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PAPER

Changes in free amino acid concentration during stabilization and aging of wines derived from garnacha and viura musts clarified by static sedimentation

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Stabilization of wine by refrigeration assures an increase in cleanliness and effective physicochemical stabilization. The objective of the present work was to study the changes in free amino acid concentrations in rosé and white wines stabilized by precipitation in the cold, filtered through non-sterilizing filters and aged for a year in bottle. The wines derived from musts clarified by static sedimentation were compared with control wines obtained from unclarified musts. The results show that the unclarified-must wines had a greater consumption of amino acids due, probably, to the greater microbial population. Copyright © 1996 Elsevier Science Ltd.

Keywords: must clarification; wine stabilization and aging; amino acid concentration changes

INTRODUCTION

Newly made wine, at the beginning of its development, exhibits an elevated turbidity because of complex physicochemical processes such as variations in the pH, in the colloidal electrical charge and, to a lesser extent, in the redox potential and temperature (Troost, 1985).

Stabilization of wine can proceed naturally or be accelerated artificially. Filtering, cooling or heating are frequently used to clarify and stabilize wine. One of the most used procedures is refrigeration which causes (Madrid, 1987): (i) precipitation of salts (potassium bitartrate and, in lesser amounts, calcium tartrate); (ii) partial insolubilization of colorant substances; (iii) flocculation of proteins; and (iv) sedimentation of proteins and metal ions in colloidal state, pectin materials and such. Therefore, the refrigeration assures an increase in cleanliness and an effective physicochemical stabilization (Ribéreau-Gayon *et al.*, 1976; Oreglia, 1979; Peynaud, 1993). Subsequent use of sterilizing filters assures the elimination of yeasts and microorganisms guaranteeing also the biological stability of the product (Oreglia, 1979; Peynaud, 1993); however, the common practice

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is the use of non-sterilizing filters that may permit the development of biological phenomena such as the yeast lysis and malolactic fermentation.

Free amino acids in the wine are excellent nitrogenous sources for the growth of microorganisms, especially the lactic acid bacteria (Zoecklein *et al.*, 1990). On the other hand, fermentation of sweet wines with yeast strains that have a strong demand for growth limiting amino acids can be a future method for stabilization (Zoecklein *et al.*, 1990). It is also known that must clarification treatments influence the consumption profile of some amino acids during fermentation (Ayestarán *et al.*, 1995) and in the final production of higher alcohols (Ancín *et al.*, 1996).

The objective of the present work was to observe the influence of static sedimentation on the amino acids present, which favour the development of microorganisms in the wine. Accordingly, the changes in amino acid concentrations were studied during the aging of rosé and white wines stabilized by precipitation in the cold and filtered through nonsterilizing filters. Two varieties of grapes were selected. Wines were prepared from untreated must and from must clarified by static sedimentation before initiating alcoholic fermentation. Original musts were taken as controls. The musts were fermented to produce the corresponding wines (rosé and white). Prefermentation treatment, fermentation and stabilization of wines were done in a pilot plant to simulate industrial conditions. All wines obtained were analysed and compared.

MATERIALS AND METHODS

Samples and vinification

Vitis vinifera var. garnacha and *Vitis vinifera* var. viura grapes of Navarra Denomination of Origin were collected; rosé and white wines were produced in a pilot plant.

Vitis vinifera var. garnacha grapes were crushed and destemmed. The skins were not removed for 17 hours. Must was later divided into two fractions. The first was treated with SO_2 (50 mg/l) but was not subjected to any prefermentative technique. The other, following refrigeration to 10°C and the addition of SO_2 (50 mg/l), was clarified by decantation. In decantation, the must remained in stainless steel tanks for 24 h before racking. Then, 400 l of the two musts were subjected to fermentation using *S. cerevisiae* at 18±2°C. Must was inoculated with 0.5 g/l of Fermivin crio active dry *Saccharomyces cerevisiae* from Gist brocades (F. Lafford & Cía., S.A., Pasajes, Spain). In all cases, fermentation was continued until the concentration of reducing sugars fell below 2.5 g/l.

