Food Chemistry 111 (2008) 965–969

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Influence of the elaboration process on the peptide fraction with angiotensin I-converting enzyme inhibitor activity in sparkling wines and red wines aged on lees

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ARTICLE INFO

Article history:
Received 14 February 2008
Received in revised form 13 March 2008
Accepted 6 May 2008

Keywords:
Sparkling wines
Malolactic fermentation
Aging with lees
ACEI activity
Peptide

ABSTRACT

The influence of some variables on the manufacturing process for sparkling and red wines on the angiotensin I-converting enzyme inhibitory (ACEI) activity has been studied. Ageing on lees significantly influenced the angiotensin I-converting enzyme inhibitor activity in sparkling wines. It reached maximum values at 9 months, decreasing afterwards. In red wines, the ACEI activity also increased in the wines aged on lees. In both wines, hydrophobic peptides were responsible for the ACEI activity. These peptides would make a much greater contribution to the total activity if present in higher proportions. It would therefore be advantageous to increase their concentrations in wines, either by using starting materials with high initial peptide contents or by using a highly autolytic yeast, giving a greater degree of hydrolysis of wine proteins, and higher concentrations of peptides with ACEI activity.

1. Introduction

There are numerous current studies aimed at increasing the contents of compounds with angiotensin enzyme converting inhibitory action (ACEI)-an enzyme involved in regulating blood pressure (Erdös, 1975; Skeggs, Kahan, & Sumway, 1956) – in food products, whether by fermentation or by enzymatic action (Okamoto, Hanagata, Matsumoto, Kawamura, Koizumi, & Yanagida, 1995; Mullally, Meisel, & FitzGerald, 1997; Nakamura, Yamamoto, Sakai, Okubo, Yamazaki, & Takano, 1995; Gobbetti, Ferranti, Smacchi, Goffredi, & Addeo, 2000). In most cases, ACEI activity is attributed to peptides isolated from foods (Hernández-Ledesma, Martín-Álvarez & Pueyo, 2003; Miguel, López-Fandino, Ramos, & Aleixandre, 2005; Gouda, Gowda, Rao, & Prakash, 2006). Despite extensive literature published on this matter, as far as we are aware only two studies have focussed on peptides with ACEI activity in wines (Pozo-Bayón, Alcaíde-Hidalgo, Polo, & Pueyo, 2007; Takayanagi & Yokotsuka, 1999). This could, at least in part, be due to the complexity of separating peptides from other wine compounds.

Sparkling wines made by the traditional method are wines in which ageing is carried out in the bottle on lees for a given time period. During this period, the yeasts autolyse, releasing, mainly, nitrogenated compounds into the medium, the most important of which are the peptides (Martínez-Rodríguez & Polo, 2000; Moreno-Arribas, Pueyo, & Polo, 1996). In the work of our research team on wine peptides with ACEI activity (Pozo-Bayón, Alcaíde, Polo, & Pueyo, 2007), this was evaluated in 15 commercial sparkling wines made by the traditional method. On the other hand, Alcaide-Hidalgo, Pueyo, Polo, and Martínez-Rodríguez (2007) have demonstrated, in a study conducted in a model wine medium, that the peptides released into the medium by *Saccharomyces cerevisae*, as a consequence of autolysis, can present ACEI activity and that this activity is attributable to the more hydrophobic peptides. However, since autolysis is a dynamic process, the peptides responsible for this activity can be hydrolysed over time by the action of proteolytic enzymes also in the medium, giving rise to peptides with reduced activity.

The peptidic composition of sparkling wines depends on the yeast used in the second fermentation (Moreno-Arribas et al., 1996), and also on the protein composition of the base wine used to make these wines. The protein composition of a wine, (both the type and concentration of proteins), also depends on the variety of grape used to make the wine (Moreno-Arribas, Cabello, Polo, Martín-Alvarez, & Pueyo, 1999; Pueyo, Dizy, & Polo, 1993) and the clarification treatments to which it is submitted (Muhlack, Nordestgaard, Waters, O'Neill, Lim, & Colby, 2006; Ferreira, Picarra-Pereira, Monteiro, Loureiro, & Teixeira, 2001; PuigDeu, López-Tamames, Buxaderas, & Torre-Boronat, 1993). The study of ACEI activity of sparkling wines has only been tackled by measuring the activity in complete samples and in peptide fractions isolated from a red and a white wine (Pozo-Bayón et al., 2007).

