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Food Chemistry 98 (2006) 300–310
Food Chemistry
www.elsevier.com/locate/foodchem

Addition of amino acids to grape juice of the Merlot variety: Effect on amino acid uptake and aroma generation during alcoholic fermentation

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Received 21 February 2005; received in revised form 31 May 2005; accepted 31 May 2005

Abstract

The effects of adding selected amino acids (phenylalanine, alanine, aspartic acid and threonine) to grape juice on the generation of aroma compounds and on amino acid uptake were studied. The fermentation kinetics varied according to the quantities of amino acids added. The fermentations finished more quickly in supplemented juices and their alcoholic content was significantly higher than in the control ($p < 0.05$). Amino acids were consumed mainly in the first quarter of fermentation. Higher alcohol formation took place at the same time as ethanol formation: with more amino acids present in the medium, more phenyl ethanol ($p = 0.01$) and benzyl alcohol were formed while isoamyl alcohol production decreased. The contents of isoamyl and phenylethyl acetates, ethyl hexanoate and ethyl octanoate, as well as most fatty acids increased during the fermentation, reaching a maximum for 10% of ethanol; with higher alcoholic contents, their concentrations decreased.

Keywords: Juice; Wine; Amino acids; Aroma; Sensorial

1. Introduction

Most active aroma compounds are produced or modified during fermentation. In the course of a fermentation, *Saccharomyces cerevisiae* not only produces ethanol but higher alcohols, esters, aldehydes, small amounts of ketones, acids, terpenes, phenols and sulphur compounds, to name but a few. Of these, esters are responsible for the fruity aromas that characterize and ostensibly improve the quality of young wines. Ferreira, Lopez, and Cacho (2000), in their study of young Spanish red wines, found that some of the most important differences between young wines of different varieties were due to the presence of isoamyl acetate, isovaleric acid and isobutyric acid and their ethyl esters, as well as the presence of fusel alcohols and methionol, all of which are by-products of yeast amino acid metabolism.

Depending on their origin, wine alcohols can be classified into two categories: those that are synthesized from an amino acid-derived α-ketoacid, and those synthesized from an α-ketoacid acting as an intermediate in cell glucose metabolism. The former include isoamyl, isobutyl and phenylethyl alcohol, tyrosol and tryptophol, which can be synthesized from leucine, isoleucine, valine, phenylalanine, tyrosine and tryptophan, respectively. The second group includes 1-butanol and 1-pentanol, whose biosynthesis is especially active under anaerobic conditions (Mauricio, Moreno, Zea, Ortega, & Medina, 1997).

According to Jiranek, Langridge, and Henschke (1995), all the amino acids, except glycine, can be removed from fermentation media to different degrees.

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doi:10.1016/j.foodchem.2005.05.073

Several authors (Cooper, 1982; Gorinstein et al., 1984; Henschke & Jiranek, 1993; Jiranek, Langridge, & Henschke, 1991; Large, 1986) have reported that the best yeast nitrogen source among the amino acids are: Glu, Gln, Asp, Asn, Thr, His, Ala, Tyr, and Arg. According to Fraile, Garrido, and Ancin (2000), the formation of different alcohols takes place at the end of the fermentation, when most of the amino acids have been consumed, whereas, according to Rapp and Versini (1991), this synthesis occurs at the same time as ethanol production. Carrying out studies of partial least-squares regression models, Hernandez Orte, Cacho, and Ferreira (2002) found that amino acid composition accounts for a high proportion of the variance in the volatile composition and indicates that the relationship between the two sets of variables is highly multivariate. Moreover, the composition of the amino acids remaining in the wine has an influence on aromas during the maturing process (Escudero, Hernández-Orte, Cacho, & Ferreira, 2000).

In the wineries, there is increasing interest in obtaining young wines with fruity aromas, (both white and red varieties). Moreover, fermentative activators are usually added, mainly ammonium salts, in order to improve the fermentation kinetics and to avoid stuck and sluggish fermentations (Blateyron & Sablayrolles, 2001; Bisson, 1999). The activators are metabolised by the yeasts and modify the consumption of nitrogen sources in the juice (amino acids and ammonium), causing a variation in the aroma of the resulting wine. As occurs with esters, the pool of intracellular nitrogen regulates the formation of higher alcohols (Large, 1986). Every day research is carried out into new additives and it is therefore of interest to know how the addition of nitrogen sources affects amino acid uptake, especially of those related to the production of higher alcohols and their corresponding esters, and how the addition affects the synthesis of these aroma compounds.

The aim of this work is to study the effects that the addition of different concentrations of aspartic acid, threonine, alanine and phenylalanine have on amino acid uptake during fermentation, as well as on the generation of aromas. This effect was studied in juices of the Merlot variety (D. O. Somontano), to which 2 combinations of different amounts of the 4 amino acids were added. The choice of the four amino acids selected for this study was based on previous work carried out in our laboratory (Hernandez Orte et al., 2002). According to PLS analysis, threonine, phenylalanine and aspartic acid are the amino acids which have most influence on the aromatic compounds obtained in the fermentation process. In the aforementioned work, a clear relationship between amino acid profile in juice and concentration of some important volatile compounds was shown.

The amounts added were calculated by taking into account the maximum and minimum concentrations found in the literature for the different amino acids (Etievant, Schliich, Bouvier, Symonds, & Bertrand, 1988; Hernandez Orte, Guitart, & Cacho, 1999; Huang & Ough, 1991).