Freshly cropped *Vitis vinifera* var. viura grapes were crushed and destemmed; the skins obtained were not removed for 7 to 8 h, and the process described above for rosé vinification was followed. Average fermentation temperature was 18±2°C. In all cases, fermentation was continued until the concentration of reducing sugars fell below 2.5 g/l.

A stainless crusher-stemmer Marzola Marzinox (Marrodán and Rezola SA, Logroño, Spain) equipped with a rubber roller was used to crush the grapes.

Vinification was carried out in stainless steel (AISI 316–18/8/2) vertical tanks. Tank dimensions were 0.76 m diameter, 1.1 m height, and the capacity was 400 l.

The obtained wines were clarified by passing the samples through a cellulose pad in a plate and frame filter (K300 Seitz) with the intention of eliminating the larger particles. Then the wines were stabilized by refrigeration submitting them to a temperature between −5°C and 0°C in a cold-storage room for 10 days. Following this period, the wines were filtered using nonsterilizing filters (EK Seitz). The level of free SO_2 in the stabilized wines was adjusted, and the wines were then bottled by hand and aged for a year.

Preparation and HPLC analysis of free amino acids

Analysis was performed with a Waters high pressure liquid chromatograph (Waters Chromatography Div., Milford, MA) equipped with two 510 pumps, a U6K injector and a 486 UV-vis detector used at 254 nm. Maxima 820 software was employed for chromatographic control. A PICO·TAG reverse phase column (300 mm and 3.9 mm id) was used with a stationary phase of dimethyloctadecylsilyl bonded to amorphous silica (Ref. Waters 10950). Derivatization was performed using a Waters PICO·TAG work station.

Standard solutions, for the analysis of the free amino acids by HPLC, were prepared of 2.5 mmol/ml concentration (Sigma Chemical Co, St Louis, MO), except cystine (1.25 mmol/ml). Internal standards were L-2-aminohexanoic acid (L-norleucine) and L-2-amino-4-[methylsulphonyl]butanoic acid (L-methionine sulphone), both from Sigma. In derivatization, phenylisothiocyanate (Pierce, Rockford, IL), methanol (Scharlau, SA, Barcelona, Spain), triethylamine (Aldrich, Milwaukee, WI) and double-distilled water were used. Mobile phases were prepared with sodium acetate, acetonitrile and methanol (Scharlau) and acetic acid (E. Merck, Darmstadt, Germany). The mobile phases were purified through a HA 0.45 μm Millipore filter. Solvents were of HPLC quality, and reagents of analytical quality.

The PICO·TAG method developed by Waters (Cohen *et al.*, 1989) was followed for amino acid analysis. Samples were cleaned by ultrafiltration with a Millipore ultrafree MC cartridge, and then L-norleucine and L-methionine sulphone were added as internal standards. Then precolumn derivatization with phenylisothiocyanate was carried out. The amount of sample injected was 5 ml.

Enological parameters

Enological parameters were obtained according to the methods described by the Office International de la Vigne et du Vin (1990). All determinations were performed in quadruplicate on representative samples of musts and wines. The results given in *Tables 1* and *2* include standard deviation (SD).

RESULTS AND DISCUSSION

General parameters

In *Table 1*, the principal parameters are presented that characterize the rosé wines recently made and those stabilized, and in *Table 2*, the corresponding white wines, derived from musts with or without static sedimentation. In both tables, it is observed that the pH of the recently obtained wines derived from clarified musts was similar to those of the control wines. In addition, the values were in the pH interval of 3.0 and 3.67 that, according to Larrechi (1986), include the optimum values for conservation.

The total acidity was less in the recently obtained wines derived from decanted musts (*Tables 1* and *2*). This can be due, in the unclarified musts, to the presence of suspended solids in the medium favouring nucleation and precipitation of potassium bitartrate. After stabilization and aging of the wines, a decrease in total acidity was produced since cooling favours the precipitation of potassium bitartrate salts and, to a lesser degree, of calcium tartrate (Correa and Polo, 1990).

The volatile acidity of the recently made wines derived from decanted musts was similar to that of the control wines. These values were in the optimum interval of 0.12 to 0.30 g of acetic acid/l described by Amerine and Ough (1976), and, as such, the wines presented no problems, neither in their conservation nor in their organoleptic characteristics. After stabilization and aging, this parameter increased roughly by 0.08 g of acetic acid/l except in the white wine derived from decanted must, where the increase was greater. The increase in volatile acidity is due, probably, to microbial alterations or an oxidation-reduction process, favoured by oxygen that penetrates via the cork during aging in bottle.