Takayanagi and Yokotsuka (1999) and Pozo-Bayón et al. (2007) have shown that the peptides isolated from red wines can also

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doi:10.1016/j.foodchem.2008.05.015

present ACEI activity. Although it has been shown that the ACEI activity of wine peptides can be negatively affected by alcoholic fermentation, the effects of other variables on this property, such as ageing time with lees and malolactic fermentation, are unknown.

The main objective of this work is, therefore, to determine how certain technological variables involved in the manufacturing process for sparkling wines made by the traditional method (yeast strain and ageing time on lees) and in the production of red wines (malolactic fermentation and ageing on lees), can affect the ACEI activity of the peptide fraction of these types of wines.

2. Material and methods

2.1. Manufacture of the wines

2.1.1. Base wine and sparkling wines

A base wine (BW) of the Malvar variety was used, manufactured in the experimental wine cellar of the Instituto Madrileño de Investigación y Desarrollo Rural Agrario y Alimentario (IMIDRA), (El Encin vineyard), located in one subzone of Madrid from the Designation of Origin Wines from Madrid (Spain). Three batches of sparkling wines were obtained by the traditional inoculation method, conducting the corresponding tirages with three different *Saccharomyces cerevisiae* yeast strains, one commercial strain EC-1118 (CS) (Lallemand, Spain S.A.), and the other two from the Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario (IMIDRA) yeast collection, *Saccharomyces cerevisiae*, strains 1S and 2S. The second fermentation (FF) and ageing were carried out at a temperature of 16 °C. Samples of the wine were taken at the end of the second fermentation and after nine and twelve months of ageing in bottle with the yeasts (9 m and 12 m, respectively).

Each experimental sample point was studied in duplicate (two different fermentations for each experimental sample). The wine samples were centrifuged at 5000*g* for 15 min and immediately refrigerated until analysis. All the analyses were conducted in triplicate.

2.1.2. Red wines

Wines used in this experiment were industrially manufactured in the Designation of Origin Navarra (Spain). The initial wine (IW) used was from the Tempranillo grape variety, manufactured in 10,000 l stainless-steel tanks. After alcoholic fermentation, this wine underwent malolactic fermentation (MLF-W). After malolactic fermentation, a part of the wine was submitted to the traditional post-fermentation treatments, racking, clarification with albumin and bentonite, cold stabilization and filtration, and was transferred to oak barrels. Another part of the MLF-W wine was transferred to oak barrels without removing the lees. Both wines were aged in barrels for 12 months (wine aged without lees: WL-AW and wine aged on lees: L-AW). Malolactic fermentation was carried out by inoculation of a commercial lactic acid bacterium, *Oenococcus oeni* (ITV 04 A1), provided by Oenofrance (Rueil-Malmaison, France). For storage of the wines during malolactic fermentation and wine ageing, 225 l new barrels of French oak (*Querqus sessilis*) were used.

All enological treatments were carried out in duplicate. The ageing experiments were done in parallel in the two duplicates after malolactic fermentation. The wine samples were centrifuged at 5000*g* for 15 min and immediately refrigerated until analysis. All the analyses were conducted in triplicate.

2.2. Analytical determinations

2.2.1. Amino and peptide nitrogen

Free amino acids were determined by the method of Doi and coworkers (Doi, Shibata, & Matoba, 1981, method 5), based on the reaction of ninhydrin/Cd with the free amino group. Absorbance was determined at 507 nm. The results were expressed as mg of free amino nitrogen/l. Similar conditions were used to determine the free amino acids plus peptides but, following the conventional method with ninhydrin (Doi et al., 1981, method 1), based on reaction of the amino group with a mix of ninhydrin/Sn. Absorbance was determined at 570 nm. A DU 70 spectrophotometer (Beckman, Fullerton, CA, USA) was used in both determinations. The peptides were quantified by the difference between the results obtained by Doi's method 1 and method 5. The results were expressed in mg of peptide nitrogen/l. The standard used was leucine (Leu) (14 g of N for each 131.17 g of Leu).

