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Influence of fertilization with foliar urea on the content of amines in wine

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Abstract

Amines are substances that could cause toxic effects in the consumer. The concentration of amines in wine depends on different factors such as grape variety, vinification conditions and nitrogen fertilization of the vines. The aim of this work was to study the influence of the application of foliar urea on the concentration of amines in wine. To carry out the study, grapevines from the Tempranillo variety were used. These grapevines were treated with foliar urea at two different concentrations (2 kg N ha^{-1} and 4 kg N ha^{-1}). Treatment with foliar urea significantly increased ($p < 0.05$) the concentration of histamine in the wines compared to the control sample (65% in the treatment with 2 kg N ha^{-1} and 93% in the treatment with 4 kg N ha^{-1}), reaching higher concentrations than the threshold level where it could provoke toxic effects in the consumer (8–20 mg l^{-1}). On the other hand, treatment with foliar urea did not increase the concentrations of other amines which could be toxic such as tyramine or phenylethylamine, nor amines such as putrescine which could enhance the toxic effect of histamine. In the case of the volatile amines containing secondary amine groups, the

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concentration of pirrolidine increased by 37% after treatment with 2 kg N ha^{-1} and 61% after treatment with 4 kg N ha^{-1}.

Keywords: foliar urea fertilization; amines; wine; Tempranillo must.

Introduction

Nitrogen is the most abundant soil–derived macronutrient for the grapevine and has a fundamental role in fermentation kinetics and on wine quality. The greater part of the geographical zones given over to grape–cultivar in Europe are in areas of soil poor in nitrogen. Habitually, the application of nitrogen comes about in the form of organic or inorganic fertilizers on the vineyard soil which is later absorbed by the roots and so brings about a correct development in both the vine and the grape itself. The problem with this fertilization is that the nitrate salts are very soluble so that the possibility of producing anion leaching is high. Furthermore, most soils have scant absorption of particles that are negatively charged. Nitrate leaching through the sub-soil could contaminate the underground aquifers. If water rich in nitrates is consumed, serious health problems could arise; nitrates could be transformed into nitrites due to the participation of some bacteria that exist in the stomach and urinary bladder. The foliar fertilization does not substitute the traditional fertilization of grape production, but it does serve to help to complete and guarantee the nutritional requirements of a cultivar which is often difficult to supply only through soil fertilization (Xia and Cheng 2004). In addition, this practice permits the application of relatively small amounts of the product in relation to the quantities which need to be supplied to the soil.

Biogenic amines in wines are formed through the action of decarboxylase enzymes produced by microorganisms from the corresponding amino acid precursor (ten Brink et

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al. 1990). There are also volatile amines in wine that, with the exception of phenylethylamine, appear to come from the amination of non–nitrogenous compounds such as aldehydes and ketones (Ough et al. 1981). Amines are formed in wine during alcoholic fermentation due to the metabolism of yeast strains (Torrea and Ancín 2002), and during malolactic fermentation due to the proliferation of lactic acid bacteria (Gerbaux and Monamy 2000) and its concentration is also modified during wine ageing in barrel (Jiménez-Moreno and Ancín-Azpilicueta 2004). Furthermore, a great variability in the concentration of amines exists between different wines as there are many factors (raw materials, amino acid concentration in the must, winemaking, vinification conditions…) which have an influence, to a greater or lesser extent, in the formation of these substances. Among these factors, the content of nitrogenous compounds in the grape stands out and depends, among other factors, on the level of maturation and on the nitrogenous fertilization. Another important factor is the winemaking method employed, since red wines show a larger concentration of amines compared to rosé or white wines (Zee et al. 1983; Radler and Fäth 1991).

The main biogenic amines implicated in food poisoning are: histamine, tyramine, and phenylethylamine. Histamine is found naturally in blood and the concentration of this amine in the blood flow is normally at between 25 to 130 mg l^{-1} (Taylor 1983). Histamine is the most studied biogenic amine and it is usually taken as an indicator of freshness and quality in many foods and has traditionally been the amine most related to the so–called wine intolerance that some sensitive people have to this product. Symptoms of intoxication by histamine are vomiting, diarrhoea, headaches, facial flushes, itching and throat inflammation (Silla–Santos 1996). Tyramine and phenylethylamine form part of the group of vasoconstrictor amines and so they can provoke hypertensive crisis and they are also responsible for alimentation migraine.