Ash values and alkalinity were similar for all of the recently obtained wines and independent of the must clarification treatment. After stabilization and aging, there was a slight decrease in ash content. The cooling process accelerated the precipitation of salts

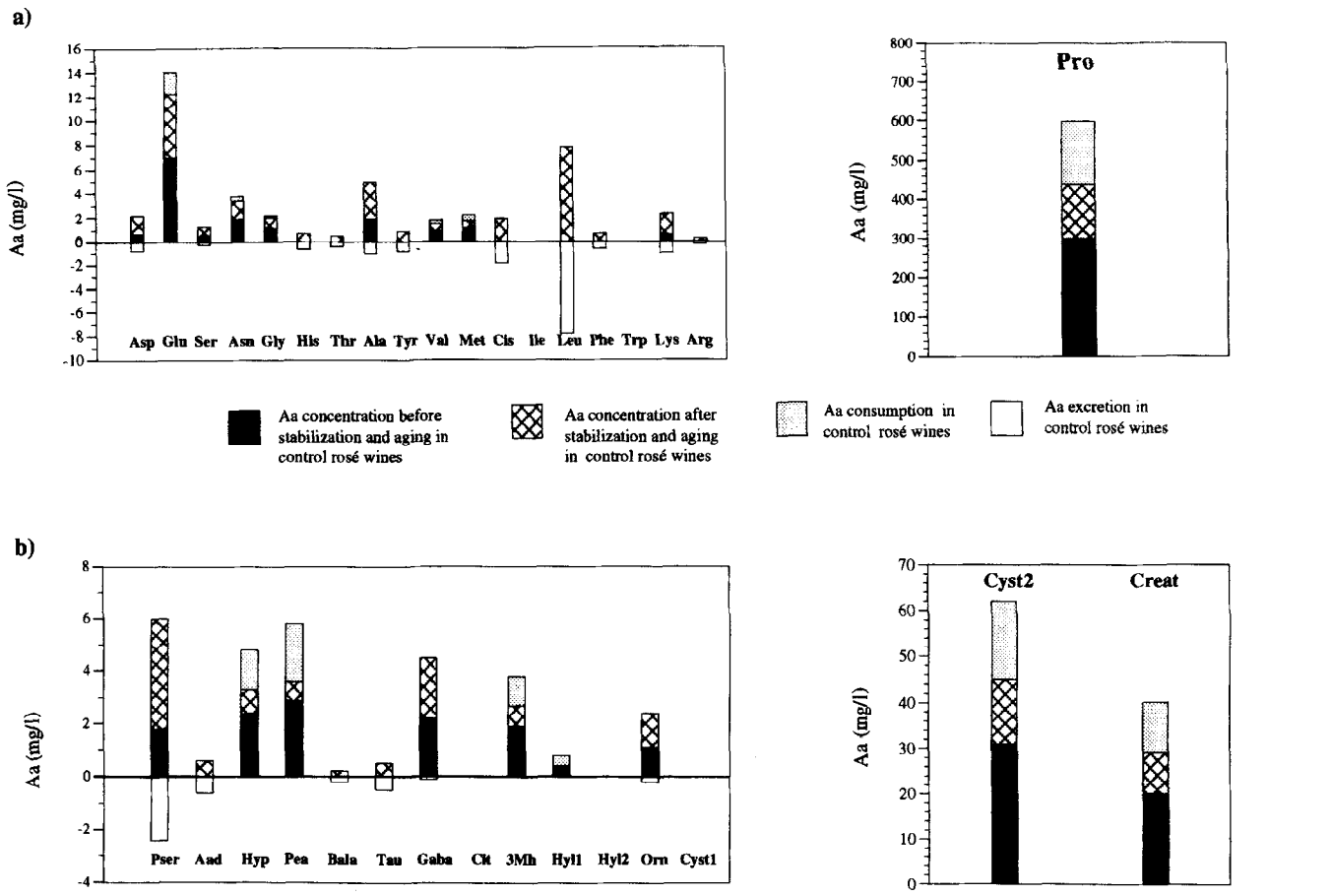

Figure 1 Concentration of amino acids in the rosé wine recently finished (derived from unclarified must) and after stabilization and aging. Consumption or excretion of amino acids: (a) amino acids found in proteins (AaP); and (b) amino acids not found in proteins (AaNP; the consumption or excretion values for each amino acid were obtained by subtracting the amino acid concentration of the stabilized and aged wine from the concentration of the recently finished wine)

and, thus, resulted in lower ash content in the stabilized wine.