2.2.2. ACE-inhibitory activity

ACE-inhibitory activity (ACEI) was determined by the method described by Cushman and Cheung (1971) and modified by Hernández-Ledesma, Martín-Álvarez, and Pueyo (2003). The method is based on the quantification of the hippuric acid formed by reaction of hippuryl-histidyl-leucine (HHL) with ACE in the presence and absence of the inhibitor. The absorbance was measured at 228 nm using a DU 70 spectrophotometer (Beckman). The interference of compounds that absorb at this wavelength was eliminated with a sample blank. ACEI activity was expressed as a percentage in the direct samples and as a ratio of the percentage of ACEI/concentration of peptide nitrogen (mg/l) for the peptide fractions obtained (Hernández-Ledesma, Miralles, Amigo, Ramos, & Recio, 2005).

2.2.3. Obtaining the fraction with molecular weight less than 4000

A volume of 450 ml of each wine was concentrated under vacuum to 25 ml. An aliquot of 20 ml of the concentrate was applied to a Sephadex LH-20 column (Amersham Biosciences, Uppsala, Sweden) 390 mm long × 25 mm i.d. swollen and packed according to the supplier's instructions. Elution was performed at room temperature under the conditions described by Acedo, Pueyo, and Polo (1994). Eluent was 0.3 M ammonium acetate buffer, pH 4. Flow rate was 0.4 ml/min, and detection was at 280 nm, using a 2138 Uvicord S detector (LKB, Uppsala, Sweden). Chromatographic data were collected with the Gold System v8.1 (Beckman) software. The void volume was calculated using dextran blue. The compounds eluted after the void volume were collected and concentrated under vacuum to 25 ml.

2.2.4. Fractionation of peptides by reversed phase low pressure chromatography

An aliquot of 20 ml of the concentrate obtained from the Sephadex LH-20 column was applied to a reversed phase open column (300 mm long × 10 mm i.d., Cosmosil 140 C_{18}-OPN, Nacalai Tesque Inc., Kyoto, Japan) that had been equilibrated with distilled water. To introduce the sample, a 50 ml superloop and a HiLoad Pump P-50 (Amersham Biosciences, Uppsala, Sweden) were used. Flow rate was 2 ml/min. Amino acids and hydrophilic peptides were eluted with water (fraction 1, F1) and 10% ethanol was used to elute hydrophobic peptides (fraction 2, F2). The elution was monitored at 280 nm, using a 2138-Uvicord S, and the eluent was changed when the base line reached the initial value. Chromatographic data were collected with the Gold System v8.1 from Beckman software. Eluted fractions 1 and 2 were concentrated to 25 ml.

2.3. Statistical methods

The statistical method used for the data analysis was one-way ANOVA and the least significant difference (LSD) test for means comparison. SPSS program for Windows (ver. 14.0, SPSS Inc., 2005. http://www.spss.com) and STATISTICA program (ver. 7.1,

StatSoft, Inc., 2006, http://www.statsoft.com) were used for data processing.