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Tyramine also reacts with drugs that inhibit the monoamine oxidase (MAOI) giving place to hypertensive crisis (Mariné–Font 1978). The use of drugs that inhibit the MAO for the treatment of mental depression interferes in the detoxification process of these amines and can produce hypertensive crisis in patients that accumulate tyramine in their blood (Blackwell 1963; Smith and Durack 1978). Putrescine and cadaverine, although they do not have any toxic activity, can inhibit the action of the monoamine oxidase (MAO) and diamine oxidase (DAO), enzymes which are responsible for the degradation of histamine, tyramine and phenylethylamine and so, they can reinforce the toxic activity of these amines. In experiments with animals, it has been proven that toxicity of histamine is increased by a factor of 10 when this amine is administered 40 min after the ingestion of putrescine (Parrot and Nicot 1966) and cadaverine (Bjedanes et al. 1978; Hui and Taylor 1985). On the other hand, those amines with secondary amino groups (spermine, spermidine, dimethylamine, pyrrolidine…) can react with the nitrous acid and its salts to form nitrosamines, compounds with carcinogenic, mutagenic and theratogenic activity (Glória et al. 1997).

The impact of different factors related to vinification on the concentration of amines in wine has been studied. However, we have found no literature about the influence of the foliar fertilization of the vineyard on the concentration of these compounds in wines. For these reasons outlined above, the aims of this research were to study a) the influence of the application of foliar urea on the concentration of amines in wine and b) the relation between the amine concentration with the concentration of its precursor amino acids and with the nitrogenous fractions of the must. To carry out the study, grapevines from the Tempranillo variety were used and these were treated with two different concentrations of foliar urea (2 kg N ha^{-1} and 4 kg N ha^{-1}).

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Material and methods

Samples and vinification

The grapes used in this study were *Vitis vinífera* var. Tempranillo (2008 vintage) from three adjacent plots within the same vineyard, located in The Rioja region, in the North of Spain. Vine plantation dates from 1990 while the planting density was 2.70 x 1.50 m. Gobelet pruning of the grapevine was carried out. The pH of the vine soil is 8.3 (diluting the soil in water 1/5 p/v). Nitrogen fertilizer of 21 kg N ha^{-1} was added to the soil of the whole vineyard. Two of the plots were treated with foliar application of urea, in one of the plots 2 kg N ha^{-1} was applied while in the other plot 4 kg N ha^{-1} was applied. The third plot was not treated with urea and this was taken as the control plot. Nitrotecnia–20® was used for foliar urea application with 20% p/p of total N from the urea. The urea treatment was first applied on the 31st of July and subsequently continued until the 9th of October at seven day intervals. This treatment was started near to veraison as the aim was for the urea nitrogen to pass directly to the grape and not to increase the foliage of the plant. The application was always carried out either early in the morning or late in the evening with the aim that the absorption would be maximized. The grapes were collected from the three plots, that is to say, the control plot and the two plots treated with foliar application of urea. After the harvest, the grapes were destemmed and crushed and afterwards they underwent pressing. Potassium metabisulphite (Aldrich, Gillingham, UK) was added to the must up to a concentration of 50 mg l^{-1} of total SO_2. Fermentation took place in stainless steel tanks with a capacity of 25 liters at controlled temperature (27 ºC). All wines were fermented to dryness (reducing sugar concentration < 2.5 g l^{-1}). Figure 1 shows a flow chart for the life cycle of the wine production steps. A study of amines was made in the must at the beginning of the alcoholic fermentation (M), must at 6 days after the start of fermentation (M6),

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must at 8 days after the start of fermentation (M8) and wine at the end of alcoholic fermentation (W). Fermentation lasted a total of 11 days. Those samples coming from the grapevines treated with 2 kg N ha^{-1} of foliar urea were given the name U2; while the samples coming from the grapevines treated with 4 kg N ha^{-1} of foliar urea were given the name U4 and the samples from the grapevines not treated with foliar urea were the control samples.

