Clarification by Vacuum Filtration of Grenache Must. Utilization of Free Amino Acids During Fermentation and Bottle-Aging of Wine

CARMEN ANCIN[1]*, BELEN AYESTARAN[2], and JULIAN GARRIDO[3]

The principal nitrogen sources in musts are ammonium ions and amino acids. Their uptake and metabolization by *Saccharomyces cerevisiae* depend not only on the strain and the physiological conditions, but also on the physical and chemical properties of the medium. In this sense, suspended solids stimulate the fermentation and intervene in nitrogen metabolism. The aim of this study was to observe the influence of vacuum filtration of Grenache must on the utilization of amino acids and other nitrogen compounds during fermentation and bottle-aging of wine. The results show that in the first phase of fermentation (up to 50% sugar consumption) the basic amino acids, with a greater general amino acid permease (GAP) affinity, were excreted or showed less consumption in the filtered must, while in the second phase (from 50% sugar consumption until the end of fermentation) most amino acids were excreted to the medium. In the process of stabilization and bottle-aging of wine, the yeast in wine made from filtered must consumed the major part of amino acids of proteins.

KEY WORDS: clarification, vacuum filtration, alcoholic fermentation, free amino acid utilization

Usually, must contains all the nutrients necessary for yeast growth during fermentation. The principal sources of carbon and energy are the sugars present in the must in quantities greater than necessary for maximum growth. On the other hand, the nitrogenous content of the must, which usually varies between 60 and 2400 mg/L (34), can be a limiting factor of yeast growth.

The nitrogenous compounds utilized by *Saccharomyces cerevisiae* include such diverse substances as ammonia, urea, amino acids, small peptides, and the purine and pyrimidine bases (8,27). Of all these, the principal nitrogen sources are ammonium ions and the amino acids present in the must which are transportable to the cell cytosol. The transport of ammonium nitrogen occurs via two specific permeases (12), through active transport not competitively inhibited by amino acids and requiring the presence of an energy source (14,38). The transport of amino acids during the first fermentation phase proceeds by specific permeases; *S. cerevisiae* contains specific transport systems for about 10 L-amino acids (16). No specific transport systems have been found for glycine, L-alanine, L-phenylalanine, or L-tryptophan, and the general amino acid permease (GAP) remains the only known transport system (27). During the final phases of the fermentation, the transport of amino acids occurs overall via a general amino acid permease that is inhibited by the presence of ammonium ion (19,49), and its activation coincides with the disappearance of this ion from the medium (39). This GAP, which is effective in transporting D- and L-isomers of basic and neutral amino acids, is synthesized by *S. cerevisiae* when grown in a medium containing a poor nitrogen source (18,40). When cells are grown in media containing a rich nitrogen source, such as ammonium ions, the GAP is not synthesized (36).

The uptake and metabolism of nitrogen compounds by *S. cerevisiae* depends not only on the strain and the physiological conditions, but also on the physical and chemical properties of the medium. In this sense, grape tissue particles stimulate the fermentation and intervene in the nitrogen metabolism, although the mechanism is not sufficiently known. Siebert *et al.* (42) suggest that suspended solids stimulate the fermentation by providing the yeast with nutritional factors (trace metals, nitrogenous substances, lipids). Recent works attribute a mechanical effect to the suspended solids, since it can act as a nucleus for the formation of CO_2 bubbles, which escape to the atmosphere when they reach a certain size, diminishing the inhibitory effect of this metabolic product on yeast growth (43). In any case, high pressures of CO_2 reduce the rate of uptake and the quantity of amino acids absorbed (35); to be specific, it has been observed that the uptake of valine, leucine, and isoleucine is progressively reduced when the quantity of CO_2 increases (24). In addition, Castelli

[1]Professor of Food Science, [2]Graduate Student, and [3]Professor of Inorganic Chemistry, Departamento de Química, Universidad Pública de Navarra, Campus Arrosaia s/n, 31006 Pamplona, Spain.

*Corresponding author.

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et at. (6) found an increase in total fatty acid content of the cell membrane from 9.4% to 14% after six hours exposure to CO_2. Further, it is apparent that membrane phospholipid composition, as well as its degree of unsaturation, are important for expression of GAP activity in *S. cerevisiae.* Thus, kinetics as well as the regulation of amino acids transport activity via GAP are sensitive to plasma-membrane lipid composition in *S. cerevisiae* (36). The elimination of the suspended solids in must, when it is adequate, enhances the wine's quality, since it eliminates substances that produce unwanted flavors. Clarified juices that contained less than 0.1% insoluble solids will ferment to dryness completely and rapidly if the insoluble solids are finely ground (20). Clarification by filtration through a rotary vacuum filter is a rapid and efficient way to clarify musts commercially, where it is fundamentally employed to filter large quantities of juice or wine loaded with solids. However, this technique presents, in many instances, the inconvenience of providing excessively clarified musts, such that it hinders the fermentation and produces poor quality wines (44).

