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PII: S0308-8146(98)00009-0

Food Chemistry, Vol. 63, No. 2, pp. 191–197, 1998

Printed in Great Britain
0308-8146/98 $19.00+0.00

Sedimentation clarification of viura musts. Utilization of amino acids during fermentation

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(Received 21 March 1997; revised version received and accepted 27 November 1997)

The influence of sedimentation on viura must's concentration of free amino acids and changes during fermentation were studied; the results were compared with an unclarified sample. The protein fraction was reduced by this treatment; the amino nitrogen increased slightly in the decanted must, and the concentration of ammonium nitrogen was similar in both samples. The decanted must had a greater concentration for most of the amino acid constituents of proteins, neutral as well as basic. This treatment did not alter the medium sufficiently to modify yeast utilization of these nutrients during fermentation; in fact, in both samples, basic, neutral, and acidic amino acids were consumed in a similar manner in the first half of fermentation (until 50% sugar consumption) and were excreted similarly in the freshly produced wine. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Nitrogenous compounds in must are very important for yeast development (Vos *et al.*, 1980; Monk *et al.*, 1986), and even some collateral metabolic products influence wine quality (Henschke and Jiranek, 1993). Ammonia, amino acids, peptides, and proteins are, quantitatively, the major nitrogenous compounds in grape must (Henschke and Jiranek, 1993) and, of these, yeast uses ammonia, amino acids and small peptides as a nitrogen source.

Ammonia disappears rapidly at the start of fermentation (Zoecklein *et al.*, 1990) and yeast uptake is by active transport using two specific permeases (Roon *et al.*, 1975; Dubois and Grenson, 1979; Egbosimba and Slaughter, 1987). Amino acids represent between 60 and 90% of total must nitrogen (Kliewer, 1970) and are the principal nitrogen source for the growth of these microorganisms. The active transport of these substances into *S. cerevisiae* occurs via general amino acid permeases (GAP) and by various specific permeases that obtain the necessary energy from degradation of glucose in the medium (Cooper, 1982; Olivera *et al.*, 1993). The GAP acts rapidly and transfers all amino acids to the cell cytosol (Cooper, 1982). The activity of this permease, in *S. cerevisiae*, is greatest in nitrogen-poor media and is inhibited by the presence of ammonia; this does not occur with specific permeases (Dubois and Grenson, 1979). Ethanol is among the factors that affect transport of nitrogenous compounds in *S. cerevisiae*, and it modifies the composition and permeability of the plasma-membrane inhibiting the transport of ammonia and amino acids (Léao and van Uden, 1984; van Uden, 1985). Carbon dioxide supersaturation of the medium modifies both the lipid content and the degree of fatty acid unsaturation in the cellular membrane, affecting the GAP activity (Calderbank *et al.*, 1984, 1985).

The variation in concentration of nitrogenous substances in must is due, principally, to varietal factors and to elaboration processes (pressing, must clarification treatments, maceration, etc.). In this respect, preclarification processes are notably influential; thus, vacuum filtration of viura and gamacha musts reduces the concentration of total protein nitrogen but does not cause a loss of ammoniacal and amino nitrogen (Ancín *et al.*, 1996a,b).

Clarified musts produce wines of excellent quality; however, slightly turbid musts ferment more vigorously and have a greater quantity of viable yeast (Siebert *et al.*, 1986). In well-clarified musts, the fatty acid concentration decreases (Bertrand and Miele, 1984; Delfini *et al.*, 1992) and, as such, the transport system for some nutrients, like amino acids, can be altered. When must clarification is excessive, CO_2 supersaturation occurs with a consequent alteration in the uptake mechanism for amino acids and for carbohydrates (Siebert *et al.*, 1986; Axcell *et al.*, 1988; Kruger *et al.*, 1992).

The objective of this work was to study the influence of sedimentation on the amino acid content in viura

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must and its utilization throughout the fermentative process. The results were compared with a control must, unclarified.