Preparation of sample and HPLC analysis of amines

Amine determination was carried out by the method described by Torrea and Ancín (2001). Samples were passed through a Millipore Ultrafree MC filter (Millipore, Cork, Ireland) to eliminate molecules of a size higher than 10,000 Da. Afterwards the precolumn derivatization of the amines was carried out through the addition of 6–aminoquinolyl–N–hydroxysuccinimidyl carbamate (AQC) from AccQ–Fluor reagent kit (Waters Chromatography Div., Milford, MA, USA). The chromatography analysis was made with a Waters high–performance liquid chromatograph equipped with a 474 fluorescence detector, using 250 and 395 nm as the excitation and emission wavelengths, respectively. A Pico·Tag™ reverse phase column (300 mm x 3.9 mm i.d.) with a stationary phase of dimethyloctadecylsilyl bonded to amorphous silica was used. For chromatographic control, Empower® 2.0 software was used. The sample injected was 10 µl. The amines under study were histamine, tyramine, putrescine, cadaverine, phenylethylamine, spermidine, spermine, dimethylamine, diethylamine, ethylamine, isobutylamine, isopropylamine, amylamine and pyrrolidine. It was not possible to separate phenylethylamine and spermidine and consequently they were quantified as one single peak. Two derivatizations were made of each sample and the samples were analyzed in duplicate (n=4).

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Preparation of sample and HPLC analysis of free amino acids

The Pico·Tag method used for amino acids determination was described by Ancín et al. (1996). Analysis was performed with a Waters high–pressure liquid chromatograph equipped with two 510 pumps, a U6K injector, and a 996 photodiode array detector used at 254 nm. A Pico·Tag™ reverse phase column (300 mm x 3.9 mm i.d.) with a stationary phase of dimethyloctadecylsilyl bonded to amorphous silica was used. Empower® 2.0 software was employed for chromatographic control. Derivatization was performed using a Telstar lyophilizer, model Cryodos–50 (Telstar Industrial, Barcelona, Spain). Internal standards were L–2–aminohexanoic acid (L–norleucine) and L–2–amino–4–[methylsulfonyl] butanoic acid (L–methionine sulfone), both from Sigma (Gillingham, UK). Two mobile phases A and B were used with the following composition: phase A, acetonitrile (2.5 %) and sodium acetate (97.5 %); phase B, a mixture of acetonitrile, water and methanol (45: 40: 15). All these reagents were from Scharlau HPLC quality (Barcelona, Spain). The mobile phases used were always purified through an HA 0.45 µm Millipore filter. The amount of sample injected was 10 µl. Two derivatizations were made of each sample and the samples were analyzed in duplicate (n=4).

Nitrogenous fractions and general enological parameters

Ammonium nitrogen and general parameters were measured using WineScan 79000 Auto (Foss Analytical, Denmark) with Fourier Transform Infrared Spectroscopy technology. The wavelength range of 240–1295 nm was used for these analyses.

Yeast assimilable nitrogen (YAN) was determined by adding amino nitrogen plus ammonium nitrogen and taking away the nitrogen from proline.

Statistical analysis

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The statistical study of the amine content in must and wine with different fertilization treatments was carried out with analysis of variance (ANOVA), and the means were compared with the Scheffé test. The probability level was 0.05. For data analysis, the software SPSS® v.17.0 was used (Chicago, Illinois, USA).

Results and Discussion

General parameters

The application of foliar urea caused a significant increase ($p < 0.05$) in the pH of the must and wines (Table 1). Bertrand et al. (1991) also found an increase in the pH in Merlot wine from nitrogen–treated vines in comparison with the same wine from non nitrogen–treated vines. Total acidity was also significantly higher ($p < 0.05$) in the Tempranillo must coming from the grape with foliar urea fertilization compared to the control must. Fertilization with foliar urea decreased the alcohol level and the total acidity of the wine (Table 1). Volatile acidity of wines was inferior in all cases to the threshold found by Peynaud (1993) as undesirable for wine aroma (0.6 g HAc l^{-1}).