The aim of this study was to observe the influence of one pre-fermentation clarification treatment, vacuum filtration, on the utilization of amino acids and the other nitrogenous compounds during fermentation and aging of produced wines. Grenache grapes were selected and the must subjected to vacuum filtration. Original must in the same batch as that taken for filtration was taken as a control. The musts were fermented to produce the corresponding rosé wines. Pre-fermentation treatment was done in a wine cellar, and the fermentation was done in a pilot plant to simulate commercial conditions. The musts and wines obtained were analyzed and compared.

Materials and Methods

Samples and vinification: *Vitis vinifera* var. Grenache grapes of Navarra Denomination of Origin were collected, crushed and destemmed in order to make rosé wines in the pilot plant. The skins were not removed for 17 hours. Must was later divided into two fractions. The first must fraction was treated with SO_2 (50 mg/L) but was not subjected to any pre-fermentation treatment. The other, following refrigeration at 10°C and addition of SO_2 (50 mg/L), was clarified by rotary vacuum filtration. Then, 400 L of the two musts were subjected to fermentation using 0.5 g/L of Fermivin active dry *S. cerevisiae* from Gist-brocades (F. Lafford & Cía., Pasajes, Spain). The temperature was controlled at 17.7°C with a standard deviation of less than 2°C. In all cases, the fermentation was continued until the concentration of reducing sugars fell below 2.5 g/L. The wines were stabilized by refrigeration at -5°C for one week, then filtered through a cellulose pad in a plate and frame filter. Finally, the stabilized wine was hand-bottled and stored for one year in the dark at ambient temperature.

A stainless crusher-stemmer, Marzola Marzinox (Marrodan & Rezola SA, Logroño, Spain), equipped with a rubber roller was used to destem and crush the grapes. The must was filtered through a rotary vacuum filter, Espal V-20 (Temavinsa, Logroño, Spain), with a 6500-L measuring barrel, equipped with a 4 hp shaking motor, a 40 hp vacuum pump, and a 7.5 hp feed pump. The diatomaceous earth filter, with a maximum particle size of 52 µm, had a surface area of 30 m^2/g and a filtration volume of 8000 to 10 000 L/hour. The turbidity of the must was determined using a model 18900 Hach turbidimeter (Hach Co. Loveland, CO), prepared for colored samples.

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

Preparation and HPLC analysis of free amino acids: Determination of free amino acids 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 × 3.9 mm i.d.) was used, with a stationary phase of dimethyloctadecylsilyl bonded to amorphous silica (Ref. Waters 10950). Derivatization was performed using a Waters PICO.TAG workstation. The PICO.TAG method developed by Waters (7) was followed. Samples were cleaned by ultrafiltration with a Millipore Ultrafree MC cartridge, and L-norleucine and L-methionine sulfone were added as internal standards. After that, precolumn derivatization with phenylisothiocyanate was carried out. The amount of sample injected was 5 µL.

Standard solutions, for the analysis of the free amino acids by HPLC, were prepared at 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-[methylsulfonyl]butanoic acid (L-methionine sulfone), both from Sigma. In derivatization, phenylisothiocyanate (Pierce, Rockford, IL), methanol (Scharlau, SA, Barcelona, Spain), triethylamine (Aldrich Chemical Co., Milwaukee, WI), and double-distilled water were used. Mobile phases were prepared with sodium acetate, acetonitrile, methanol (Scharlau) and acetic acid (E. Merck, Darmstadt, Germany). The mobile phases used were always purified through an HA 0.45-µm Millipore filter. Solvents were of HPLC quality and reagents of analytical quality.

Nitrogen contents and enological parameters: The initial must proteins were quantified by two methods: by precipitation with trichloroacetic acid followed by protein nitrogen determination; and by Bradford's modified method (47). With the former, total protein nitrogen was determined. Must proteins were precipitated with 55% aqueous trichloroacetic acid, using 1 mL for every 10 mL of must. Precipitation was performed at 0°C, and then the must was centrifuged at 4000 rpm. The supernatant was decanted and the ni-

trogen content in the residue was analyzed using the method described by the Office International de la Vigne et du Vin (31) modified by the addition of $CuSO_4$ and K_2SO_4 as catalyst instead of Se and $HgSO_4$. Nitrogen analysis of the soluble proteins in wines was performed by Bradford's modified method.

Total nitrogen, ammonium nitrogen, and enological parameters were measured according to the methods described by the Office International de la Vigne et du Vin (31). Distillation of the total and ammoniacal nitrogen was performed with Tecator automatic steam equipment (Tecator AB, S-26321 Höganäs, Sweden).

All determinations were performed in quadruplicate on representative samples of musts and wines. The results given in Tables are with standard errors (SE); to improve clarity, the results represented in histograms do not include SE; however, the coefficients of variation for amino acid data obtained by the method described were between 3% and 12.5%.

Results and Discussion

General characteristics of musts and wines: The turbidity value (Table 1) in the control must was 1460 NTU, and in the must clarified by vacuum filtration, it was 66 NTU. Therefore, 95.5% of the turbidity was eliminated. The different residual turbidity in both musts suggests that the vacuum filtration modifies the initial hydrophilic colloidal composition. The hydrophilic colloids are the most sensitive to clarification treatments (21,29). Their elimination by clarification depends on their varied macromolecular sizes (polysaccharides, proteins, pectins, and polyphenols) (45). The modification of the colloidal composition of the must has enological consequences, since it directly influences the development, the finalization of the alcohol fermentation (32) and the quality of the wine obtained (22). The filtered musts fermented much slower than control musts (2). Thus, the control must took two days to consume 50% of the sugars, while the filtered must required five days. The remaining sugar in the control was consumed in seven days, but 29 days were required for the filtered must.