The reducing sugars of the recently finished wines were less than 5 g/l indicating that fermentation had reached dryness. The decrease of reducing sugars after stabilization and aging of the wines could indicate microbial activity due, probably, to the presence of lactic and acetic bacteria. In this respect, Davis *et al.* (1986) stated that growth and metabolism of lactic acid bacteria during aging decreased the concentrations of hexose and pentose sugars. Drysdale and Fleet (1988) indicated that some species of *Acetobacter* and *Gluconobacter* could metabolize hexoses and pentoses by means of hexose monophosphate. On the other hand, the amount of alcohol in the recently obtained wines derived from clarified musts was identical to those of the control musts; thus, it does not appear to depend on the clarification treatment applied to the initial must.

Changes in free amino acid concentrations

Rosé wines

Amino acids found in proteins. In *Figure 1a* it is observed that, in stabilization and aging of the control rosé wine, the amino acids found in protein were excreted except for glutamic, asparagine, glycine, valine, methionine and proline, which were consumed; tryptophan and isoleucine were not detected. Proline was the only amino acid found in protein that was greatly consumed (160 mg/l). Notable also was the excretion of leucine, cystine and lysine. In stabilization and aging of clarified-must rosé (*Figure 2a*), something similar occurred: the amino acids found in protein were excreted except glycine, threonine, alanine, methionine, tryptophan and proline, which were consumed; glutamic and isoleucine were not detected. Notable again was the excretion of leucine ($\approx$2 mg/l, much less than in the control wine), cystine and lysine. In regard to proline, it was consumed in almost the same quantity in the stabilization of the control wine and in that derived from decanted must, and it was always consumed in quantities much greater than the rest of the amino acids.

It is possible that the excreted amino acids are produced by autolysis of residual yeast (Feuillat and Charpentier, 1982; Colagrande *et al.*, 1984; Silva *et al.*, 1987; Leroy *et al.*, 1990). On the other hand, the consumption of some amino acids and, in particular, the large quantity of proline consumed could be due to residual biological activity that requires the contribution of nitrogenous sources. Drysdale and