3. Results and discussion

3.1. Study of changes in the nitrogenated fraction and evaluation of ACEI activity of the base wine and sparkling wines

Table 1 shows the changes observed in the nitrogenated compounds analysed, both for BW of Malvar variety and for the corresponding sparkling wines obtained with the three yeast strains used, FF and at 9 m and 12 m. Significant differences were not obtained for amino and peptide nitrogen in relation to the yeast strain used. This behaviour could be due to the fact that yeast strains used in the manufacture of sparkling wines are often subject to strict selection criteria which could result in their being rather similar (Martínez-Rodríguez, Carrascosa, Barcenilla, Pozo-Bayón, & Polo, 2001). The concentration of free amino nitrogen does not vary significantly during the second fermentation and ageing, although it is lower than the concentration of amino acids present in the base wine. However, there are significant differences in peptide nitrogen as a function of ageing time. Its concentration decrease during the second fermentation because the yeast uses that compound as a nitrogen source and increases significantly after the second fermentation and remains constant from 9 to 12 months of ageing, reaching similar values to the base wine. In previous works carried out by our group, we have shown that peptides are consumed during the fermentation and that they are the main compounds released by yeasts during the autolysis that takes place during the ageing of sparkling wines made by the traditional method (Martínez-Rodríguez & Polo, 2000; Moreno-Arribas et al., 1996). The results obtained here would, therefore, indicate that, in these wines, yeast autolysis has been triggered before 9 months of ageing.

Table 1 also shows the results of the study of ACEI activity of the base wine and sparkling wines. The percentage of ACEI activity of the base wine was lower than that of the sparkling wines these produced, and there was no significant difference between the yeast strain used or the ageing time of the wine. The ACEI rose from 46.6% in the base wine to a mean value of 63.2% for the nine sparkling wines, corresponding to an increase of 15.7%. Since peptides are the main compounds released by the yeasts during wine ageing, the results obtained could indicate that these peptides, formed during the second fermentation and ageing with yeasts, help to increase the ACEI activity of these wines. It has recently been shown that the peptide fraction released by *Saccharomyces cerevisiae* during autolysis induced in a model wine medium significantly contributed to the ACEI activity of the autolysate obtained (Alcaide-Hidalgo et al., 2007).

Table 1
Mean values ± standard deviation (SD) of free amino nitrogen, peptide nitrogen and % ACEI, of the base wine (BW), after final second fermentation (FF) and of the sparkling wines after ageing for 9 and 12 months with the yeast (9 m and 12 m, respectively) for the three strain of yeast studied (commercial strain EC-1118 = CS, two strains of *Saccharomyces cerevisiae* from IMIDRA = 1S and 2S) and mean values and SD for the three pooled strains

Samples	BW	Strain yeast	FF	9 m	12 m
Free amino nitrogen (mg/l)	98.0	CS	80.7 ± 4.2	78.2 ± 7.0	78.4 ± 2.4
		1S	89.1 ± 3.6	87.9 ± 5.0	82.3 ± 3.0
		2S	84.0 ± 2.4	79.8 ± 2.8	79.8 ± 2.2
		Mean ± SD for the three strain			
			84.6[a] ± 4.4	81.9[a] ± 5.0	80.1[a] ± 2.1
Peptide nitrogen (mg/l)	42.4	CS	35.9 ± 3.1	47.8 ± 4.8	49.6 ± 2.0
		1S	31.7 ± 1.7	40.0 ± 3.5	46.7 ± 2.5
		2S	33.6 ± 1.5	47.2 ± 4.0	47.2 ± 2.6
		Mean ± SD for the three strain			
			33.7[a] ± 3.4	45.0[b] ± 3.3	47.9[b] ± 2.1
% ACEI	46.6	CS	61.2 ± 1.9	67.0 ± 2.1	64.1 ± 1.9
		1S	62.4 ± 2.1	62.3 ± 2.5	64.6 ± 2.6
		2S	63.2 ± 1.7	60.4 ± 1.8	63.9 ± 2.1
		Mean ± SD for the three strain			
			62.3[a] ± 2.6	63.2[a] ± 3.2	64.2[a] ± 1.4

(a–b) Means in the same row with different letter are significantly different ($P < 0.05$).

3.2. Isolation and fractionation of the peptides of the base wine and the sparkling wines and evaluation of ACEI activity

The peptides do not all contribute equally to ACEI activity, and there may also be another series of compounds that can present this activity (Actis-Goretta, Ottaviani, & Fraga, 2006; Yang, Chen, Li, & Tsai, 2007), the next objective was to study, in depth, the role of the peptides on this activity. To do this, the peptide fraction of the wines was isolated.

The peptides were isolated from BW and also from the corresponding sparkling wines, FF, 9 m and 12 m. Since significant differences were not obtained in relation to the yeast strain used, samples obtained with the commercial strain, CS, were selected for fractionation.