MATERIALS AND METHODS

Samples and vinification

Vitis vinifera var. viura grapes of Navarra Denomination of Origin were crushed and de-stemmed to make must for subsequent production of white wine in a pilot plant. The skins were not removed for 5–8 h. Must was later divided into two fractions. The first was treated with SO_2 (50 mg/litre) but was not subjected to any prefermentation technique. The other, following refrigeration to 10°C and addition of SO_2 (50 mg/litre), was clarified by sedimentation for 24 hours. Then 400 litres of the two musts were each subjected to fermentation using 0.5 g/litre of Fermivin active dry *S. cerevisiae* from Gist-brocades (F. Lafford and Cia., Pasajes, Spain). The temperature was controlled at 18 ± 2°C. In both cases, the fermentation was continued until the concentration of reducing sugars fell below 2.5 g/litre.

A stainless crusher-stemmer, Marzola Marzinox (Marrodan and Rezola SA, Logroño, Spain), equipped with a rubber roller was used to de-stem and crush the grapes. Vinification and sedimentation were 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 litres.

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 (Cohen *et al.*, 1989) was followed. Samples were cleaned by ultrafiltration with a Millipore ultrafree MC cartridge, and then 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, internal standards and mobile phases were the same as those reported by Ayestarán *et al.* (1995).

Nitrogen contents

The initial must proteins were quantified via two methods: precipitation with trichloroacetic acid followed by protein nitrogen determination and by Bradford's modified method (Waters *et al.*, 1991). 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 the must was then centrifuged at 4000 rpm. The supematant was decanted and the nitrogen content in the residue was analyzed using the method described by the Office International de la Vigne et du Vin (1990) modified by the addition of $CuSO_4$ and K_2SO_4 as catalyst instead of Se and $HgSO_4$. Nitrogen analysis of soluble proteins in wines was performed by Bradford's modified method.

Total nitrogen and ammonium nitrogen were measured according to the methods described by the Office International de la Vigne et du Vin (1990). Distillation of the total and ammonium nitrogen was performed with Tecator automatic steam equipment (Tecator AB, S-26321 Höganäs, Sweden).

Turbidity and enological parameters

Enological parameters were obtained according to the methods described by the Office International de la Vigne et du Vin (1990). Must turbidity was determined using a 18900 Hach turbidimeter (Hach Co., Loveland, Colorado, USA) prepared for coloured samples.

All determinations were performed in quadruplicate on representative samples of musts and wines. The results given in the tables and figures include standard errors (SE).

RESULTS AND DISCUSSION

General characteristics of musts and wines

Sedimentation of must reduced turbidity 72% with respect to the control (Table 1). This clarification tratment did not modify the initial content of reducing sugars, ash, nor pH. The wines produced had similar values for alcoholic degree and volatile acidity.

Nitrogenous content in the musts and wines

Sedimentation reduced total nitrogen concentration 10.6% with respect to the control must (Table 2). The nitrogenous fraction most sensitive to sedimentation was total protein nitrogen since it decreased by 23.4%; this was due to the elimination of protein fractions with high molecular weight (Vos and Gray, 1979). Must proteins are important for the development and finalization of fermentation due to their capacity to adsorb inhibitors of this process (Ollivier *et al.*, 1987), such as decanoic acid, octanoic acid, and their corresponding ethyl esters (Lafon-Lafourcade *et al.*, 1984). Protein elimination in decanted must could be one of the factors that induced different average daily sugar consumption

Table 1. General parameters of decanted and control viura musts, and the wines produced from fermentation ($n = 4$, SE = standard error)