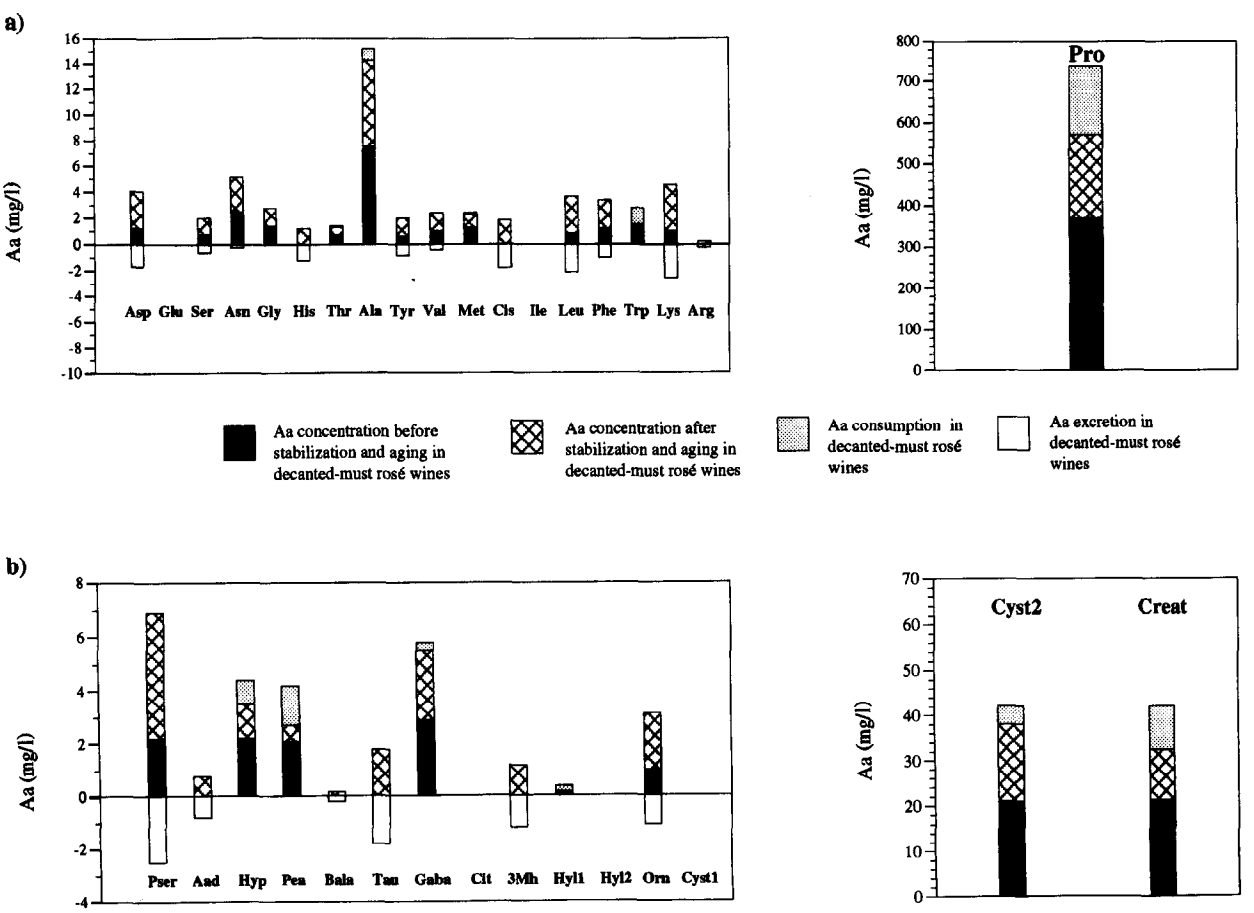

Figure 2 Concentration of amino acids in the rosé wine recently finished (derived from decanted must) and after stabilization and aging. Consumption or excretion of amino acids: (a) amino acids found in proteins (AaP); and (b) amino acids not found in proteins (AaNP; the consumption or excretion values for each amino acid were obtained by subtracting the amino acid concentration of the stabilized and aged wine from the concentration of the recently finished wine)

Fleet (1988) stated that proline is one of the amino acids that stimulates growth in acetic bacteria.

Amino acids not found in proteins. In stabilization of the control wine and that derived from clarified must (*Figures 1b* and *2b*), the excretion of phosphoserine was exceptional as well as the consumption of hydroxyproline and phosphoethanolamine. The more abundant amino acids (cystathionine-2 and creatinine) were consumed during stabilization of the control wine (17 and 12 mg/l, respectively) although the consumption was much less in the clarified-must wines.

In summation, there was little consumption of amino acids found during protein in stabilization of the rosés studied except for proline, which was amply consumed. Most of the amino acids found in protein were excreted to the media during the stabilization of the rosé wines. Leucine and, to a lesser extent, lysine were the protein amino acids most excreted. Among the nonprotein amino acids, the most consumed were creatinine and cystathionine-2, although the latter was consumed much more during stabilization of the control wine than in the wine derived from clarified must.

White wines

Amino acids found in proteins. After stabilization and aging of the control white wine (*Figure 3a*), the consumption of glutamic acid ($\approx$2.8 mg/l) was exceptional and, to a lesser extent, that of asparagine, glycine, tryptophan and arginine. The remaining amino acids, except proline, were not consumed or were excreted. Among those excreted, cystine (1.9 mg/l), leucine (1.0 mg/l) and lysine (1.4 mg/l) were notable. Proline, for its part, was consumed in quantities similar to the control rosé wine (some 150 mg/l). In white wines derived from clarified musts (*Figure 4a*), the consumption of glutamic acid (3.8 mg/l) and the excretion of asparagine, cystine, leucine, phenylalanine, lysine and tyrosine was noteworthy; with respect to proline, it is necessary to indicate the small quantity of this amino acid consumed during stabilization and aging of this wine in comparison with the control wines and with the clarified-must rosé.

