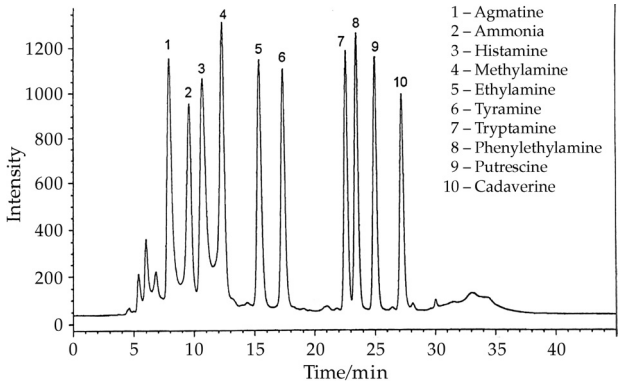

Fig.1. Chromatogram of standard mixture of biogenic amines

mg/L, histamine of 0.250–0.989 and 0.276–2.626 mg/L and tyramine of 0–1.294 and 0.524–1.583 mg/L in white and red wines respectively.

As in most reports, the quantities of biogenic amines in white and red wines were found to be different even if a statistically significant difference was determined only for methylamine (p=0.028). The order of biogenic amines in white wines from the highest to the lowest was determined to be (in mg/L): putrescine (4.44±1.9) > agmatine (0.827±1.7) > ethylamine (0.492±0.51) > histamine (0.490±0.98) > methylamine (0.143±0.12), while for the red wines the order was determined to be: putrescine (4.73±0.82) > histamine (1.15±1.2) > ethylamine (0.683±0.45) > methylamine (0.617±0.34) > tyramine (0.318±0.57) > cadaverine (0.116±0.20) > tryptamine (0.027±0.067). Red wines contained higher amounts of biogenic amines than white wines, except agmatine.

Evaluating the colour of organic and non-organic wines, definite results were obtained. The quantities of most biogenic amines in red wines predominated over those of white ones for both types of wines (Fig. 2), as determined in other studies (*5*,*9*,*17*,*18*). The stated opinion that white wines, which are generally more acidic, contain lower biogenic amine concentrations than red wines (*16*,*19*) has been confirmed by our study.

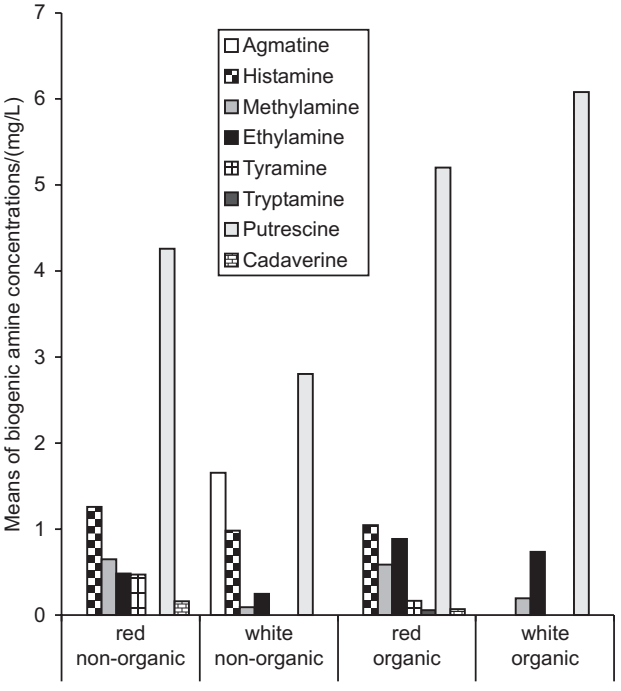

Fig. 2. Means of biogenic amine concentrations regarding wine types and colours

The concentration of biogenic amines in organic and non-organic wines could be seen in Fig. 3. Putrescine content in organic wines was significantly greater than in non-organic ones (p=0.008). The highest average values for non-organic wines were found as follows (in mg/L): putrescine (3.68±0.89), histamine (1.14±0.80), agmatine (0.662±1.5), methylamine (0.425±0.34) and ethylamine (0.388±0.16). In organic wines the highest levels (in mg/L) were found for putrescine (5.55±0.83), ethylamine (0.825± 0.56), histamine (0.628±1.4) and methylamine (0.430±0.41). The differences in quantities of biogenic amines between organic and non-organic wines could be explained by differences in their production steps (spontaneous fermentation/pure culture, pressing process, quantities of