2. Materials and methods

2.1. Reagents and standards

The pure reference compounds used in the quantitative analysis of volatile components were purchased from Aldrich (Gillingham, UK), Sigma (St. Louis, MO, USA), Fluka (Buchs, Switzerland), Poly Sciences (Niles, IL, USA), Lancaster (Strasbourg, France), and Chemservice (West Chester, PA, USA). Individual amino acids were from Sigma. Diammonium phosphate and formaldehyde were obtained from Panreac S.A. (Barcelona, Spain). Water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Samples and vinification

Juice from *vitis vinifera* grapes of the Merlot variety with a fermentable sugar content of 222 g/l was used. The pH of the juice was 3.42, and the total acidity, expressed as tartaric acid, was 6.8 g/l. The easily-assimilable nitrogen concentration was 291.2 mgN/l. In order to obtain the juice, the de-stemmed and crushed grapes were introduced into the fermentation tank, 30 mg/l of SO_2 were added, and, after 4 h of maceration, the skins and seeds were removed. The juice was then settled at 4 °C for 8 h and decanted.

Three batches of 2×250 ml of juice were used: (A) non-supplemented juice, which was used as control; (B) juice supplemented with: phenylanine (20 mg/l); aspartic acid (40 mg/l), threonine (50 mg/l) and alanine (50 mg/l), labelled as (Ad1); and (C) juice supplemented with phenylanine (37.5 mg/l), aspartic acid (40 mg/l), threonine (50 mg/l) and alanine (98.5 mg/l), labelled as (Ad2). The fermentations were carried out in 500 mL erlenmeyer flasks closed by Muller fermentation locks. The fermentation temperature was kept constant at 20 °C in a CLIMAS incubator (Barcelona, Spain). The fermentation vessels were inoculated with active dry yeasts *S. cerevisiae* (Stellevin NT116) obtained from DSM Food Specialties Oenology S.A.S. (France).

The yeasts were inoculated into the juice at a concentration of 0.3 g/l. To this end, 0.5 g of dry yeast were rehydrated in a sterile flask in 6 ml of sterile deionised water with 0.5 g of glucose, maintaining the resulting solution at 35 °C for 15 min.

The fermentations were performed in duplicate. At the beginning of the fermentation, samples were taken every 12 h, and, once 41 h had elapsed, samples were

obtained every 24 h. The samples were taken after the same period of time in all cases, for comparison. Monitoring of the fermentation was carried out by daily weighing.

2.3. General enological parameters

Methods of analysis for general parameters were according to the Office International de la Vigne et du Vin (OIV, 1990). Easily-assimilable nitrogen (which includes nitrogen from ammonia, and all the α-amino acids, except proline) was measured by formol titration (Aerny, 1996). α-Amino nitrogen was calculated by taking into account the amount of nitrogen from free α-amino acids, except proline, quantified by HPLC, following the procedure described by Hernandez Orte, Ibarz, Cacho, and Ferreira (2003). The volatile compounds were analysed using the procedure proposed by Ortega, Lopez, Cacho, and Ferreira (2001).

2.4. Chemometric study

One-way ANOVA and stepwise multiple linear regression analysis were carried out with the Statview program (SAS Institute, Cary, NC, USA). The probability of the F quotient was taken as criterion to enter ($p < 0.05$).

2.5. Sensory evaluation

The wines were evaluated on the same day that the fermentation finished by a 10-member expert taste panel. All replicates of each treatment were tasted. The wines were coded randomly and were arbitrarily presented to the panel. The evaluation consisted in describing the aromatic notes found in the samples. Data were analysed by grouping the notes into eight categories (sulphur notes, vegetal, fusel, floral, reduction, spices, lactic and sweet fruits).

3. Results and discussion

3.1. Course of the fermentation

The course of the fermentation, expressed as rate of CO_2 versus fermentation time, is given in Fig. 1. As can be seen, the maximum fermentation rate is in the sample containing the highest level of amino acids, Ad2. The effect of the addition of amino acids is also apparent in the main compositional parameters of the wines, given in Table 1.

The reducing sugars are lower in the control than in the supplemented samples, although, in all cases, the sugar content is below 2 g/l. ANOVA revealed a significant effect ($p < 0.05$) of the amino acid supplementation on the pH and alcohol content but not on total acidity, volatile acidity or reducing sugars.

Table 1
Enological parameters of wines obtained in the fermentation of the control and supplemented Ad1 and Ad2 juices

	Control	Ad1	Ad2
Total acidity (g tartaric acid/l)	7.02	7.10	7.00
Volatile acidity (g/l)	0.18	0.20	0.17
pH*	3.29	3.30	3.32
Reducing sugars (g/l)	0.85	1.70	1.35
Degree alcoholic (v/v)*	12.05	12.37	13.04
Assimilable nitrogen (mgN/l)	25.2	25.7	28.1

Values are expressed as means of two replicates.
* Indicates a significant difference ($p < 0.05$) between the fermentations assayed, using an ANOVA test.

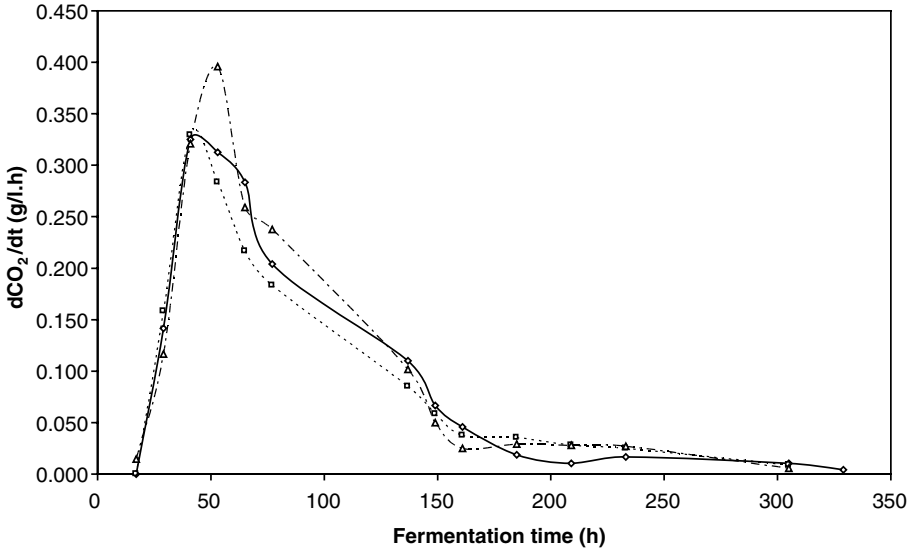

Fig. 1. Comparison of the fermentation kinetics of Merlot juices (control and supplemented juices Ad1 and Ad2). Symbols used are: control sample (-◇-); sample Ad1 (-□-); sample Ad2 (-·-△-·-). Data are the mean of two replicate samples.