Amino acids not found in proteins. The consumption of citrulline (8 mg/l) in stabilization and aging of the control wine was exceptional (*Figure 3b*); however, this amino acid was not detected in the clarified-must wine (*Figure 4b*). In addition, the consumption of

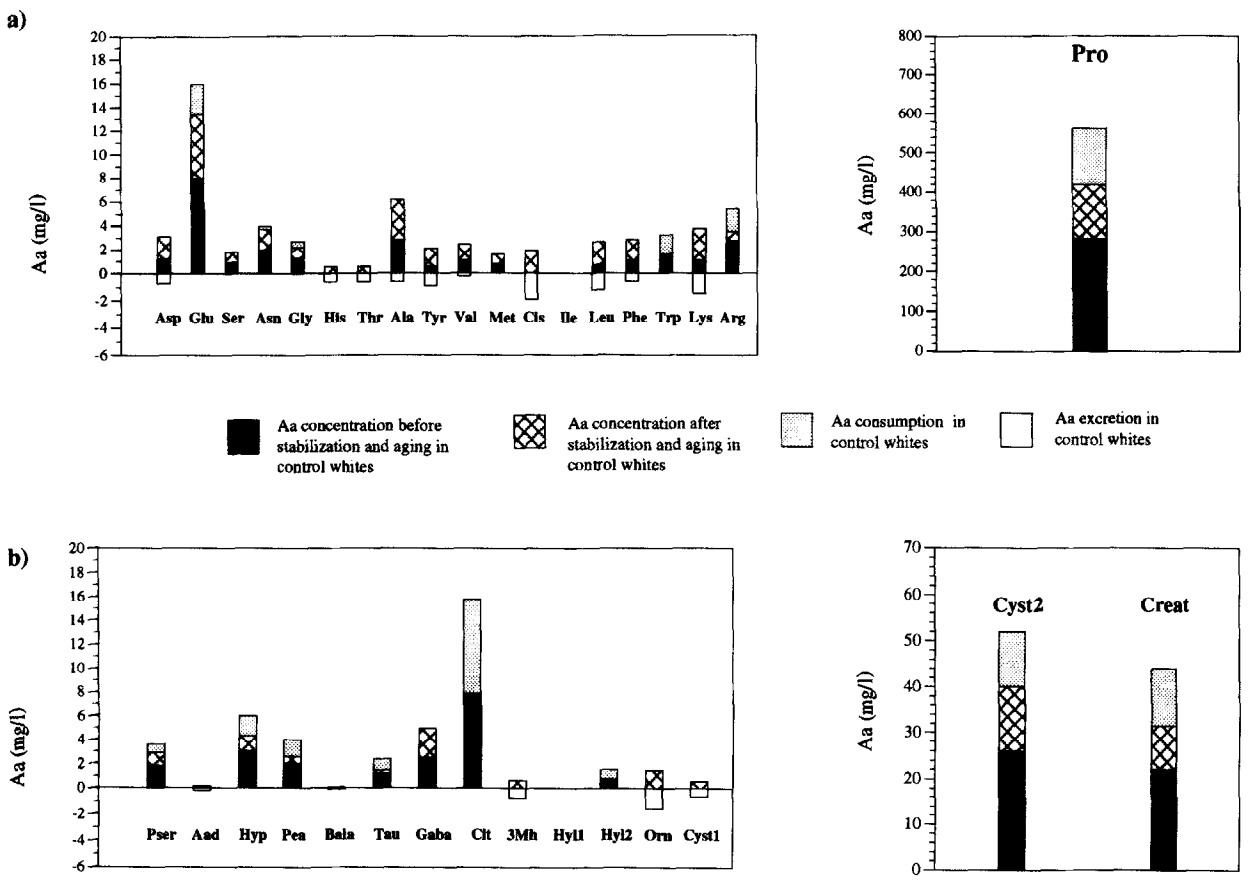

Figure 3 Concentration of amino acids in the white wine recently finished (derived from unclarified must) and after stabilization and aging. Consumption or excretion of amino acids: (a) amino acids found in proteins (AaP); and (b) amino acids not found in proteins (AaNP; the consumption or excretion values for each amino acid were obtained by subtracting the amino acid concentration of the stabilized and aged wine from the concentration of the recently finished wine)