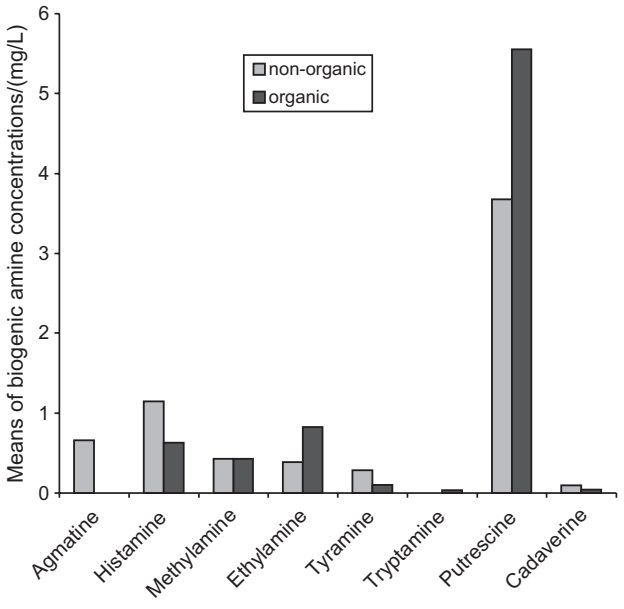

Fig. 3. Means of biogenic amine concentrations in organic and non-organic wines

SO_2, fining process). Different fermentation types led to different bacterial microflora in organic and non-organic wines. It is known that the decarboxylating capacity of bacteria is very variable depending on the origin of bacteria (*3*). The use of pressing machine in non-organic wines allowed the extraction of more phenolic compounds. It is well known that most phenolic compounds possess an antimicrobial activity, which can change the microflora of the initial must. The relatively low level of sulphur dioxide, phenolic compounds and indigenous microflora in organic wines could be the reason for high quantities of ethylamine and putrescine.

Evaluating the grape varieties used in the production of organic and non-organic wines, statistically significant differences were obtained between grape varieties for histamine, methylamine, tyramine and cadaverine at $p<0.05$. Considering histamine, a significant difference was found between Merlot and Semillon ($p=0.038$); for methylamine, significant differences were found between Merlot and Semillon ($p=0.005$), Merlot and Cabernet Sauvignon ($p=0.018$), Merlot and Carignan ($p=0.018$), and Merlot and Colombard ($p=0.003$). For tyramine significant differences were found between Merlot and Semillon ($p=0.023$), Merlot and Cabernet Sauvignon ($p=0.023$), Merlot and Carignan ($p=0.023$), and Merlot and Colombard ($p=0.023$). Evaluating cadaverine, significant differences were determined between Cabernet Sauvignon and Semillon ($p=0.011$), Cabernet Sauvignon and Merlot ($p=0.011$), Cabernet Sauvignon and Carignan ($p=0.011$), and Cabernet Sauvignon and Colombard ($p=0.011$).

The formation of biogenic amines in wines is related to the wine microflora and to the levels of amino acids after alcoholic fermentation. The composition of amino acids in wines depends on the yeast metabolism, grape varieties and vine growth conditions (*6,19*). In studies concerning hybrid grapes, different varieties have been studied (*18,20*). During the malolactic fermentation of wines, indigenous or added lactic acid bacteria cause the formation of biogenic amines through the decarboxylation of free amino acids present in wine. Some of the biogenic amines may also result from grape must. In our study, the malolactic fermentation was not allowed for neither type of wines (organic or non-organic), and the presence of different concentrations of biogenic amines demonstrated the importance of grape varieties. The pH of the wine is among the main factors affecting the activity of bacteria. The pH values of grape varieties were different (Colombard 3.38, Semillon 3.62, Cabernet Sauvignon 3.48, Carignane 3.58, Merlot 3.82), which could be the plausible explanation for the differences among biogenic amine levels.