In Table 1, the elevated volatile acidity of the wine obtained from the filtered must stands out (0.66 g acetic acid/L). This value is a consequence, among other factors, of the slower fermentation rate of the more clarified musts; this permits an accumulation of CO_2 that will have greater difficulty escaping in the absence of suspended solids (43). The accumulation of CO_2 reduces to a minimum the synthesis of long chain fatty acids, suggesting a greater concentration of acetyl-CoA that, upon hydrolysis, produces high concentrations of acetic acid (21).

It is also notable that vacuum filtration of the must did not modify the pH, nor the quantity of reducing sugars. The ash content was lower in the filtered must, indicating that the filter removed salts. The ash alkalinity was lower in the clarified sample, due to the retention of organic acid salts in the filter. Finally, it is observed that the quantity of alcohol was the same in the two wines, and it was not affected by the degree of clarification of the original must.

Nitrogenous content of musts and wines: Table 2 presents the total nitrogen content as well as values for the nitrogenous fractions. Total nitrogen was greater in the control must than in that clarified by vacuum filtration, dropping by 13.4% in the latter. During the fermentation process the total nitrogen diminished by 32.7% in wine made from filtered must and 41.7% in wine from control must. The decrease of assimilated nitrogen in wine made from filtered must with respect to the control is 32.1%; however, in both processes the consumption of nitrogen occurs within the interval (between 30% and 46%) found by Gorinstein *et at.* (17) after fermentation of Semillon and Carignane musts. Cold stabilization and aging led to a much larger control wine nitrogen consumption (41.8%) than found in the wine obtained from filtered must (8.4%). The lower consumption may be related to certain metabolic activity of the residual bacteria population (11), which was confirmed through microbiological analysis, demonstrating the existence of Gram (+) and catalase (-) bacteria in the wine produced from the filtered must.

Table 1. Characteristics of musts and corresponding wines (all parameters listed with their standard error, SE).

	Initial must		Midpoint of fermentation must		Wine before aging		Stabilized and aged wine	
	Filtered	Control	Filtered	Control	Filtered	Control	Filtered	Control
Turbidity (NTU ± SE)	66 ± 1	1460 ± 14	—	—	—	—	—	—
Reducing sugar (g/L ± SE)	204 ± 3	205 ± 1	93.1 ± 0.2	98 ± 3	1.27 ± 0.04	0.86 ± 0.04	0.77 ± 0.02	0.96 ± 0.01
pH ± SE	3.31 ± 0.01	3.31 ± 0.01	3.21 ± 0.01	3.15 ± 0.01	3.25 ± 0.01	3.11 ± 0.01	3.33 ± 0.01	3.12 ± 0.01
Total acidity (g/L[a] ± SE)	4.50 ± 0.01	4.80 ± 0.03	3.79 ± 0.01	3.89 ± 0.06	4.93 ± 0.02	5.36 ± 0.01	4.50 ± 0.01	4.65 ± 0.01
Volatile acidity (g/L[b] ± SE)	—	—	—	—	0.66 ± 0.05	0.19 ± 0.03	0.75 ± 0.03	0.27 ± 0.04
Ash (g/L ± SE)	2.42 ± 0.06	2.9 ± 0.3	2.61 ± 0.04	2.69 ± 0.01	1.61 ± 0.07	2.24 ± 0.06	1.06 ± 0.06	1.06 ± 0.04
Ash alkalinity (meq/L ± SE)	28.5 ± 0.5	35 ± 1	35.3 ± 0.3	32.6 ± 0.2	19.5 ± 0.7	30.0 ± 0.7	21.50 ± 0.01	24.8 ± 0.4
Alcohol (v/v ± SE)	—	—	—	—	12.8 ± 0.1	12.3 ± 0.1	12.8 ± 0.1	12.4 ± 0.1

[a]As g/L tartaric acid. [b]As g/L acetic acid.

Table 2. Total nitrogen and nitrogen concentrations in the nitrogenous fractions of musts and corresponding wines (all parameters listed with their standard error,SE).