It should be noted that a higher ethanol content is obtained in amino acid-supplemented juices and the more amino acids are added, the higher is the alcoholic grade content. This would seem to indicate that the addition of these amino acids enables the yeast to work more efficiently, resulting in a higher fermentation rate (Fig. 1) and better performance with regard to the production of alcohol. These results were confirmed by other experiments done under the same experimental conditions but using other yeasts (data not given).

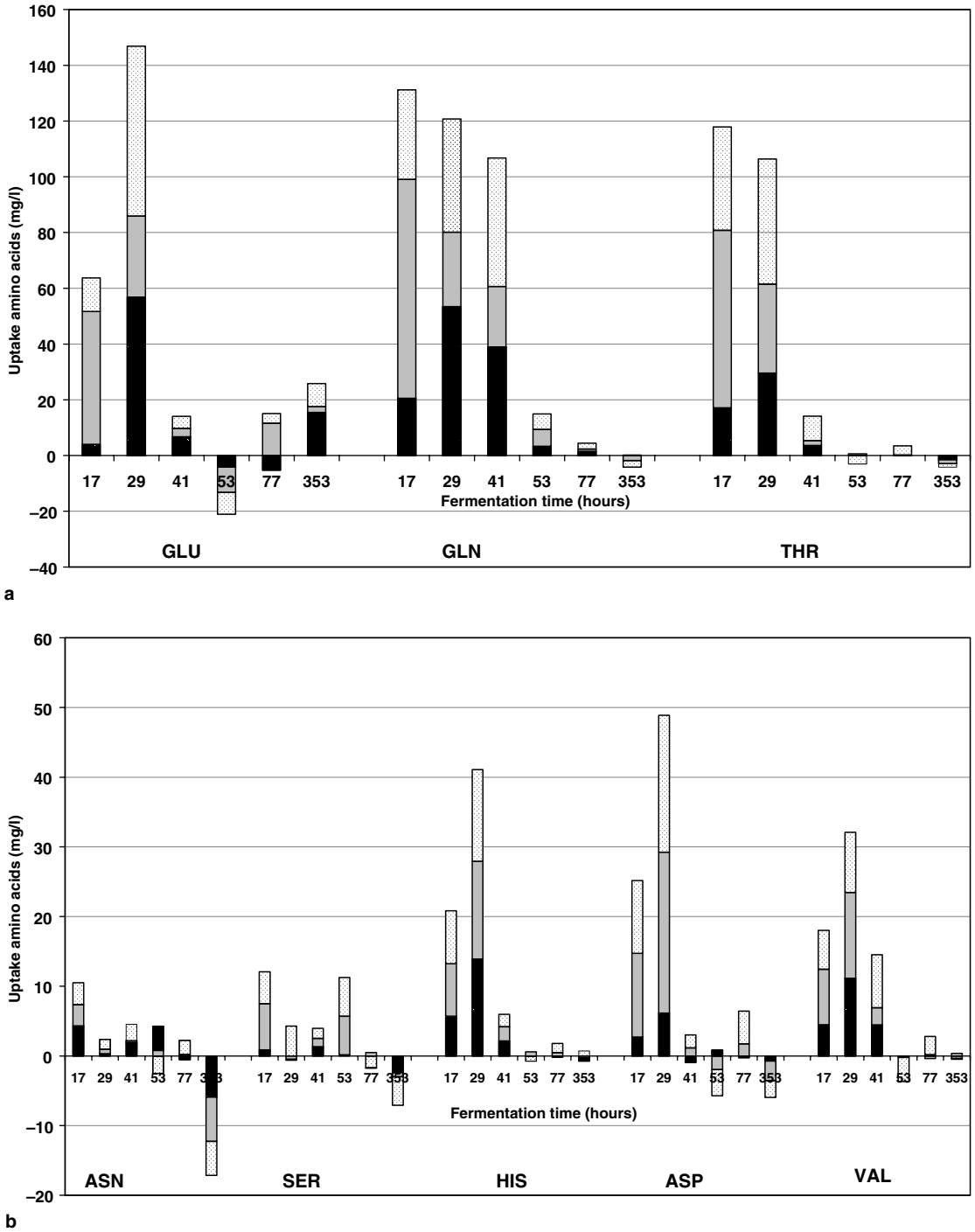

Fig. 2. Use of free amino acids during fermentation of Merlot juices. In the graph, are positive values of the amino acid uptake (consumption), and negative amino acid uptake (excretion). The samples were taken at 17, 29, 41, 53, 77 and 353 h for both the control (■) and the supplemented samples: Ad1 (▩) and Ad2 (⋮⋮). The consumption was calculated for each amino acid as the difference between the value at a given hour and the value 1 h later. If it is positive the yeasts absorb amino acids from the medium during that interval and if negative they excrete amino acids to the medium. Data are the means of two replicate samples.