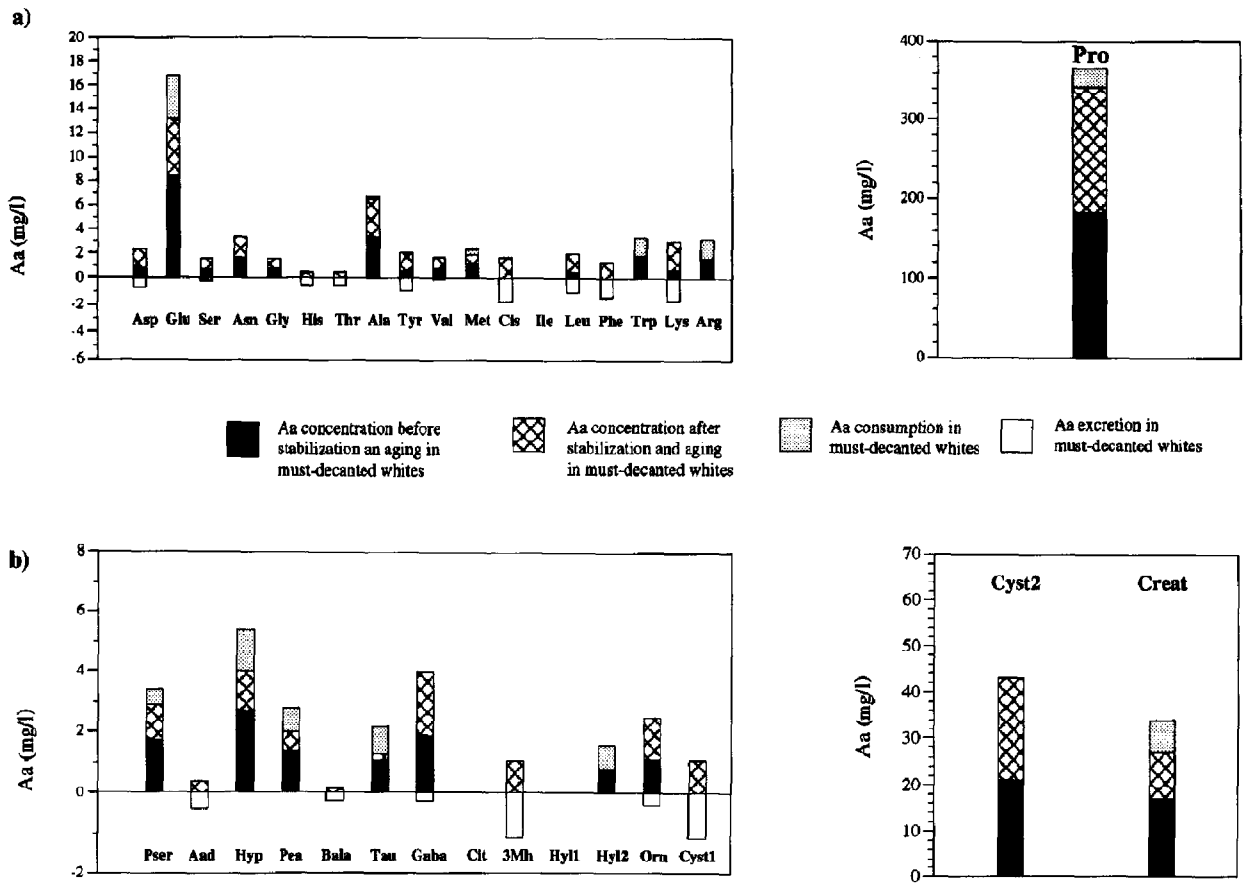

Figure 4 Concentration of amino acids in the white wine recently finished (derived from decanted must) and after stabilization and aging. Consumption or excretion of amino acids: (a) amino acids found in proteins (AaP); and (b) amino acids not found in proteins (AaNP; the consumption or excretion values for each amino acid were obtained by subtracting the amino acid concentration of the stabilized and aged wine from the concentration of the recently finished wine)