	Initial must		Midpoint of fermentation must		Wine before aging		Stabilized and aged wine	
	Filtered	Control	Filtered	Control	Filtered	Control	Filtered	Control
Total nitrogen (mg/L ± SE)	504.0 ± 0.5	582.0 ± 0.6	492± 1	635 ± 1	339 ± 1	339 ± 9	310.5 ± 0.2	197.5 ± 0.5
Ammonium nitrogen (mg/L ± SE)	116.0 ± 0.7	107.4 ± 0.7	[a]	[a]	5.5 ± 0.1	1.9 ± 0.1	24.4 ± 0.1	4.7 ± 0.1
Protein nitrogen (mg/L ± SE)	[b]24 ± 3/ [c]15 ± 2	[b]174.2 ± 0.4/ [c]18.0 ± 0.4	[c]6.8 ± 0.5	[c]17.0 ± 0.4	[c]2.4 ± 0.3	[c]14.2 ± 0.2	[c]13.5 ± 0.1	[c]19.8 0.4
Amino nitrogen (mg/L ± SE)	183.1 ± 0.6	168.3 ± 0.2	110.5 ± 0.2	19.3 ± 0.1	217.4 ± 0.6	65.02 ± 0.05	59.3 ± 0.2	54.0 ± 0.4
Other[d] nitrogens (mg/L ± SE)	180.6	132.8	375.2	598.5	95.02	257.9	213.3	118.4

[a]Not detected. [b]Trichloroacetic method. [c]Bradford method. [d]Obtained by difference.

The ammonium nitrogen values were similar in both musts, independent of the clarification treatment, and in both cases greater than those encountered by Ough (33) with Californian grapes, and practically double those reported by Bely *et at.* (3) in French musts. This nitrogen is consumed in the first half of the fermentation, since it is the principal source of assimilable inorganic nitrogen for the yeast in its growth phase (8). However, in our case, while this nitrogen was consumed in the first hours of fermentation in the control must, it remained in the filtered must for four days. In the filtered must, a lower fermentation rate resulted in the yeast having less energy for active transport of ammonia. In the last phase of fermentation, ammonia was liberated from the media, especially in the filtered must sample (5.5 mg/L). In the bottle-aging process, the ammoniacal nitrogen greatly increased in wine made from filtered must (24.4 mg/L); this larger liberation likely results from the deamination of amino acids that have served as nitrogenous sources for the residual lactic bacteria which have survived during aging period (26).

With respect to protein nitrogen, it is evident that there is an important reduction of proteins in the vacuum filtered must due to the retention of macromolecules or colloids formed principally by high molecular weight protein fractions (46) and polysaccharides; however, there was practically no change in the fraction of soluble proteins (Table 2). During the fermentation process, the protein nitrogen precipitates in both musts. During bottle-aging, the protein nitrogen concentration increased overall in the wine produced from the clarified must; this is probably due to proteins or polypeptides liberated by autolysis of the residual microorganisms (37).

The amino nitrogen increased in the filtered must with respect to the control, suggesting that the hydrolytic activity of the plant (grape) proteases acts not only during the time of skin-must contact but also during the clarification (13). In the filtered must, the yeast used only 39.7% of this amino nitrogen during the first phase of fermentation, probable due to oversaturation of CO_2 affecting the transport of amino acids (25,36). In the second phase of fermentation, amino nitrogen was liberated possibly by excretion of amino acids from the intracellular pool as a response to the absence of glucose in the medium (4,30), and to the yeast's proteolytic activity, a process favored by CO_2 saturation (25).

Evolution of amino acids during the fermentation and bottle-aging of the wine: The vacuum filtration process increased the must's concentration of amino acids of proteins (AaP), probably as a result of the action of plant (grape) proteases which pass into the must from the pulp. This increase in AaP did not produce an increase in the must fermentation rate. However, the amino acids not found in proteins (AaNP) were hardly modified by filtration with respect to the control must (2).

First phase of fermentation (up to 50% of sugar consumption): The initial must concentrations, the fermentation midpoint values and the consumption or excretion of AaP (Fig. 1A) and of AaNP (Fig. 1B) are represented graphically; the values of arginine, proline and glutamic acid are given in Table 3. Evidently, the basic amino acids (histidine, lysine, ornithine, γ-aminobutyric acid, creatinine, hydroxylysine-2, and arginine), with a greater GAP affinity (50), were excreted or showed less consumption in the filtered must than in the control, with the exception of lysine. Noteworthy is the high consumption of arginine in the control must (99.7%) and the low consumption of this amino acid in the filtered must (37.6%). Probably this difference coincides with the transport of this amino acid being different in both musts; in the control must, ammonium disappeared rapidly from the medium, implying that the yeast, besides obtaining this amino acid by specific permeases (utilized when there is ammonium in the medium), utilized GAP. In the filtered must, the longer duration of ammonium in the medium increased the time the specific permeases acted alone. In the more clarified musts, the GAP, besides the delayed action, is less active, since it is sensitive to the changes in lipid composition of the plasmatic membrane of *S. cerevisiae;* these changes are due, among other factors, to the oversaturation of CO_2 existing in the well-clarified musts (25,36,42,,43). The different means of transport in both musts will probably cause distinct metabolic pathways; in the case

Table 3. Evolution of arginine, proline, and glutamic acid during fermentation of musts and aging of wine

Amino acids	Concentration (mg/L)				[a]Assimilation/excretion[b] (mg/L)		
	Initial must	Midpoint of fermentation must	Wine before aging	Stabilized and aged wine	1st phase of fermentation	2nd phase of fermentation	Aging
Control							
Arginine	358 ± 10	1.10 ± 0.07	2.6 ± 0.3	2.6 ± 0.2	-357	+1.5	0
Proline	174 ± 5	82 ± 4	373.4 ± 0.7	239 ± 15	-92	+291	-134
Glutamic acid	28.3 ± 0.7	10.6 ± 0.7	16.3 ± 0.3	11.0 ± 0.4	-17.7	+5.7	-5.3
Filtered							
Arginine	358 ± 11	224 ± 3	463 ± 33	112 ± 3	-134	+239	-351
Proline	175 ± 4	127 ± 7	223 ± 15	66 ± 8	-48	+96	-157
Glutamic acid	60 ± 2	1.9 ± 0.7	10.0 ± 0.8	5.6 ± 0.5	-58	+8.1	-4.4

[a]Assimilation = negative value. [b]Excretion = positive value.