Fig. 1 shows the concentration of peptide nitrogen and the ratio ACEI activity/peptide nitrogen concentration for each of the fractions obtained, F1 and F2, corresponding to BW, FF, 9 m and 12 m, respectively. In the studied samples the sum of F1 and F2, the peptide concentrations are lower than in the whole samples. This is because some big peptides elute with the void volume in the process of peptide isolation and then they are quantified in the whole samples. As can be observed, the peptide contents in the F1 fraction are higher than the peptide contents of the F2 fraction (18.1–38.8 mg/l for F1

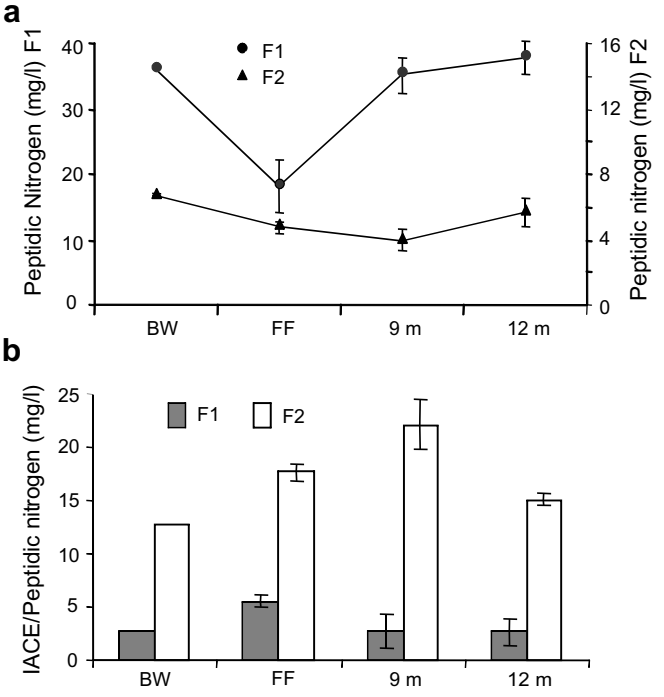

Fig. 1. Mean values ± standard deviation (SD) of (a) peptide nitrogen and (b) ACEI/peptide nitrogen concentration of the F1 and F2 fractions obtained in the Cosmosil-140 C18 OPN column, of the base wine (BW), after final second fermentation (FF) and of the sparkling wines after ageing for 9 and 12 months with the yeast (9 m and 12 m, respectively) obtained with the commercial strain EC-1118 (CS).

and 4.0–6.7 mg/l for F2). The F1 fraction presented similar behaviour to that observed for the whole wine (Table 1), which was only to be expected, given the small contribution of F2 to the global concentration. Peptides of the F2 fraction also diminished as a consequence of the second fermentation from 6.7 mg/l to 4.8 mg/l, but, at 9 and 12 months of ageing with yeasts, the peptide concentration of F2 did not change. On the contrary, F1 increased at 9 month of aging. The simultaneous process of peptide hydrolysis and formation that occurs during autolysis could explain the behaviour observed (Martínez-Rodríguez & Polo, 2000).

Although peptide concentration in F2 fraction is lower than in F1, its activity is higher (Fig. 1). This means that F2 contains peptides with higher ACEI activity. Similar results have been found by others (Alcaide-Hidalgo et al., 2007; Takayanagi & Yokotsuka, 1999).

The ratio ACEI/peptide concentration in the F2 fraction changed from 12.8 in BW to 22.1 in 9 m, reaching, at this point, its maximum value. It decreased again in 12 m to 15.1. These results show that, although the peptide concentration in F2 remained constant between the second fermentation and aging, the peptide composition of F2 changed. Peptides present at 9 months of aging in F2 were more active than were peptides corresponding to FF and after 12 months of aging with the yeast. This behaviour shows that the hydrolytic process that takes place during aging is very dynamic and continuous. Hydrophobic peptides are formed and/or hydrolysed simultaneously, increasing the peptide concentration in F1 for 9 m (Martínez-Rodríguez & Polo, 2000), and modifying some properties, such as the ACEI activity.