Table 1 General parameters for rosé wines recently finished, and stabilized and aging

	Control rosés		Decanted-must rosés	
	Recently finished	Stabilized and aging	Recently finished	Stabilized and aging
pH ± SD	3.17 ± 0.01	3.21 ± 0.01	3.21 ± 0.01	3.16 ± 0.01
Total acidity (tartaric acid g/l ± SD)	6.44 ± 0.03	5.22 ± 0.05	5.66 ± 0.02	4.19 ± 0.01
Volatile acidity (acetic acid g/l ± SD)	0.15 ± 0.01	0.23 ± 0.01	0.21 ± 0.05	0.28 ± 0.01
Ash (g/l ± SD)	1.46 ± 0.05	1.05 ± 0.03	1.50 ± 0.03	1.1 ± 0.1
Ash alkalinity (mequiv/L ± SD)	25.5 ± 0.7	22.0 ± 0.71	27 ± 2	25.3 ± 0.4
Reducing sugars (g/l ± SD)	1.27 ± 0.01	0.67 ± 0.02	1.1 ± 0.1	0.54 ± 0.03
Alcohol (v/v % ± SD)	12.2 ± 0.1	12.2 ± 0.1	12.25 ± 0.05	12.3 ± 0.1

Table 2 General parameters for white wines recently finished, and stabilized and aging

	Control whites		Decanted-must whites	
	Recently finished	Stabilized and aging	Recently finished	Stabilized and aging
pH ± SD	3.28 ± 0.01	3.35 ± 0.01	3.26 ± 0.01	3.33 ± 0.01
Total acidity (tartaric acid g/l ± SD)	4.85 ± 0.02	3.72 ± 0.02	4.60 ± 0.01	3.89 ± 0.01
Volatile acidity (acetic acid g/l ± SD)	0.14 ± 0.01	0.23 ± 0.02	0.11 ± 0.01	0.27 ± 0.01
Ash (g/l ± SD)	1.6 ± 0.1	1.4 ± 0.1	2.1 ± 0.4	1.2 ± 0.1
Ash alkalinity (mequiv/l ± SD)	27 ± 1	30.00 ± 0.01	32 ± 6	25.3 ± 0.3
Reducing sugars (g/l ± SD)	0.45 ± 0.08	0.27 ± 0.02	0.72 ± 0.02	0.71 ± 0.01
Alcohol (v/v % ± SD)	10.7 ± 0.1	10.7 ± 0.1	10.7 ± 0.1	10.7 ± 0.1

cystathionine-2 and creatinine was greater during the stabilization of the control wine (12.5 and 12 mg/l, respectively; *Figure 3b*) than in the wine derived from decanted must (6.5 mg/l for creatinine; cystathionine-2 was excreted; *Figure 4b*). In the white control wine, there was a greater consumption of the abundant amino acids (proline, creatinine and cystathionine-2) than in the clarified-must white wine. It seems that in the clarified-must white wine, there is less biological demand for nitrogenous sources, including proline. This suggests the existence of a smaller number of postfermentative bacteria, especially lactic bacteria, in this wine.

CONCLUSIONS

The results obtained suggest the presence of biological activity, in different degrees, during aging of the wines studied. It is possible that this activity is due to post-fermentative growth of lactic and acetic bacteria (van Wyk, 1976; Beelman *et al.*, 1982; Davis *et al.*, 1986). In general, most of the amino acids present, in very small concentrations, in the recently finished wines were not consumed or were excreted slightly, but proline was consumed in much greater quantities. This notable consumption of proline, which is considered a poor nitrogenous source (Henschke and Jiranek, 1993), would indicate the need to supply nitrogen for the post-fermentative microorganisms when there is a shortage of better nitrogenous sources; that is, it would indicate the presence of biological activity during aging of the wines studied. Lastly, in aging of stabilized wines derived from clarified musts and especially in the white wine, there was less consumption of amino nitrogen, whether found in proteins or not. This fact suggests a lesser bacterial development in the clarified must wines since the applied technological treatment eliminates microbial populations; this agrees with the observations of other authors (Fornachon, 1957; van Wyk, 1976). Thus, the wines derived from clarified musts should exhibit a greater microbiological stability during aging than the wines derived from unclarified musts.

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