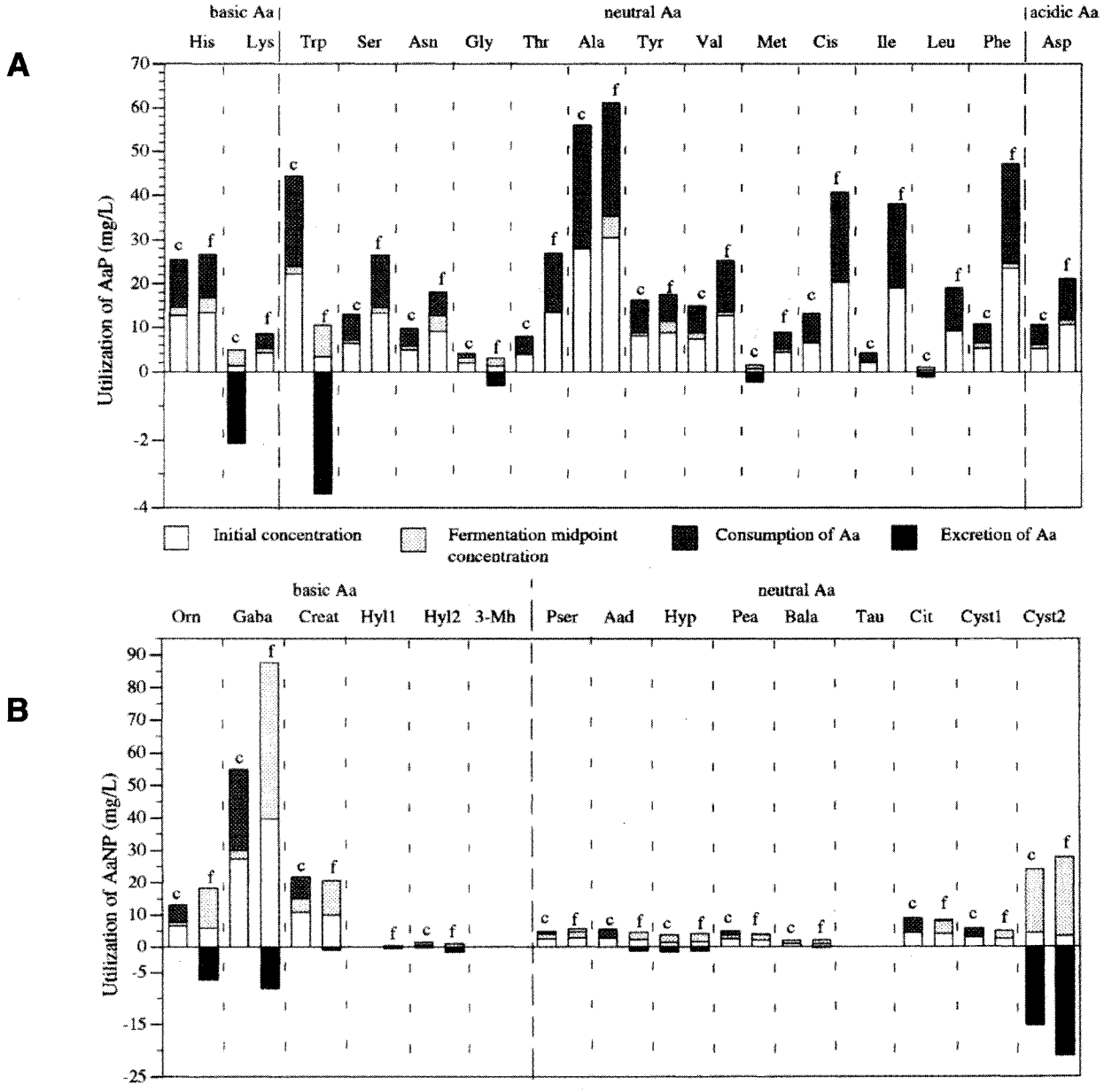

Fig. 1. Initial concentration of amino acids in the musts and at fermentation midpoint. Assimilation or excretion of amino acids during the first phase of fermentation: (**A**) amino acids of proteins (AaP); (**B**) amino acids not found in proteins (AaNP); (**c**) control must sample; (**f**) filtered must sample).

of arginine, procurement via basic amino acid permeases will assure an anabolic route (8), while if it is transported by GAP, it will be catabolized and/or accumulated in the vacuoles. With histidine something similar occurred, although the difference in the control must (86.1%) and in the filtered must (73.8%) were not so marked. In the filtered must, the activation time of the two histidine specific permeases (8) was greater than in the control must, where GAP was active sooner. This effect will be a consequence of the differing velocities for the disappearance of ammonium in the medium.