Nitrogen compounds in must and its relation with the total biogenic amines in wines

When foliar urea was applied, the concentration of amino nitrogen and YAN (yeast assimilable nitrogen) significantly increased ($p < 0.05$) in the must (Figure 2a). In the control Tempranillo must, the concentration of amino nitrogen (201 mg l^{-1}) was similar to the average value (199 mg l^{-1}) found by Henschke and Jiranek (1993) for seven Australian varieties of grapes. It was observed that foliar urea application increased the concentration of amino nitrogen in must by 29% when 2 kg N ha^{-1} was applied and by 104% when 4 kg N ha^{-1} was applied (Figure 2a). Yeasts assimilable nitrogen was also much higher in the must from the grape treated with 4 kg N ha^{-1} of foliar urea (500 mg l^{-1}) and in the must from the grape treated with 2 kg N ha^{-1} of foliar urea (300 mg l^{-1})

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than in the control must (242 mg l^{-1}). These results indicate that must treated with urea accumulated a greater quantity of arginine in relation to proline than in the control must. Furthermore, the consumption both of amino nitrogen as well as of YAN during fermentation was significantly higher in the musts treated with foliar urea than in the control must (Figure 2b). The concentration of total biogenic amines did not show any significant differences ($p < 0.05$) between the three wine samples (control, 33.5 mg l^{-1}; U2, 34.7 mg l^{-1}; U4, 30.9 mg l^{-1}). Consequently, no correlation was found between the concentration of yeasts assimilable nitrogen in the must and its consumption, with the concentration of total biogenic amines in the wine.

Biogenic amines in wines

In the Tempranillo must used in this study, small quantities of histamine, tyramine, putrescine and spermine were found. The rest of the amines were formed above all in the second half of alcoholic fermentation, although the concentration of cadaverine decreased at the end of fermentation (Figure 3). Some amines are normal constituents of grapes, and the amounts varied with grape variety, soil type, fertilization and climate conditions (Millán et al. 2007).

The concentrations of histamine, cadaverine and spermine (Figures 3a, 3d and 3e) were significantly higher ($p < 0.05$) in the wine coming from the must treated with the higher concentration of foliar urea (U4 wine) than in the control wine as well as in the U2 wine. The concentrations of histamine and spermine also increased with the application of 2 kg N ha^{-1} of urea in comparison with the control sample. The histamine concentration in both wines from musts that had been treated with urea is within the range of concentrations (8–20 mg l^{-1}) where this amine could provoke toxic effects in the consumer (Soufleros et al. 1998). The toxicity of histamine on the general population is very difficult to establish since in some cases toxic effects have been

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associated with even small amounts of this amine (Wantke et al. 1996), as there are some individuals who are especially sensitive to the action of histamine. Generally, these individuals show a deficiency of DAO or are being treated with MAOI drugs. There also exist strengtheners of the toxicity of histamine, such as other biogenic amines which are present in food (tyramine, phenylethylamine, tryptamine, putrescine or cadaverine) which compete for the same endogen metabolic pathways and strengthen the absorption of histamine in the intestine (Veciana-Nogués and Vidal-Carou 2000). Likewise, it has been observed that some compounds present in food and drinks such as alcoholic ones or the acetaldehyde of wine or the polyphenols from tea all strengthen the toxic action of histamine. Bertrand et al. (1991) found that histamine, putrescine and cadaverine concentrations in wines from nitrogen–treated vines were 1.3 to 3.8 times higher than those of wines from untreated vines. Concentration of putrescine (Figure 3c) was significantly inferior ($p < 0.05$) in the wine from the must treated with the higher concentration of foliar urea (U4 wine) than in the control and in the U2 wines. The concentration of phenylethylamine+spermidine (Figure 3f) also decreased in the wine on applying foliar urea. The yeasts have a mechanism known as general amino acid control (Hinnesbuch 1988). The precursor amino acids of histamine, cadaverine and putrescine (histidine, lysine and arginine respectively) are all under general amino acid control, so that their metabolic pathways are corelated and depend on the concentration of each of them. High concentration of arginine stimulates arginase activity, this regulatory strategy allows a rapid switch from arginine biosynthesis to arginine catabolism. However, *S. cerevisiae* uses the nitrogen compounds at the start of fermentation without modification. This yeast, once it has covered its specific demands for each amino acid, assimilates those that are in excess and uses them as nitrogen sources (Bisson 1991). In our case, the concentration of arginine was very high (Table

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2) in the U4 must. In this sample, arginine would have been preferably used as a nitrogen source and, to a lesser extent than in the other samples, its decarboxylation would have been produced to form putrescine. Thus, the concentration of putrescine in the U4 wine is significantly lower ($p < 0.05$) than in the control wine and in the U2 wine. On the other hand, in the U4 must, where the yeasts had higher amounts of a good nitrogen source such as arginine, lysine and histidine would not have followed the biosynthesis pathways and they would have decarboxylated in greater measure to produce their corresponding biogenic amines (cadaverine and histamine) than in the other samples.