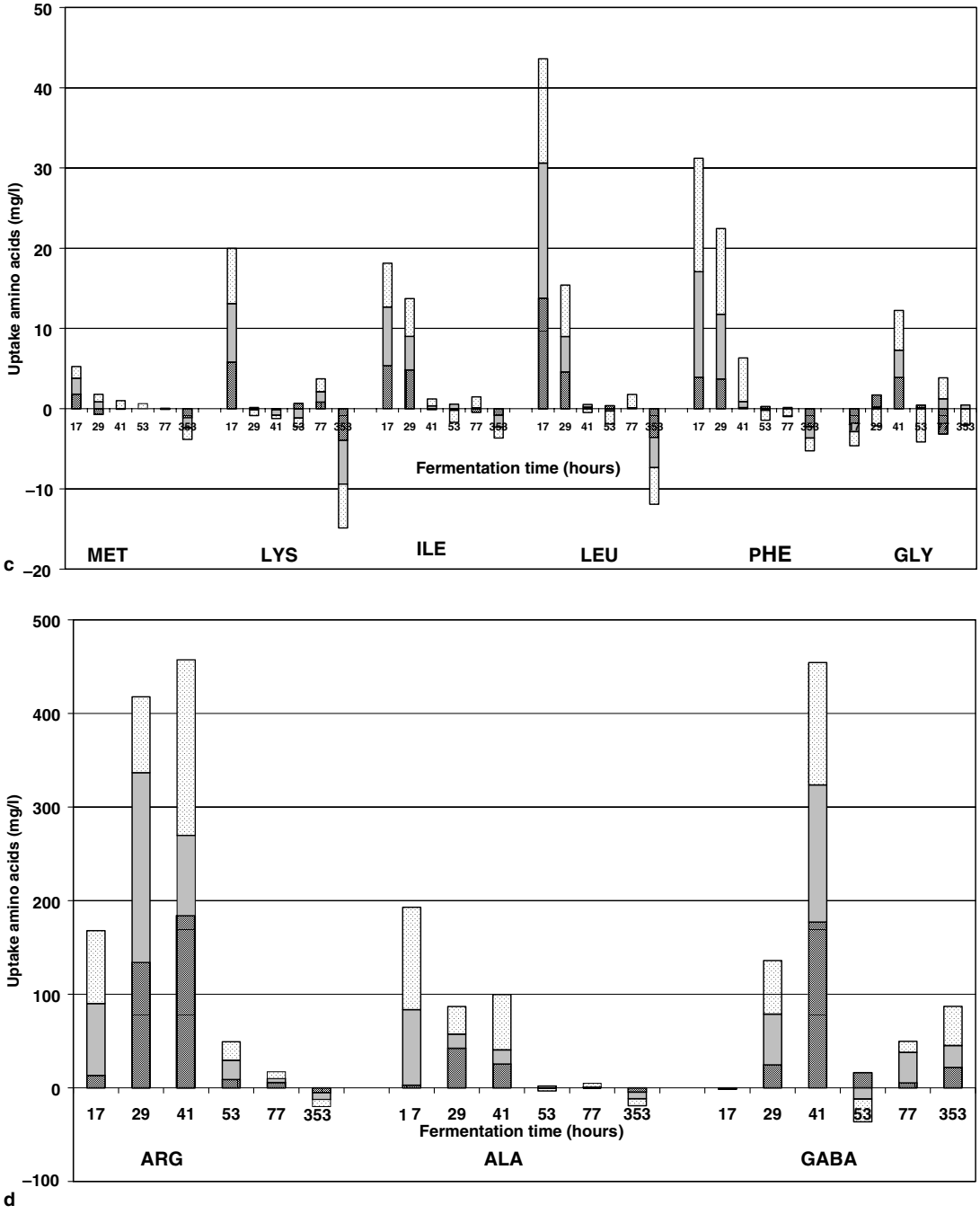

Fig. 2 (*continued*)

3.2. Course of consumption of amino acids

Fig. 2a to d show the consumption of amino acids during the course of fermentation. The highest consumption occurs during the first 41 h for most of the amino acids, after which the variation is very small, the amino acids being excreted to the medium as the alcohol content increases.

During the first 17 h of fermentation, Met, Lys, Asn, Ile, Leu, Glu, Gln, Thr and Phe were largely consumed in all the samples. In the next 12 h, Ile, Leu and Phe disappeared almost completely although, in the case of Phe, the absorption finished after 41 h in the samples with maximum levels of this compound. Between 17 and 29 h, Asp, Glu, Thr, His and Val were entirely consumed. In the period between the 29th and the 41st h, Gaba, barely metabolised in the initial hours, was almost totally consumed, and 90% of Arg disappeared. This amino acid was only metabolised after the yeast had metabolised others, an observation reported by Mckelvey, Rai, and Cooper (1990).

After 53 h, the excretion of Ser, Val, Glu, Lys, Ile, Leu and Phe began, and took place to a greater extent in supplemented juices (Ad2). In the interval between the 53rd and the 200th h, the variation in amino acid content was negligible, since ethanol inactivated permeases. After 200 h, the excretion of Glu, Gly, Thr, Lys, Ile and Leu increased, with relative final concentrations, regardless of those present in the initial juice. The release of amino acids during the last phase of the fermentation is essentially due to the high concentration of ethanol (10% after 150 h and 12.2% after 353 h). This metabolite produces an increase in the permeability of the plasma membrane (Ferreras, Iglesias, & Girbes, 1989), which leads to an increase in the excretion of amino acids present in the cytoplasmatic pool of the wine by means of a passive process (Bidan, Feuillat, & Moulin, 1986).

In the finished wine (Table 2), the concentrations of all the amino acids are higher than those measured after 77 h of fermentation (data not given). Only Pro and Gly were present at higher concentrations in the wine than in the juice. As can be seen in Table 2, at the end of fermentation, the concentrations of amino acids in the different wines were similar but more amino acids were excreted in the wines from supplemented juices than in the control, significant differences being found for Gln, Met and Arg ($p < 0.05$) and for Lys and Ser ($p < 0.1$). Ser and Lys were hardly metabolized at all and Arg and Gaba were consumed later than other amino acids (see Fig. 2d); that is to say, the amino acids present at highest concentrations in the Ad2 wines were either those which do not metabolize or those that metabolize at a later stage. It should be pointed out that the differences in amino acid profile were minimal and, surprisingly, the amino acids added (Asp, Thr and Phe) had disappeared completely and there were only 2 mg/l more of Ala remaining than in the control.