3.3. Evaluation of ACEI activity in red wines

Table 2 shows the changes that occur during malolactic fermentation and ageing in barrel of free amino nitrogen and peptide nitrogen. As can be observed, the amino nitrogen rose after malolactic fermentation, although the peptide nitrogen decreased. It has been claimed that malolactic bacteria hydrolyze peptides to amino acids in order to cover their nutritional requirements, producing an accumulation of amino acids not consumed by bacteria (Manca de Nadra, Farías, Moreno-Arribas, Pueyo, & Polo, 1997; Manca de Nadra, Farías, Moreno-Arribas, Pueyo, & Polo, 1999). Both amino acids and peptides increased during ageing. In the case of WL-AW, the peptide concentration after 12 months of ageing was significantly lower than was the peptide concentration found in L-AW. This result could be due to the proteolytic activity attributable to the enzymes released during malolactic fermentation while, in L-AW, the enzyme content of the media is higher, as a result of autolysis of the lees, as occurs in sparkling wines.

Table 2 also shows the change in ACEI activity, expressed as a%, in all the red wine samples studied. The ACEI activity in IW was 88.7%, and this dropped during MLF (64.1%). During wine ageing, there was, once again, an increase in the activity studied, which was significantly greater in WL-AW (94.2%) and smaller in L-AW (80.7%). These data present the same profile as those obtained for the peptides of these wines mentioned previously, decreasing during MLF and increasing during ageing in barrel.

Table 2
Mean values ± standard deviation (SD) of free amino nitrogen, peptide nitrogen and% ACEI of the initial wine (IW), of the wine after malolactic fermentation (MLF-W) and of the wines after ageing for 12 months in the barrel with lees (L-AW) or without lees (WL-AW)

Samples	IW	MLF-W	L-AW	WL-AW
Free amino nitrogen (mg/l)	33.2	$36.5^{a} \pm 0.7$	$39.4^{b} \pm 0.3$	$38.5^{b} \pm 0.6$
Peptide nitrogen (mg/l)	19.4	$16.3^{a} \pm 1.7$	$24.2^{c} \pm 1.7$	$21.4^{b} \pm 1.0$
% ACEI	88.7	$64.1^{a} \pm 7.6$	$80.7^{b} \pm 5.5$	$94.2^{c} \pm 3.5$

(a–c) means in the same row with different letter are significantly different ($P < 0.05$).

3.4. Isolation and fractionation of red wine peptides and evaluation of their ACEI activity

In red wines, there are other compounds, in higher concentration than in white wines that could have ACEI activity. We, therefore, subjected these wines to fractionation, as with the sparkling wines, to isolate the peptides and to study, in greater depth, their role in this functional activity.

The concentration of peptides and the ratio, ACEI activity/peptide nitrogen concentration, of the fractions studied are shown in Fig. 2. The peptide concentration in the F1 fraction dropped during MLF, following the same pattern as for the peptides in the whole sample, and also those in the sparkling wines, passing from a value of 15.0 mg/l to 9.3 mg/l. After this, there was a significant rise during ageing (12.9 mg/l for L-AW and 16.1 mg/l for WL-AW), and the difference between the two wines aged in barrels was also significant.

The sample with the higher peptide concentration in the F2 fraction corresponded to MLF-W (2.9 mg/l), presenting values higher than those of IW (2.5 mg/l). During ageing, this tends to decrease, and the peptide content was significantly higher in the fraction F2 of L-AW (2.0 mg/l). In contrast, the F2 fraction of WL-AW (1.0 mg/l) presented a significantly lower peptide concentration. In all samples, the peptide contents were higher in the F1 than the F2 fraction, as also occurred in the previously studied sparkling wines.