The concentration of tyramine (Figure 3b) did not show any significant differences ($p < 0.05$) between the control sample and the wine samples from grape with foliar urea fertilization. The concentration of the precursor amino acid of this amine, tyrosine, did not show any significant differences ($p < 0.05$) either between any of the three must samples (Table 2). In all the samples, the concentrations of tyramine were found to be below the range at which wines could have toxic effects for the consumer (10–80 mg l^{-1}), so that these wines do not present any toxic risk due to this amine. Phenylethylamine+spermidine were formed during alcoholic fermentation and its concentration was significantly lower ($p < 0.05$) in the wines coming from the must treated with urea than in the control sample (Figure 3f). The precursor amino acid of phenylethylamine (phenylalanine) disappeared in the must coming from the grapes treated with foliar urea (Table 2). In all the wine samples, the concentration of phenylethylamine+spermidine was so low that it would be necessary to drink more than two litres of these wines to ingest a dose of 3 mg, above which phenylethylamine causes migraines (Sandler et al. 1974).

Volatile amines

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Pyrrolidine, ethylamine and diethylamine were found in all the musts (Figure 4). The concentration of these three amines in the must was significantly higher ($p < 0.05$) in the U4 must than in the other two musts. The rest of the volatile amines were formed during alcoholic fermentation (Figure 4). Pyrrolidine and diethylamine (Figures 4d and 4f) were found in greater concentration in the U4 wine. In the case of dimethylamine (Figure 4a), its concentration decreased in the wines coming from grape treated with urea. Dimethylamine, pyrrolidine and diethylamine contain secondary amino groups in their molecular structure and so they can react with nitrous acid and their salts to form nitrosamines, substances that have carcinogenic action (Glória et al. 1997)

Within the volatile amines which do not show secondary amine groups, the concentrations of isobutylamine and amylamine (Figures 4c and 4g) hardly showed any significant differences ($p < 0.05$) after the treatment with foliar urea. Only the concentration of isobutylamine was significantly lower ($p < 0.05$) in the U2 wine than in the control wine. The concentrations of ethylamine and hexylamine (Figure 4e and 4h), both decreased after foliar fertilization. On the contrary, the concentration of isopropylamine (Figure 4b) increased with foliar fertilization, while no significant differences ($p < 0.05$) were observed between the different doses of treatment with foliar urea.

Conclusions

Treatment with foliar urea (2 kg N ha^{-1} and 4 kg N ha^{-1}) increased significantly ($p < 0.05$) the concentrations of histamine and spermine in the wine. The concentrations of histamine in the wines coming from the grape treated with urea were above the threshold of concentration where this amine could provoke toxic effects in the consumer. On the other hand, treatment with foliar urea did not increase the concentrations of tyramine, nor putrescine nor phenylethylamine+spermidine. As for the

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volatile amines with secondary amino groups, the concentrations of pyrrolidine and diethylamine increased to a significant degree after treatment with foliar urea. Consequently, from the results obtained in this study, it may be concluded that wines coming from grapevines treated with foliar urea could present toxic problems due to histamine in a part of the general population. The exact impact is difficult to establish as poisoning from this amine in wine would depend on personal factors such as the presence of other substances in the diet that could strengthen the toxic action of histamine.

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Table 1. Enological parameters of musts and wines. All parameters listed with standard deviation, n=4. Means within the same row followed by different letters are significantly different ($p < 0.05$).