In Figure 1B, the evolution of ornithine is very different in the filtered must (where it was excreted, 108.6%) and the control must (where it was consumed, 81.4%). The liberation of ornithine in the filtered must could be due to a lack of substrate acceptor (α-ketoglutarate) for ornithine's amino group, a necessity for this amino acid to follow its metabolic route to glutamate semialdehyde. The production of this acceptor depends on the degradation of glucose via pyruvate (50); therefore, the slower rate of sugar uptake in the growth phase of the filtered must will imply a lesser quantity of the acceptor with which the ornithine, formed from arginine (34), joins and would be accumulated and/or excreted to the medium.

Also noteworthy are the values of lysine which exhibits a different tendency than the rest of the basic

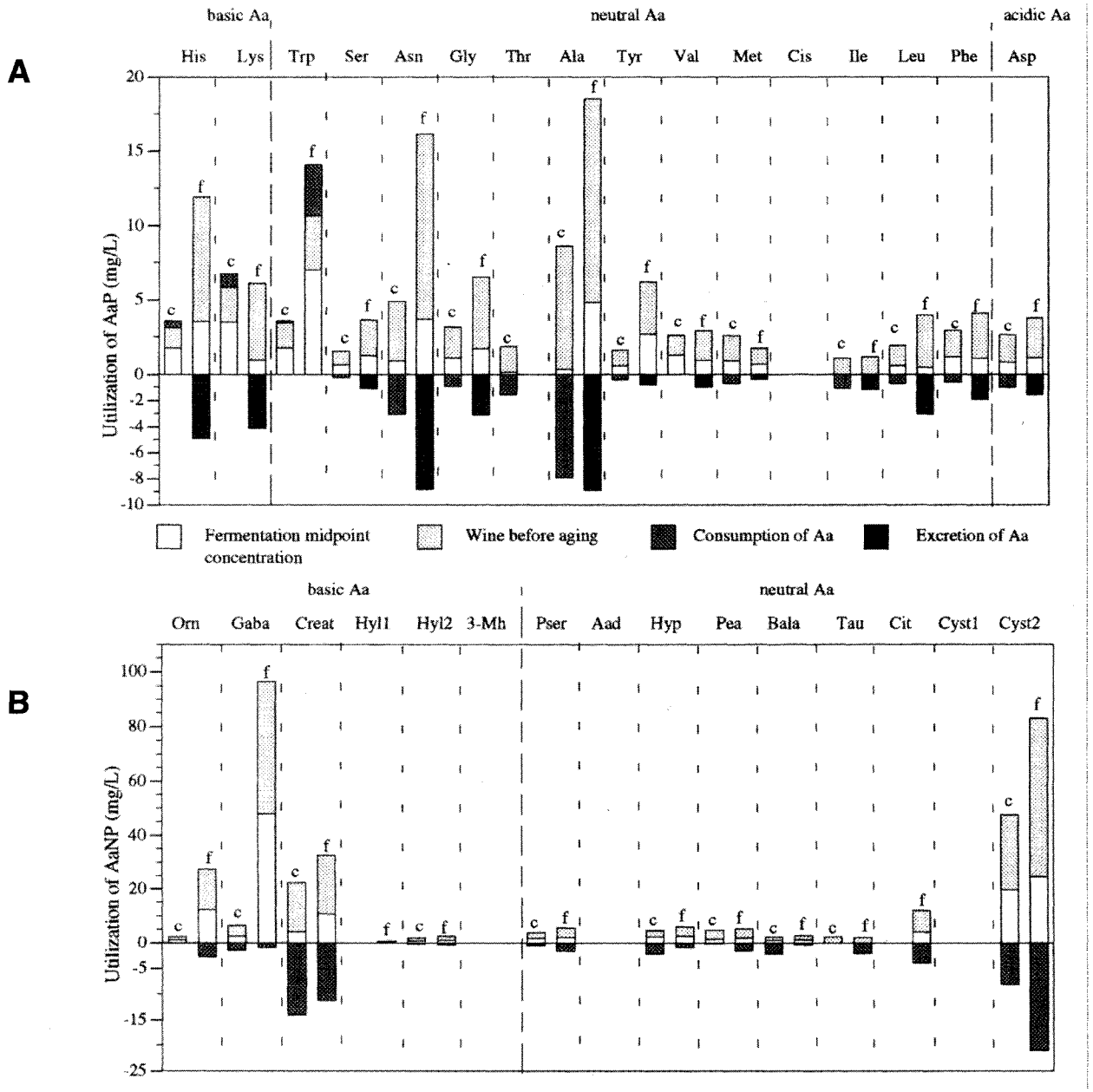

Fig. 2. Concentration of amino acids in the must at the midpoint of fermentation and in the wine before aging. Assimilation or excretion of amino acids during the second phase of fermentation: (**A**) amino acids of proteins (AaP); (**B**) amino acids not found in proteins (AaNP); (**c**) control must sample; (**f**) filtered must sample).

amino acids, since it was consumed in the filtered must (76.8%), while it was excreted in the control must (148.6%). Various authors have found that 90% of lysine remains as a reservoir in the vacuolar pool (9). It cannot be fully utilized because *S. cerevisiae* does not possess the necessary enzymes for its complete degradation as sole nitrogen source (30). The increase of lysine in the control must will be due to the yeast's capacity to transport and hydrolyze small dipeptides and tripeptides within the cell (8,28) and to the interchange of this amino acid between the contents of the intracellular pool and the external medium (30). Finally, γ-aminobutyric acid and creatinine were consumed in the control must while their content increased in the filtered must.