When a two-parameter ANOVA (“addition” and “hours of fermentation”) was performed with all the amino acids consumed by the yeasts, significant differences were only observed in the consumption of Asp ($p = 0.0002$), Ala ($p = 0.042$) and Phe ($p < 0.0001$) for the factor “addition”. For the “hours of fermentation” factor, all the amino acids showed significant differences between one given time and another, except for Gly and Pro. The most interesting aspect was the interaction between the two factors, with a high level of significance for Asp, Ser, Arg, Ala and Phe ($p < 0.001$) and for Thr, Val, Met, Ile, and Leu ($p < 0.05$), which shows that the amino acid uptake at different times was affected by the concentration of the other amino acids.

3.3. Course of formation of alcohols

The formation of 1-butanol, isobutanol, isoamyl alcohol, β-phenylethanol, 1-hexanol, benzyl alcohol and methionol during the fermentation was also studied (Fig. 3).

The higher alcohols isoamyl, isobutanol and β-phenylethanol were generated throughout the entire

Table 2
Mean concentrations of amino acids in the initial juice (control) and in the recently finished wines from fermentation of the control and the supplemented juices (Ad1 and Ad2)

	Control juice (mg/l)	Wine (mg/l)			Probability (p)
		Control	Ad1	Ad2	
ASP	16.2	9.20	9.13	9.81	ns*
ASN	15.1	13.5	14.1	13.3	ns
SER	15.6	10.9	10.7	13.1	0.093
GLU	85.1	11.4	11.2	11.4	ns
HIS	29.4	7.53	7.64	8.01	ns
GLN	131	12.7	12.8	13.5	0.047
GLY	5.23	5.54	5.74	6.92	ns
ARG	400	17.4	17.8	19.5	0.049
THR	54.0	5.30	5.46	5.58	ns
ALA	68.8	10.1	10.5	12.4	ns
PRO	1840	1902	1911	2033	ns
GABA	190	9.86	13.7	16.0	ns
TYR	4.72	4.00	3.82	4.40	ns
VAL	24.8	5.02	5.14	5.44	ns
MET	3.52	1.96	2.19	2.42	0.045
LYS	8.01	6.44	6.65	7.96	0.082
ILE	10.5	1.37	1.45	1.77	ns
LEU	18.9	4.20	4.07	5.11	ns
PHE	14.6	9.18	9.12	9.74	ns
mgN/l (without PRO)	231	25.6	26.6	29.0	

1-factor ANOVA comparing the amino acid contents of the wines.
* ns: No significant differences were observed at 90% of probability.