	Turbidity (NTU ± SE)	Reducing sugars (g/litre ± SE)	pH ± SE	Volatile acidity (g/litre[a] ± SE)	Ash (g/litre ± SE)	SO_2 total (mg/litre ± SE)	Alcohol (v/v % ± SE)
Decanted must	195 ± 7	181 ± 0.5	3.51 ± 0.01	—	3.33 ± 0.05	48 ± 2	—
Midpoint of fermentation		89.5 ± 0.4	3.31 ± 0.01	—	3.0 ± 0.1	49 ± 2	—
Wine recently produced		0.72 ± 0.02	3.26 ± 0.01	0.11 ± 0.03	2.1 ± 0.4	49 ± 1	10.7 ± 0.1
Control must	695 ± 7	180 ± 1.7	3.51 ± 0.01	—	3.4 ± 0.1	48.5 ± 0.2	—
Midpoint of fermentation		74 ± 2	3.32 ± 0.01	—	3.1 ± 0.2	48.9 ± 0.2	—
Wine recently produced		0.45 ± 0.08	3.28 ± 0.01	0.14 ± 0.01	1.6 ± 0.1	55.6 ± 0.9	10.7 ± 0.1

[a]As g/litre acetic acid.

Table 2. Total nitrogen and nitrogen concentrations in musts and corresponding wines ($n = 4$, SE = standard error)

	Initial must		Midpoint of fermentation		Recently produced wine	
	Decanted	Control	Decanted	Control	Decanted	Control
Total nitrogen (mg/litre ± SE)	369 ± 0.5	413 ± 1	171 ± 1	210 ± 1	111 ± 3	125 ± 4
Ammonium nitrogen (mg/litre ± SE)	110 ± 7	101 ± 10	[a]	[a]	[a]	[a]
Protein nitrogen (mg/litre ± SE)	[b]61.7 ± 0.3/17.5 ± 0.03[c]	[b]80.5 ± 0.2/12 ± 2[c]	15.0 ± 0.3[c]	11.8 ± 0.1[c]	1.4 ± 0.1[c]	2.7 ± 0.3[c]
Amino nitrogen (mg/litre ± SE)	117 ± 1	96.3 ± 0.6	11.4 ± 0.05	12.8 ± 0.04	35.7 ± 0.2	52.6 ± 0.2
Other[d] nitrogens (mg/litre ± SE)	80.6	135	144.6	185.4	73.9	69.7

[a]Not detected.
[b]Total protein.
[c]Soluble protein.
[d]Obtained by difference.

percentages in the ranges 5–50% (V_{f5-50}) and 0–99% (V_{f0-99}) of total sugar consumed (Houtman and Du Plessis, 1985); in the range 5 to 50%, the amount of sugar consumed was greater in the control must (31.8%/day) than in the decanted (22.5%/day) and, similarly, the value (V_{f0-99}) was lesser in the decanted (5.6%/day) than in the control (14.1%/day) (Ancín *et al.*, 1996*c*). In contrast to protein nitrogen, amino nitrogen slightly increased in decanted must (18%) with respect to the control, and ammonium nitrogen was similar in both samples.

At the end of fermentation, total nitrogen decreased equally in both wines (≅69%). The disappearance of soluble protein nitrogen was greater in wine produced from decanted must (92%) than in the control (77.5%). On the other hand, amino nitrogen was utilized in greater quantity in the decanted sample (69.5%) than in the control (45.4%).

Basic amino acids in the must and their utilization during the fermentative process

The total concentration of these amino acids was slightly greater in decanted must (244 mg/litre) than in control must (188 mg/litre). The clarification process increased the concentration of arginine and creatinine, but did not alter the concentration of histidine, lysine, ornithine, γ-aminobutyric acid, and hydroxylysine2 (Fig. 1(a) and Table 3). The presence of these amino acids varied greatly in viura must since their concentrations ranged from 2.7 to 191 mg/litre in the decanted sample and from 2.7 to 148 mg/litre in the control. In both cases, lysine had the lowest concentration and arginine together with γ-aminobutyric acid constituted the majority of basic amino acids; this agrees with the results reported by other authors for different musts (Kliewer, 1970; Juhász *et al.*, 1984; Ooghe and Kastelijn, 1988).