For the neutral amino acids, there is no clear tendency, such as that in the filtered must; less alanine, tyrosine, citrulline, cystathionine-1, and proline were consumed than in the control, but more of tryptophan, serine, asparagine, threonine, valine, cystathionine, isoleucine, and phenylalanine. As for glycine and aminoadipic acid, they were excreted in the filtered must and consumed in the control. Finally, methionine and leucine were excreted in the control and consumed in the filtered must (Fig. 1A, 1B; Table 3). Of these results, the minor consumption of the first group in the filtered must is noteworthy, probably equal to that in the basic amino acids, and it will be a consequence of the delayed activation of GAP by the presence of ammo-

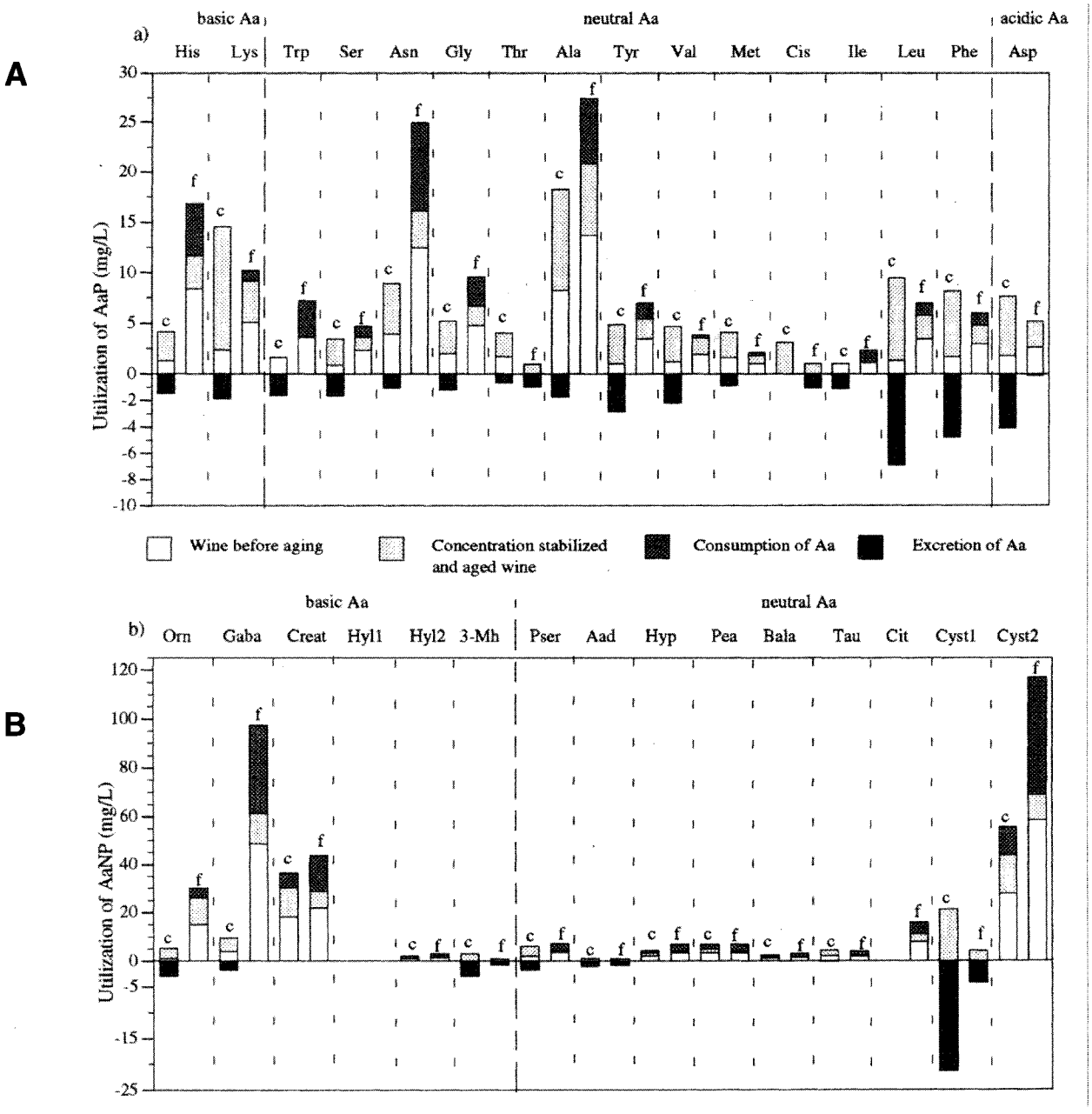

Fig. 3. Concentration of amino acids in wines before aging and in wines aged for a year. Assimilation or excretion of amino acids during the aging and stabilization process: (**A**) amino acids of proteins (AaP); (**B**) amino acids not found in proteins (AaNP); (**c**) control must sample; (**f**) filtered must sample).