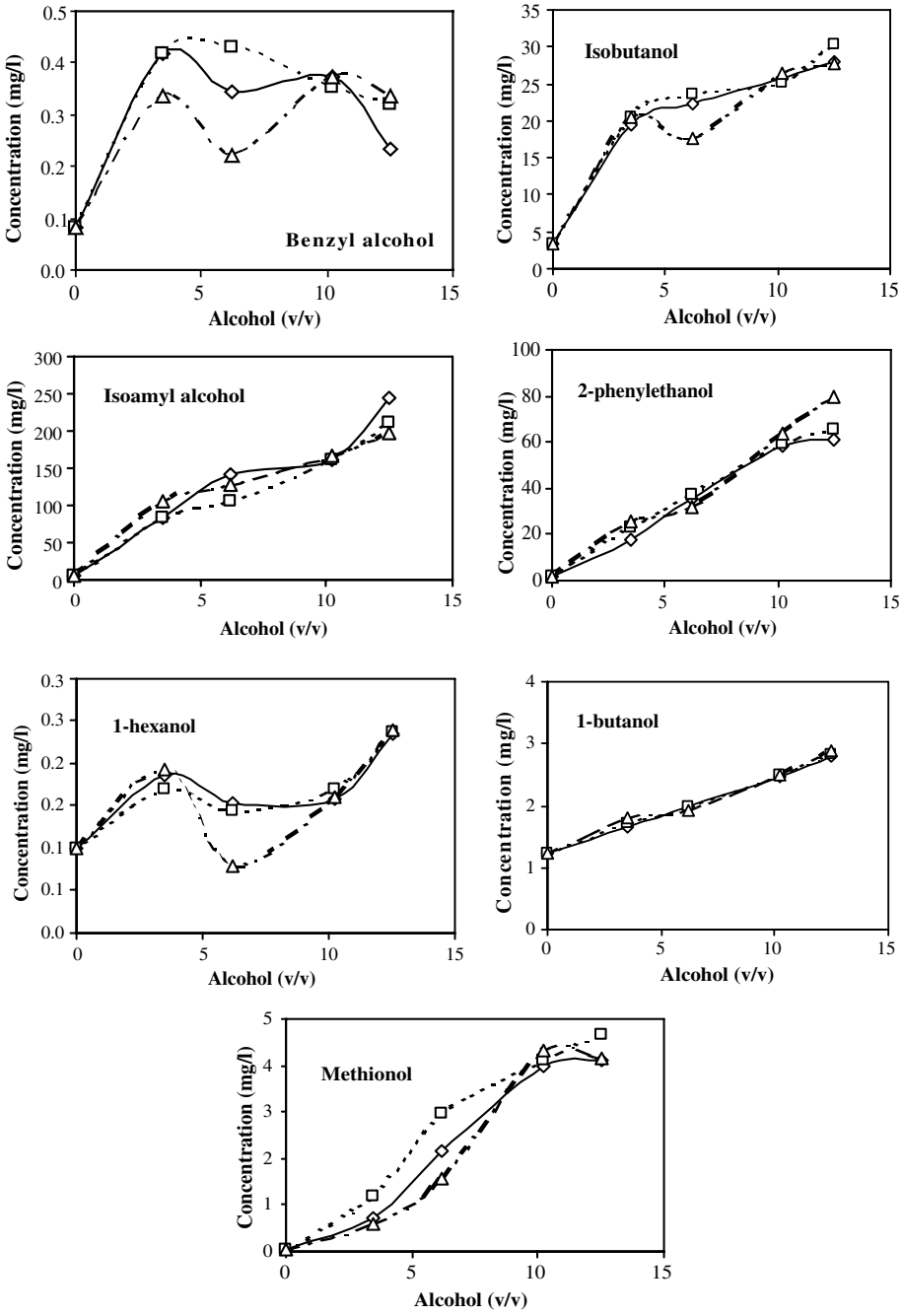

Fig. 3. Formation of higher alcohols during fermentation of Merlot juices. Control sample (-◇-); sample Ad1 (-□-); sample Ad2 (-·-△-·-). Data are the mean of two replicate samples.

fermentation. Smaller amounts of isoamyl alcohol were formed with higher concentrations of amino acids in the juice (although the difference is not significant), while benzyl alcohol and β-phenylethanol ($p = 0.011$) increased when amino acids were added to the juice. The level of β-phenylethanol was closely related to the level of phenylalanine (Hernandez Orte et al., 2002). Methionol increased during the fermentation in all cases. With the addition of Ad1 more methionol was formed than in the control whereas, with Ad2, the concentration was very similar to the control ($p = 0.052$).

1-Hexanol and 1-butanol were initially present in the juice. Their final concentrations were the same in the three types of test, which seems to indicate that the concentrations of these compounds do not depend on the nitrogen concentration of the juice.

3.4. Course of formation of esters

The formation of the acetates of the higher alcohols (Fig. 4) as well as the ethyl esters of fatty acids during the fermentation was also studied (Fig. 5).

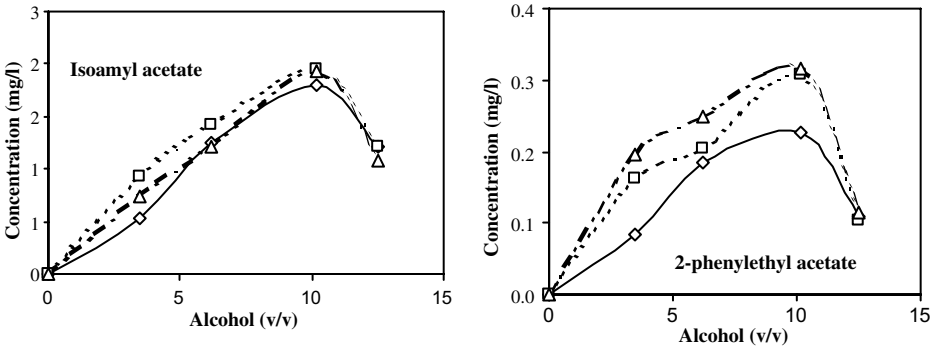

Fig. 4. Formation of isoamyl acetate and β-phenylethyl acetate during fermentation of Merlot juices. Control sample (-◇-); sample Ad1 (-□-); sample Ad2 (-·-△-·-). Data are the means of two replicate samples.

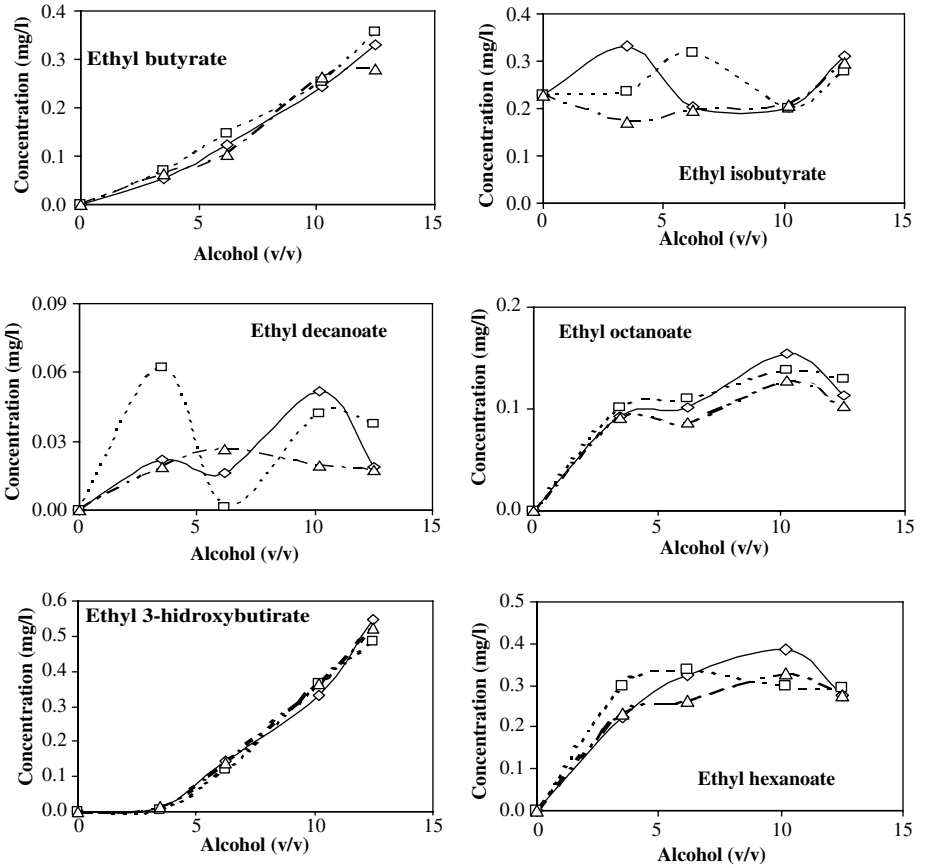

Fig. 5. Formation of ethyl esters during fermentation of Merlot juices. Control sample (-◇-); sample Ad1 (-□-); sample Ad2 (-·-△-·-).