Regarding different biogenic amines of all wines, positive correlation (Pearson r method) was obtained between methylamine and histamine contents ($r=0.687$, $p=0.028$) and between methylamine and tyramine levels ($r=0.646$, $p=0.043$).

The results of cluster analysis demonstrated the relations between analyzed parameters and wine types (Figs. 4 and 5, respectively). Evaluating biogenic amines three main clusters were obtained: histamine, methylamine and tyramine; ethylamine and putrescine; agmatine, tryptamine and cadaverine. Considering wine types, two clusters were formed: organic Colombard, Cabernet

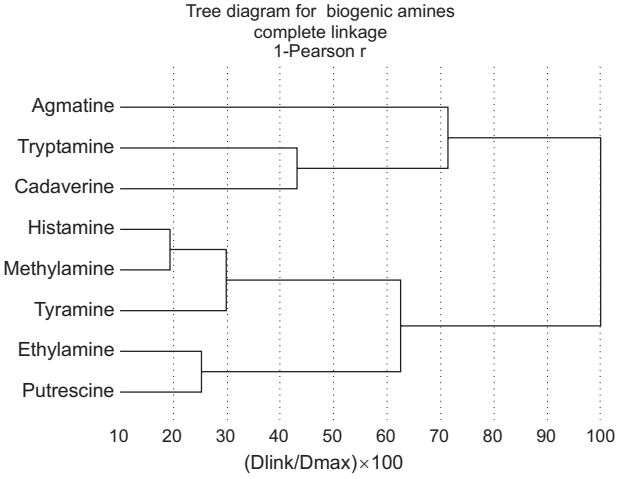

Fig. 4. Cluster analysis of biogenic amines in wines

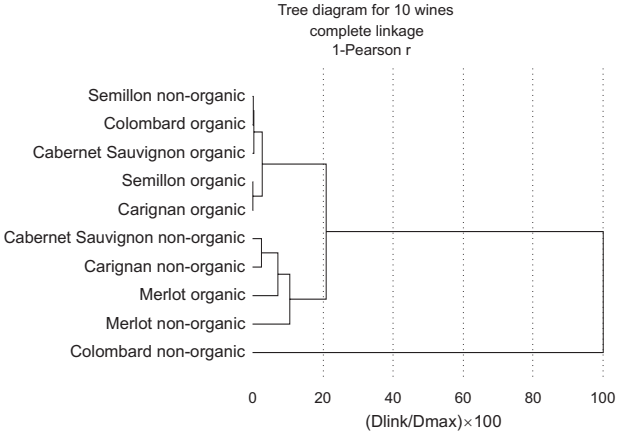

Fig. 5. Cluster analysis of organic and non-organic wines

Sauvignon and Semillon; non-organic Cabernet Sauvignon, non-organic Carignan, organic and non-organic Merlot.

Regarding wine types (organic and non-organic) and grape origin, eight principal components were extracted. The eigenvalues of correlation matrix for the first three factors explained the 76.05 % (36.18 % × 22.80 % × 17.07 %) of the total variance. Therefore, by using factor coordinates these principal components were expressed in 3-dimensional scale. The results of PCA were given in two figures, one with distribution of wines (Fig. 6) and the other with distribution of analyzed parameters (Fig. 7).