nium in the medium and of this transport enzyme's sensitivity to lipid composition changes in the plasma membrane of *S. cerevisiae* caused by oversaturation of CO_2 in the clarified musts (25,36,42,43). The consumption of proline in the control must was 52.9%, while in the filtered it was 27.2%. Among the factors that contribute to the decreased consumption of proline in the filtered must, the decreased turbidity is remarkable; low values of turbidity favor more anaerobic conditions impeding the activation of the proline-degrading mitochondrial enzymes (23).

In Figure 1A and in Table 3, there is greater consumption of dicarboxylic amino acids (aspartic and glutamic) in the filtered must. Notable is the large consumption of glutamic acid in the filtered must (96.8%) in contrast to the control (62.8%). The elevated utilization of this amino acid confirms the results obtained by Cooper (8), who considers it a good nitrogenous source for a large number of yeasts since, in the presence of ammonium, it enters directly into the intracellular pool during the yeast's growth (16,27). The greater duration of ammonium in the filtered must allows the two glutamate specific permeases, the one with high affinity (K_m = 20 µmoles) and the one with low affinity (K_m = 3.3 mmoles) (5), to function alone for a longer period. In the filtered must (with slower sugar consumption), the greater utilization of these amino acids will be due to their providing for the immediate nitrogen necessities of the cells while requiring less energetic force, since they are found principally in the cytoplasmatic pool (48).

Second phase of fermentation (from 50% sugar consumption until the end of fermentation): In general, it is observed (Fig. 2A, 2B; Table 3) that in this phase, in contrast to the first, a majority of the amino acids were excreted to the medium in a quantity independent of the initial must concentration. This coincides with the results obtained by Monteiro and Bisson (30), who observed that the relative concentration of free amino acids did not seem to be related with their initial concentration in the must.

This liberation in the last phase of the fermentation is fundamentally due to the high concentration of ethanol; this metabolic product increases the plasma membrane's permeability (15,41). The increase in the permeability causes excretion of amino acids from the cytoplasmic pool to the medium by a passive process (4). Since there is practically no sugar remaining at the end of the fermentation, the nitrogen compounds are not reabsorbed and remain in the wine influencing the quality (41). In the wine derived from the filtered must, a greater content of amino acid constituents of proteins was excreted than in the control (Fig. 2A) with the exception of methionine and proline; on the other hand, the excretions of amino acids which do not comprise part of the proteins do not show a clear tendency (Fig. 2B). The major liberation of amino acids in the wine from filtered must is probably due to the lower turbidity diminishing the tolerance of the cell membrane to ethanol; this means greater permeability for the membrane. Supplementing the clarified must with combinations of unsaturated fatty acids, vitamins and proteins increases the tolerance of the cellular membrane for ethanol (10). Another possible cause for the increased liberation of amino acids in the filtered must would be the oversaturation of CO_2 that increases the proteolytic activity of the yeast (25).

Process of stabilization and bottle-aging of the wine: In general, the process of stabilization and bottle-aging showed a diminution of amino nitrogen (Table 2). Figures 3A and 3B represent the consumption (or excretion) of amino acids in both wines. In Figure 3A, it is observed that the yeasts in wine made from filtered must consumed the major part of the amino acids of proteins with the exception of threonine, cystine and aspartic acid. In the wine produced from the control must, they were excreted, with the exception of cystine. In Figure 3B, it is observed that in both wines, certain amino acids were consumed which are not found in proteins, such as creatinine, hydroxylysine-1, hydroxyproline, phosphoethanolamine, ß-alanine, taurine, and cystathionine-2; however, 3-methylhistidine, α-aminoadipic acid, and cystathionine-1 were excreted. In the wine from filtered must, unlike the control, ornithine, γ-aminobutyric acid, and phosphoserine were consumed. The high consumption of amino acid proteins in the filtered must wine is probably due to increased growth of the residual bacteria population favored by the elevated assimilable nitrogen concentration that has remained in this wine (11,26) and by the liberation to the medium of constituent compounds from yeast autolysis (1,16).

Conclusions

From the results of this work, it can be concluded that in Grenache musts, highly clarified by vacuum filtration, the utilization of nitrogenous compounds by yeast follows a different behavior from that of the unclarified control must. Among these differences, the low consumption of basic amino acids in the first half of fermentation with respect to the control must is noteworthy. Also, it was observed that in the latter stage of fermentation the excretion of amino acids to the medium was much greater in the clarified must than in the control. Probably, these differences were due to various factors. The low rate of fermentation of the filtered must would be the cause of yeast having less energy, at a given moment, for the active transport of nitrogenous substances. Similarly, in well-clarified media, CO_2 is retained in high concentrations: this metabolic product being responsible for altering different systems related to nitrogenous metabolism. Further, the absence of suspended solids in the must may make the yeast more sensitive to the toxic effect of ethanol.

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