It can be seen in Fig. 4 that isoamyl acetate and 2-phenylethyl acetate were formed simultaneously with their respective higher alcohols until an alcoholic content of 10% (v/v) was obtained. In the supplemented juices, significantly more phenylethyl acetate is formed ($p = 0.048$) than in the control until the alcohol concentration reached 10%. The concentration of both esters decreased from that point onwards. At the end of the fermentation, the concentration obtained was smaller and very similar in the three tests. This decrease is probably due to hydrolysis via the action of cellular esterases, their activity increasing at the end of the fermentation (Mauricio et al., 1993).

Ethyl butyrate and ethyl 3-hydroxybutyrate (Fig. 5) increased slowly during the fermentation, almost regardless of the amount of amino acids added. Their synthesis took place mainly from a 5% ethanol concentration onwards. Ethyl hexanoate and ethyl octanoate were formed largely during the first stages of the fermentation, reaching a maximum at 10% ethanol, and decreasing in the final

stage. They reached similar final concentrations after the different additions, despite the fact that, during the fermentation, significant differences in the formation of ethyl octanoate ($p = 0.0163$) were observed, depending on the amino acids added. Ethyl isobutyrate and ethyl decanoate showed very different behaviour throughout the fermentation, reaching very similar concentrations at the end of the process in all three cases. Ethyl lactate and diethyl succinate were synthesized towards the end of the fermentation (graphs not shown). The concentrations of ethyl lactate obtained were 1.72, 1.90 and 2 mg/l in the control, Ad1 and Ad2, respectively. The final concentration of ethyl succinate were the same in all three cases (0.52 mg/l).

3.5. Course of formation of acids

The formation of propanoic, butyric, hexanoic, octanoic, decanoic, isovaleric and isobutyric acids was analyzed (Fig. 6). Only isovaleric acid increased continuously during the fermentation. The other acids followed a complex evolution with time, most of them decreasing from 10% ethanol onwards. Little difference was found between the supplemented wines and the control. The decrease in the levels of fatty acids during the last phase of fermentation is in good agreement with the results reported by Fraile et al. (2000); this decrease was attributed to their absorption within the cell walls and to their being used by the yeast.

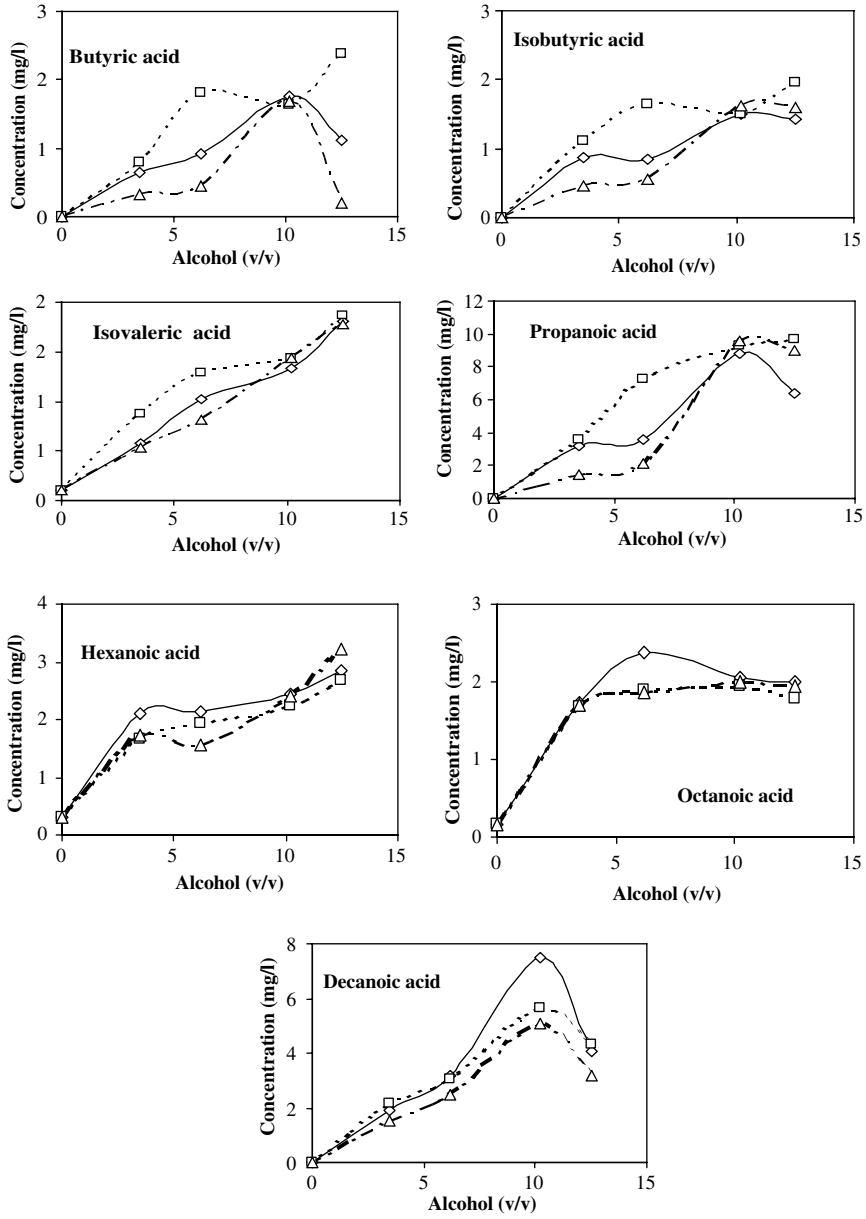

Fig. 6. Formation of acids during the fermentation of Merlot juices. control sample (-◇-); sample Ad1 (-□-); sample Ad2 (-·-△-·-). Data are the means of two replicate samples.