During the first half of fermentation (until 50% sugar consumption), there was a very large consumption of basic amino acids (Fig. 1(b) and Table 3), and it was similar in both samples (decanted, 95.6%; control, 94.6%). The high consumption of arginine in both musts was remarkable (decanted, 99.7%; control, 99.5%); this amino acid provided yeast with 27% of nitrogen consumed in decanted must and 25.7% in the control. This is similar to the results of Henschke and Jiranek (1993) who observed that arginine satisfied between 30 and 50% of yeast's nitrogenous requirements. In Fig. 1(b) and in Table 3, it is also observed that the concentration of the majority of basic amino acids (histidine, ornithine, γ-aminobutyric acid, creatinine, hydroxilysine2 and arginine) evolved similarly in both samples. This agrees with the fact that, independent

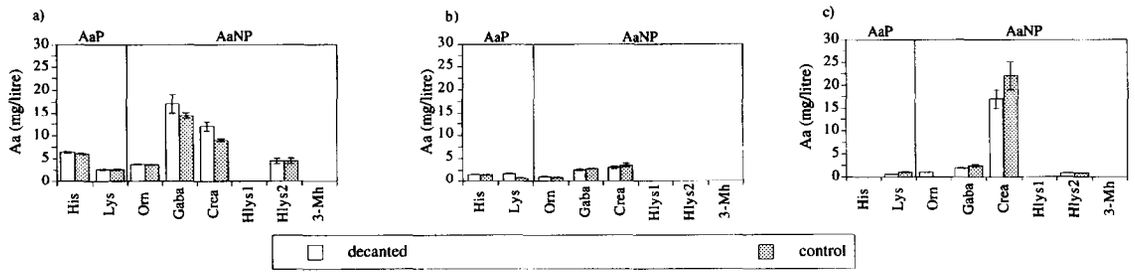

Fig. 1. Concentration of basic amino acids in decanted and control samples: (a) in viura must; (b) at midpoint of fermentation; (c) in wine recently produced. AaP: amino acid constituent of proteins. AaNP: amino acid not constituent of proteins.

Table 3. Evolution of arginine and proline during fermentation of musts ($n = 4$, SE = standard error)

Amino acids	Concentration (mg/litre)			Assimilation[a]/excretion (mg/litre)[b]	
	Initial must	Midpoint of fermentation	Recently produced wine	First half of fermentation	Second half of fermentation
Decanted					
Arginine	191 ± 16	0.59 ± 0.06	1.6 ± 0.5	−190.4	+1
Proline	175 ± 14	38 ± 5	183 ± 19	−137	+145
Control					
Arginine	148 ± 11	0.71 ± 0.04	2.7 ± 0.9	−147.3	+2
Proline	147 ± 10	48 ± 5	282 ± 22	−99	+234

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

of the degree of must clarification, ammonia was consumed in the first hours of fermentation. Ammonia inhibits GAP activity (Dubois *et al.*, 1974; Dubois and Grenson, 1979), which has a great affinity for basic amino acids (Woodward and Cirillo, 1977), so that this transport system will act analogously in both musts. Sedimentation eliminated solids in suspension which act as nuclei for CO_2 bubble formation (Thomas *et al.*, 1994). However, it does not seem that the clarification was so drastic that high concentrations of CO_2 (which would cause changes in lipidic composition of the plasma-membrane of *S. cerevisiae*) (Prasad and Rose, 1986; Siebert *et al.*, 1986; Kruger *et al.*, 1992), altering the activity of GAP, existed in the must. In contrast to the rest of the basic amino acids, lysine was poorly consumed in decanted must (37%). This corroborates the results of Monteiro and Bisson (1991*a*,*b*), who considered this amino acid a poor nitrogenous source for *S. cerevisiae* since the yeast does not possess the necessary enzymatic machinery for complete degradation.

In the second half of fermentation (from 50% sugar consumption until the end), there was no uniform tendency in utilization of basic amino acids (Fig. 1(c) and Table 3) since some were excreted and others were consumed. In both samples, the high liberation of creatinine was noteworthy, reaching values superior to the initial concentration. Both hydroxylysine2 and arginine were liberated in small concentrations in both wines; in contrast, histidine was consumed in both samples. The fact that greater concentrations of basic amino acids were not liberated in clarified must than in the control confirms that sedimentation did not excessively clarify the must. In excessively clarified musts, plasma-membranes of yeast can be altered as these are more sensitive to the toxic action of ethanol (D'Amore and Stewart, 1987; Salgueiro *et al.*, 1988).