During MLF, the malolactic bacteria preferentially consume hydrophilic peptides and, in contrast, there is also a simultaneous hydrolysis of proteins in the medium that gives rise to the formation of more hydrophobic peptides, similar to the situation occur-

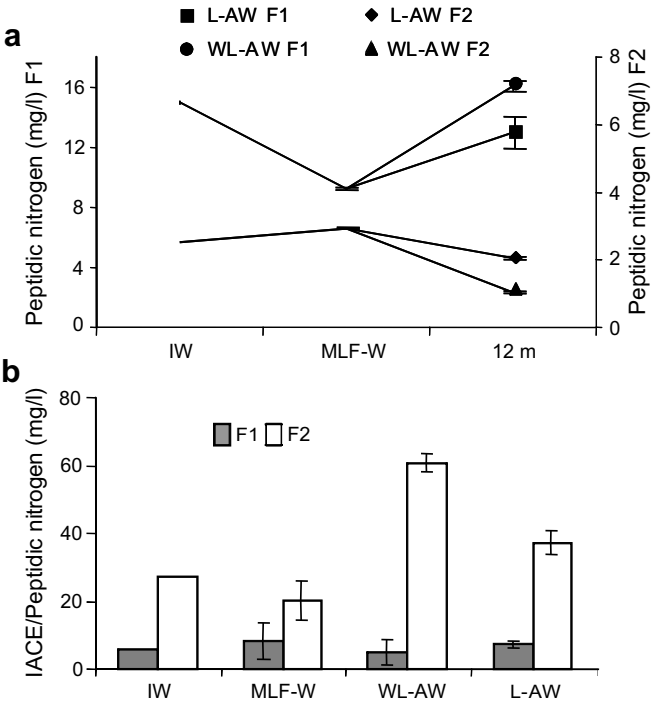

Fig. 2. Mean values ± standard deviation (SD) of (a) peptide nitrogen and (b) ACEI/peptide nitrogen concentration of the F1 and F2 fraction obtained in the Cosmosil-140 C18 OPN column, of the initial wine (IW), of the wine after malolactic fermentation (MLF-W) and of the wines after ageing for 12 months in the barrel without lees (WL-AW) or with lees (L-AW).

ring during the production of sparkling wines (Martínez-Rodríguez & Polo, 2000). However, during ageing, there was only enzymatic degradation of hydrophobic peptides to produce a rise in hydrophilic peptides (Fig. 2).

By expressing the results of ACEI activity of both wine fractions relative to their peptide nitrogen levels (Fig. 2), we can observe that this is also significantly higher in the F2 fraction than in the F1 fraction. During ageing in barrel, the ratio ACEI/peptide nitrogen increased significantly in the F2 fraction, reaching the maximum value in WL-AW. However, the peptide concentration was twice greater in L-AW than in WL-AW, and thus the global ACEI activity was high for L-AW (74.6% and 60.8%, respectively). The ageing of the wines in barrel improves the ACEI activity in red wines, it being still better when ageing is developed with lees.

We can conclude that these results demonstrate the importance of hydrophobic peptides in the ACEI activity detected in wines. After malolactic fermentation, in both the manufacture of sparkling wines and of red wines aged on lees, yeasts and lactic acid bacteria can be observed to play a key role in the ACEI activity attributable to wine peptides.

These peptides would make a much greater contribution to the total activity if they were present in higher proportions, since, as observed in wine, they are only present (especially the hydrophobic peptides) in very low concentrations. It is, therefore, advantageous to increase their concentrations in wines, either by using starting materials with high initial peptide contents or by using highly autolytic yeasts that permit a greater degree of hydrolysis of wine proteins, giving rise to higher concentrations of peptides with ACEI activity.

These studies should continue, carrying out experiments with wines made with different grape varieties, to establish how each of these contributes to the peptide compositions of the corresponding wines. Also, studies should be conducted on still wines aged on lees since, in still wine production, there is a greater versatility in the choice of the yeast strain responsible for the alcoholic fermentation, which is much more limited in the manufacture of sparkling wines.

Acknowledgements

This work has been supported by the Comunidad Autónoma de Madrid, projects CM S-0505/AGR/0153-CAM. J.M. Alcaide-Hidalgo was the recipient of a fellowship from Danone Institute, Spain. The authors wish to thank to Mª Victoria Moreno and Pilar Hidalgo for the samples.

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