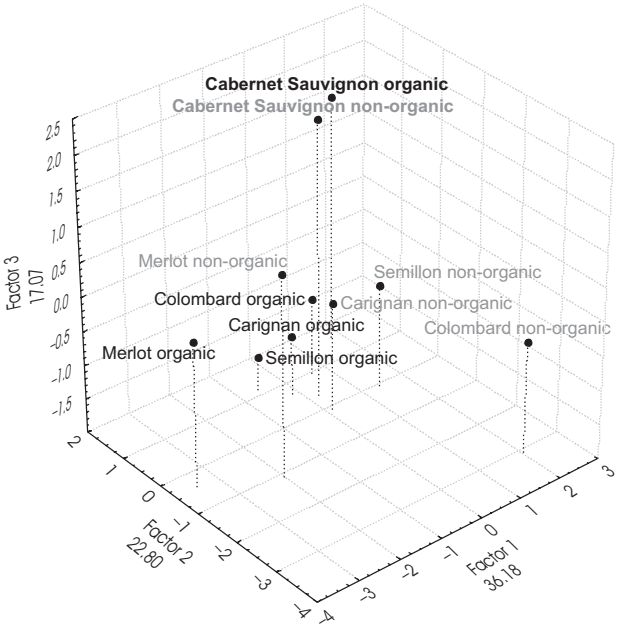

Fig. 6. Principal component analysis of biogenic amines with distribution of wines

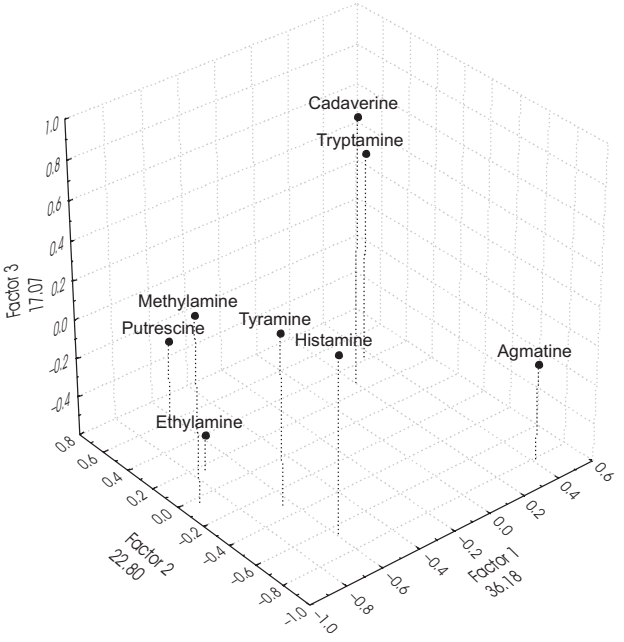

Fig. 7. Principal component analysis of biogenic amines with distribution of analysed parameters

As could be seen from Fig. 6, three main wine groups were formed: Cabernet Sauvignon wines (organic and non-organic), Colombard wine (non-organic), and all other wines. Since the first two groups have completely different coordinates from all other wines, they could be evaluated as the samples with different characteristics. The projection of analyzed parameters is seen in Fig. 7. As in the Fig. 6, three main groups could be differentiated: agmatine, tryptamine and cadaverine and all other biogenic amines. The studied biogenic amines were found to correspond to the following wine groups: agmatine to non-organic Colombard, and tryptamine and cadaverine to organic and non-organic Cabernet Sauvignon, which can be seen in Figs. 6 and 7.

Conclusions

The order of biogenic amines in wines from the highest to the lowest quantities was determined as follows: putrescine > histamine > ethylamine > methylamine > agmatine > tyramine > cadaverine > tryptamine. The quantities of biogenic amines in red wines predominated over those in white ones for both organic and non-organic wines. In organic wines, higher levels (in mg/L) were obtained for putrescine (5.55±0.83), ethylamine (0.825±0.56), histamine (0.628±1.4) and methylamine (0.430±0.41). Regarding non-organic wines, higher levels (in mg/L) were obtained for putrescine (3.68±0.89), histamine (1.14±0.80), agmatine (0.662±1.5), methylamine (0.425±0.34) and ethylamine (0.388±0.16). Putrescine and ethylamine concentrations in organic wines were found greater than those in non-organic wines. There was a significant difference between putrescine levels. Evaluating the grape varieties used in the production of organic and non-organic wines, statistically significant differences were obtained between grape varieties for some biogenic amines (histamine, methylamine, tyramine and cadaverine). Principal component analysis results demonstrated the close relations between the following biogenic amines and wines: agmatine and non-organic Colombard; tryptamine, cadaverine and organic and non-organic Cabernet Sauvignon wines.

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