Neutral amino acids in must and their utilization during the fermentative process

In Fig. 2(a) and in Table 3, it is observed that there is a large range of values for these amino acids, from the concentration of β-alanine (decanted, 0.57 mg/litre; control, 0.4 mg/litre) to that of proline (decanted, 147 mg/litre; control, 175.1 mg/litre). Proline and alanine were the major neutral amino acids in both samples, which agrees with the results of diverse authors for different varieties of must (Kliewer, 1970; Juhász *et al.*, 1984; Ooghe and Kastelijn, 1988). The majority of neutral amino acids which are building blocks of proteins (tryptophan, serine, asparagine, glycine, threonine, tyrosine, valine, methionine, cystine, isoleucine, leucine, phenylalanine), were present in greater concentrations in decanted must than in the control. Equally, as with arginine, the increase for the majority of the neutral amino acids in decanted must was due to the activity of grape vegetal protease during clarification. On the other hand, concentrations of neutral amino acids which are not constituents of proteins (phosphoserine, α-aminoadipic acid, hydroxyproline, phosphoethanolamine, β-alanine, citrulline, cystathioninel, cystathionine2), were

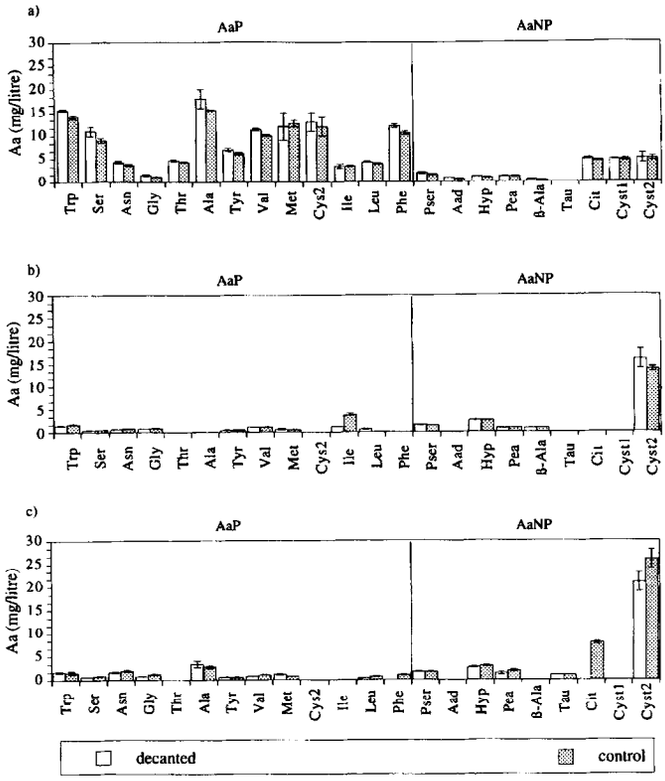

Fig. 2. Concentration of neutral amino acids in decanted and control samples: (a) in viura must; (b) at midpoint of fermentation; (c) in wine recently produced. AaP: amino acid constituent of proteins. AaNP: amino acid not constituent of proteins.

similar in both musts but inferior to the aforementioned neutral amino acids (Ayestarán *et al.*, 1995).

In the first half of fermentation (Fig. 2(b) and Table 3), total consumption of neutral amino acids was comparable in both samples (decanted, 78.5%; control, 71.4%) although less than in the basic amino acids. This confirms that GAP has a greater affinity for basic amino acids than for neutral and dicarboxylies (Woodward and Cirillo, 1977). In both samples, the elevated consumption of these amino acids (between 80 and 100%), with the exception of glycine, isoleucine, phosphoserine, and phosphoethanolamine that were consumed between 0 and 50%, was remarkable. A particularly good nitrogenous source was alanine since it was present in major concentrations and was consumed totally. This result agrees with those of various authors (Cooper, 1982; Large, 1986; Jiranek *et al.*, 1990) whose works demonstrated that alanine, as well as other amino acids, was a good nitrogenous source. Similarly, tryptophan, serine, valine, methionine, cystine, and phenylalanine were, for yeast, important nitrogenous sources in both samples since they were present in elevated concentrations and were greatly consumed (between 90 and 100%). In contrast, glycine and isoleucine were hardly consumed in both musts.