Musts	control	U2 (2 kg N ha^{-1})	U4 (4 kg N ha^{-1})
pH	3.57 ± 0.00 [a]	3.66 ± 0.00[b]	3.78 ± 0.00 [c]
Glucose + fructose (g l^{-1})	223.3 ± 0.1 [a]	223.5 ± 0.2 [a]	212.9 ± 0.2 [b]
Total acidity (g l^{-1})	2.80 ± 0.05 [a]	2.96 ± 0.04 [b]	3.15 ± 0.04 [c]
Malic acid (g l^{-1})	3.5 ± 0.1 [a]	3.8 ± 0.1 [b]	3.6 ± 0.0 [ab]
Wines	control	U2 (2 kg N ha^{-1})	U4 (4 kg N ha^{-1})
pH	3.74 ± 0.00 [a]	3.90 ± 0.00 [b]	3.96 ± 0.00 [c]
Alcohol content (% v/v)	12.75 ± 0.01 [a]	12.65 ± 0.00 [b]	11.82 ± 0.02 [c]
Total acidity (g l^{-1})	5.50 ± 0.02 [a]	5.35 ± 0.03 [b]	5.27 ± 0.01 [c]
Volatile acidity (g l^{-1})	0.26 ± 0.01 [a]	0.30 ± 0.00 [b]	0.28 ± 0.01 [a]

Note: Total acidity expressed as tartaric acid.

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Table 2. Concentration of amino acids precursors of biogenic amines. All data listed with standard deviation, n=4. Means within the same row followed by different letters are significantly different ($p < 0.05$).

Musts	control	U2 (2 kg N ha^{-1})	U4 (4 kg N ha^{-1})
Histidine	119.2 ± 3.8[a]	192.6 ± 2.6 [b]	266.8 ± 0.9 [c]
Arginine	242.9 ± 21.9 [a]	507.6 ± 67.25 [b]	735.0 ± 63.0 [c]
Tyrosine	17.0 ± 2.9 [a]	16.2 ± 4.5 [a]	15.6 ± 1.5 [a]
Phenilalanine	5.8 ± 0.4 [a]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]
Lysine	2.5 ± 0.3 [a]	36.0 ± 2.9 [b]	36.1 ± 2.4 [b]

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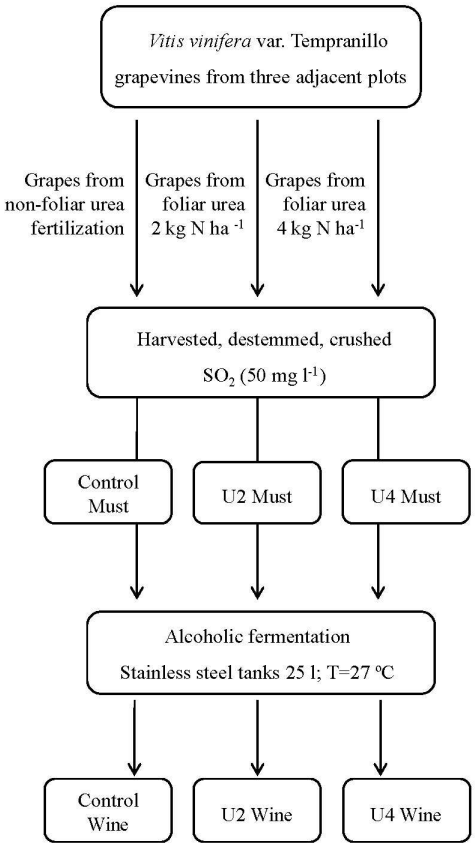

Figure 1. Flow chart for the life cycle of the wine production steps.
190x254mm (200 x 200 DPI)