3.6. Course of formation of acetaldehyde, acetoin, diacetyl and γ-butyrolactone

Acetaldehyde and acetoin (Fig. 7) are two of the earliest metabolic subproducts in the fermentation. In the fermentations studied, no great differences were found in the course of formation of acetaldehyde and it was therefore deduced that the nitrogen concentration did not have a great influence on its synthesis. Acetaldehyde disappeared during the fermentation, due fundamentally to its combination with anthocyanins and sulphurous compounds.

Acetoin, however, displayed very different behaviour. It was produced in larger amounts in the control, up to a 3.5% level of ethanol (v/v). In the final stage of fermentation, this compound disappeared, and similar concentrations were obtained in all cases. It should be stressed that less generation of acetoin as a fermentation sub-product takes place when amino acids are added, probably due to greater efficiency of the fermentation process with regard to ethanol production. The concentration of γ-butyrolactone increased from the beginning, but at a greater rate from 6% of ethanol onwards and in the supplemented juices. With regard to diacetyl (graph not shown), this component only appeared in quantifiable concentrations towards the end of the fermentation, apparently unaffected by the addition of the amino acids. Acetic acid and ethyl acetate mainly appeared towards the end of fermentation, as occured with ethyl lactate, diethyl succinate, and diacetyl.

3.7. Sensory analysis

The addition of certain amino acids before the fermentation process modified the sensory profile of the wines obtained. As can be seen in Fig. 8, most affected were the sulphured, vegetal, fusel, floral, lactic and reduction notes and the least altered were spices and sweet fruits.

The addition of amino acids to Ad2 produced a significant decrease in the sulphured ($p = 0{,}043$), reduction ($p = 0.019$) and lactic ($p = 0.033$) notes and much less noticeable increase in the fusel and floral notes. The Ad1 amino acid addition also increased the fusel and floral notes, as well as the vegetal notes, keeping the sulphur-associated note at the level of the control sample. The addition of amino acids improved the sensorial profile of the wines obtained.

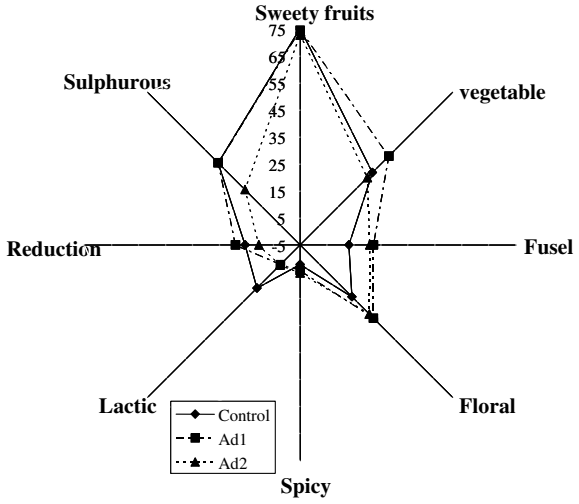

Fig. 8. Modification of the sensory impression of the wines obtained from the control juice and supplemented juices of the Merlot variety. Control sample (-◆-); sample Ad1 (···■···); sample Ad2 (···▲···). Data are the means of two replicate samples.

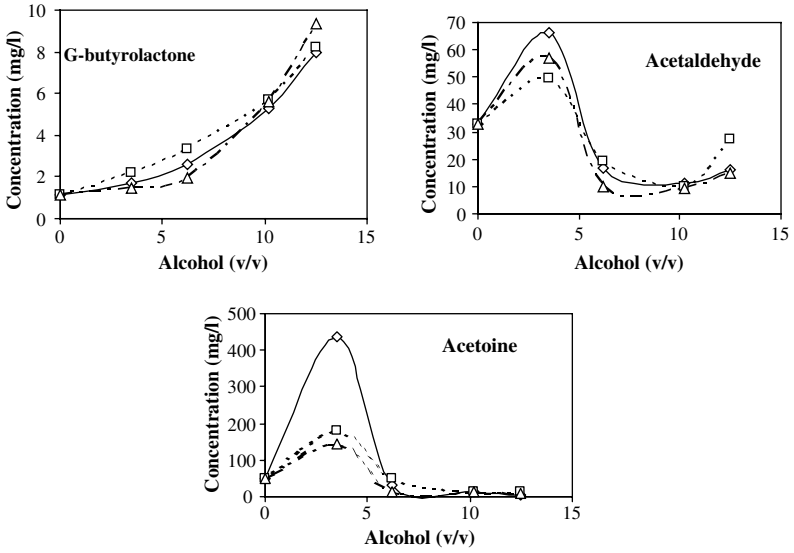

Fig. 7. Formation of acetaldehyde, acetoin and γ-butyrolactone content during fermentation of Merlot juices. Control sample (-◇-); sample Ad1 (-□-); sample Ad2 (-·-△-·-). Data are the means of two replicate samples.

4. Conclusions

The addition of amino acids produced differences in fermentation kinetics. The fermentations finished earlier in supplemented juices and the alcohol contents of these juices were notably higher than those of the control. Amino acids were mainly consumed during the first 41 h of fermentation and excreted to the medium. The final concentration of each amino acid was independent on its initial concentration in the juice.

Higher alcohols were generated at the same time as ethanol. In the presence of greater concentrations of amino acids more β-phenylethanol ($p = 0.0119$) and benzyl alcohol were produced but less isoamyl alcohol. Isoamyl acetate, β-phenylethyl acetate, ethyl hexanoate, ethyl octanoate and some fatty acids increased, during the fermentation, reaching a maximum at 10% of ethanol, after which they decreased. The supplemented juices produced considerably less acetoin than the control ($p < 0.001$) when 3.5% of ethanol had been generated.

From the sensorial point of view, the wines obtained with more amino acids were better valued by the panel of tasters because the sulphured notes decreased significantly while the floral notes increased.

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