In clarified must, proline was consumed 78.3% and in the control 67.3% (Table 3). The utilization of this amino acid in both samples was related to the elevated consumption of the rest of the amino acids that, when present in the medium, inhibit the specific transport system of proline (Duteurtre *et al.*, 1971; Lasko and Brandiss, 1981; Jiranek *et al.*, 1990). Furthermore, at the start of fermentation, the conditions are not so anaerobic as to impede activation of the mitochondrial degradative enzymes of proline (Ingledew *et al.*, 1987).

Among the neutral amino acids (Fig. 2), most of those with sulfur (methionine, cystine, and cystahioninel) were consumed in both samples (between 90 and 100%) with cystahionine2 the exception; it was excreted. This agrees, partially, with the observation of Bidan and Collon (1985) who reported that *S. cerevisiae* consumed methionine but not cystine.

In the second half of fermentation (Fig. 2(c) and Table 3), most of the amino acids in both samples were liberated with a notable and important excretion of alanine and proline; in contrast, valine and isoleucine were consumed. This evolution, similar to the basic amino acids and independent of the degree of must clarification, confirms that sedimentation was not so drastic as to diminish the yeast's tolerance to ethanol and provoke a high liberation of amino acids to the medium.

Acidic amino acids in must and their utilization during the fermentative process

In Fig. 3(a) it is observed that, in both musts, aspartic acid and glutamic acid were present in comparable

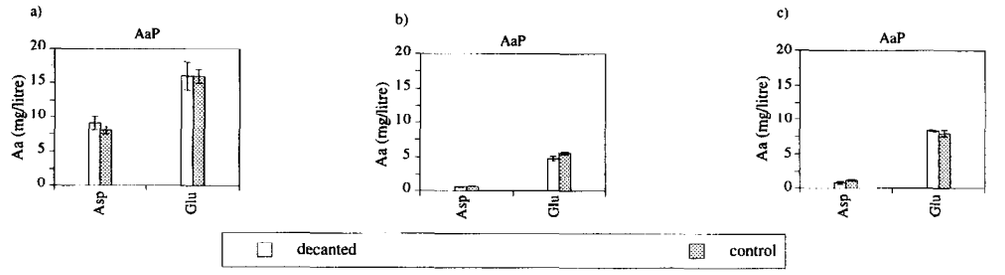

Fig. 3. Concentration of acidic amino acids in decanted and control samples: (a) in viura must; (b) at midpoint of fermentation; (c) in wine recently produced. AaP: amino acid constituent of proteins. AaNP: amino acid not constituent of proteins.

concentration and independent of the sedimentation treatment. As various authors have observed for different varietal musts (Kliewer, 1970; Juhász *et al.*, 1984; Ooghe and Kastelijn, 1988) glutamic acid was the most prevalent acidic amino acid in viura must.

In the first half of fermentation (Fig. 3(b)), acidic amino acids were consumed in similar percentages in both samples (decanted, 78.4%; control, 74%). The high consumption of aspartic acid and glutamic acid was due to these amino acids, as well as leucine, meeting with less energetic effort, the immediate cellular necessities being, principally, in the cytoplasmatic pool (Watson, 1976).

In the second half of fermentation (Fig. 3(c)), glutamic acid liberation in both samples was greater than aspartic acid. As with the neutral and basic amino acids, the concentration of acidic amino acids evolved similarly in this step and independently of the degree of clarification. This confirms that sedimentation of the must did not reduce turbidity so much that ethanol alters the permeability of the cellular membrane of yeast.

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