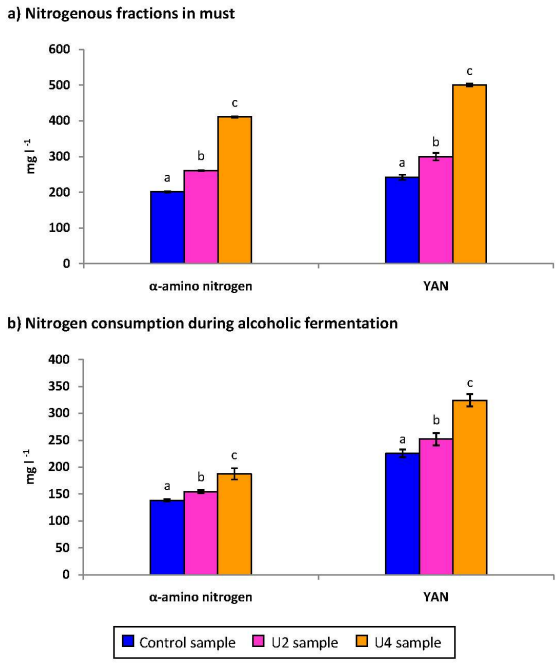

Figure 2. Nitrogenous fractions in must and nitrogen consumption during alcoholic fermentation. Bars with different letters are significantly different ($p < 0.05$). Note: Yeast Assimilable Nitrogen (YAN).
210x297mm (200 x 200 DPI)

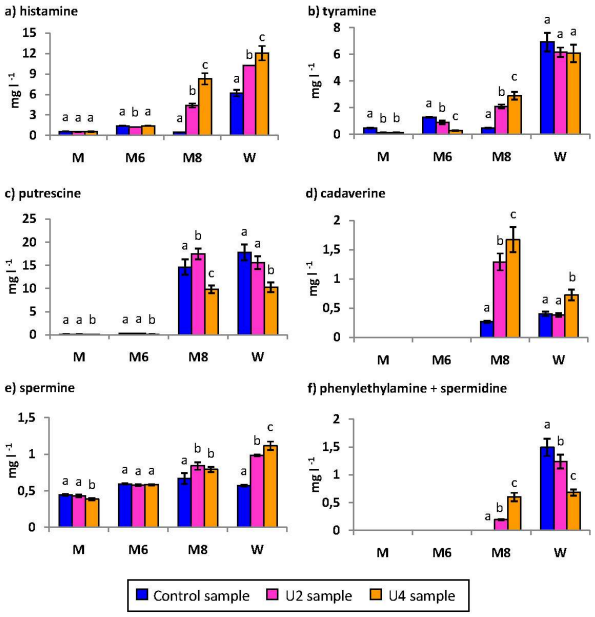

Figure 3. Evolution of the concentration of biogenic amines during the alcoholic fermentation. Bars with different letters are significantly different ($p < 0.05$).
210x297mm (200 x 200 DPI)

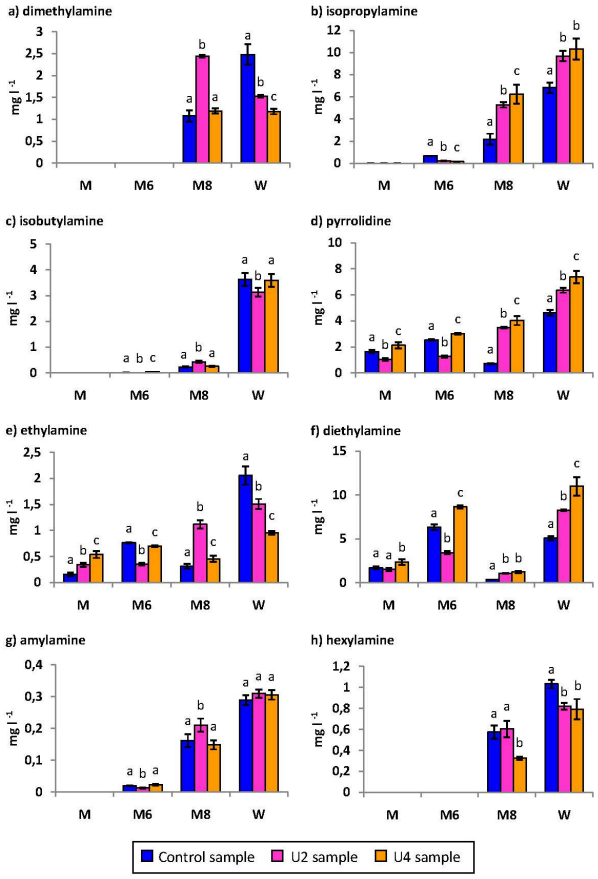

Figure 4. Evolution of the concentration of volatile amines during the alcoholic fermentation. Bars with different letters are significantly different ($p < 0.05$).
210x297mm (200 x 200 DPI)