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Partial characterization of peptides from red wines. Changes during malolactic fermentation and ageing with lees

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Abstract

The peptide fraction of an industrially manufactured red wine has been studied during malolactic fermentation, carried out in stainless-steel tanks or in the barrel and ageing in the barrel, with or without lees, for 12 months. Peptides were fractionated using Sephadex LH-20 and Cosmosil 140 C_{18}-OPN columns, giving two fractions in relation to peptide polarity. The most important changes were detected during malolactic fermentation and during the ageing in barrel with lees. The peptides present in the wine could be glycopeptides from grape or yeast. Most amino acids in the most polar peptides were aspartic acid and/or asparagine, glutamic acid and/or glutamine, serine, glycine, α-alanine and tyrosine and, in the less polar fraction, were glycine, α-alanine and leucine. The amino acid distribution is most different in the most polar fraction, among the studied wines, owing to autolysis and hydrolysis of the polypeptides and proteins.

Keywords: Red wine; Peptides; Malolactic fermentation; Ageing with lees; Glycopeptides

1. Introduction

Peptides are the least known nitrogenated compounds of wine despite their diverse properties, such as their tensioactivity (González-Llano, Herraiz, & Polo, 2004), sensorial activity (Desportes, Charpentier, Duteurtre, Maujean, & Duchiron, 2001) and antihypertensive activity (Pozo-Bayón, Alcaíde, Polo, & Pueyo, 2007), as well as being their nutrients for yeasts and bacteria (Alexandre et al., 2001; Manca de Nadra, Farías, Pueyo, & Polo, 2005; Remize et al., 2006). The main reason for the dearth of studies on this fraction is due to the complexity and lack of specificity of the techniques used for its analysis. To characterize this fraction, the sample must be treated in successive fractionation steps to eliminate compounds that may interfere in the analysis (Desportes et al., 2001; Moreno-Arribas, Pueyo, & Polo, 1996). The few studies that have been carried out on the peptide fraction of wine have focussed on white wines, including table wines (Desportes et al., 2001), sparkling wines (Moreno-Arribas et al., 1996; Moreno-Arribas, Pueyo, Polo, & Martín-Alvarez, 1998) and biologically aged wines (Dos Santos, Feuillat, & Charpentier, 2000). Also, release of peptides into wine from yeasts during autolysis has been studied during the manufacture of sparkling wines (Martínez-Rodriguez & Polo, 2000). However, to our knowledge, to date no study has been carried out on the peptide fraction of red wines or on the changes occurring during malolactic fermentation and ageing in the barrel, with or without lees.

Ageing of wines with lees is a common practice in white wines (Dos Santos et al., 2000) and in sparkling wines (Feuillat & Charpentier, 1982; Martínez-Rodríguez, Carrascosa, Martín-Álvarez, Moreno-Arribas, & Polo, 2002; Moreno-Arribas et al., 1996) in which yeast autolysis occurs, with release of its components into the wine, altering its organoleptic characteristics. This practice has been extended throughout wine cellars in order to increase the diversity and complexity of the wines (Feuillat, Escot,

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Charpentier, & Dulau, 2001; Doco, Vuchot, Cheynier, & Moutounet, 2003). Few studies have been carried out on the effect of this practice on the composition of red wines, and research to date has centred on the polysaccharide fraction (Doco et al., 2003) and its interaction with phenolic compounds and the effect on colour stability (Feuillat et al., 2001; Hernández, Estrella, Carlavilla, Martin-Alvarez, & Moreno-Arribas, 2006). One wine fraction that can be significantly altered with this technique is the nitrogenated fraction. The effect of different ways of carrying out malolactic fermentation (in tanks or in the barrel) and ageing, with or without lees, on free amino acids and amines was studied by Alcaide-Hidalgo, Moreno-Arribas, Martín-Álvarez, and Polo (2007), who demonstrated an increase in free amino acids on ageing with lees that could result in a risk of biogenic amine formation.

We began this research work, owing to the lack of studies on red wine peptides in general and, more specifically, on their evolution during malolactic fermentation and their changes due to ageing on lees. Starting with one red wine obtained industrially, we made three red wines using different technological conditions: malolactic fermentation in barrel or in stainless-steel tanks and ageing for 12 months in oak barrels, with or without lees. Malolactic fermentation in stainless-steel tanks and ageing in barrel without lees is the technique traditionally used in the wineries, so the wine obtained this way can be considered as the control wine.

2. Materials and methods

2.1. Manufacture of the wine

A red wine was industrially manufactured and submitted to different experiments in a winery. The initial wine (Iw) used was a quality red wine from the AOC Navarra (Spain), from *Vitis vinifera* L., c.v Tempranillo grapes, manufactured in 10,000 l stainless-steel tanks. After alcoholic fermentation, part of this wine (approximately 7000 l) underwent malolactic fermentation in the tank (MLF-T). After this second fermentation had finished a part of the wine was submitted to the traditional postfermentation treatments, racking, clarification with albumin and bentonite, cold stabilisation and filtration, and was transferred to oak barrels. This treated wine was considered as the control wine. Another part of the MLF-T wine was transferred to oak barrels without removing the lees. The rest of the wine (approximately 3000 l) underwent malolactic fermentation in oak barrels with yeasts lees (MLF-B). All the wines were aged in barrels for 12 months (T-C-12; T-L-12, and B-L-12). During ageing in barrels with lees, the wines were stirred weekly. 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 (*Quercus sessilis*) were used.

Table 1 summarises the main steps in the manufacturing process of the wines and the sample keys. 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 for 15 min at 5000*g* and immediately refrigerated prior to analysis. All the analyses were conducted in duplicate.

2.2. Chemical analysis

Total nitrogen was determined by the Kjeldahl method with a DK 20 Heating Digestor System and an UDK 142 Automatic Distillation Unit from VELP Scientifica (Italy) and a 702 SM Tritino Unit from Metrohn (Switzerland). Free amino nitrogen was quantified by the Cd-ninhydrin method (Doi, Shibata, & Matoba, 1981, method C). Peptide nitrogen was estimated as the difference between the total nitrogen and the free amino nitrogen.

2.3. Obtaining the fraction of molecular weight less than 4000

Four hundred and fifty ml quantities of each wine were 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 from LKB (Uppsala, Sweden). Chromatographic data were collected with the Gold System v8.1 (Beckman Instruments Inc., Fullerton, CA, USA) 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.

2.4. Fractionation of peptides by reversed-phase low pressure chromatography

Twenty ml of the concentrate obtained from the Sephadex LH-20 column were 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,

Table 1
Technological procedures used for the manufacture of wines and key labels

Red wine Tempranillo variety Iw	Wine that underwent MLF in stainless-steel tank MLF-T	Wine aged 12 months without lees T-C-12 (Control wine)
		Wine aged 12 months with lees T-L-12
	Wine that underwent MLF in barrel MLF-B	Wine aged 12 months with lees B-L-12

a 50 ml superloop and a HiLoad Pump P-50 from Amersham Biosciences, Uppsala, Sweden were used. Flow rate was 2 ml/min. Amino acids and more polar peptides were eluted with water (fraction 1, F1) and 10% ethanol was used to elute less polar peptides (fraction 2, F2). The elution was monitored at 280 nm using a 2138-Uvicord S detector from LKB (Uppsala, Sweden) 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.

2.5. Peptide analysis by high performance liquid chromatography

Twenty μl from fractions F1 and F2 obtained from the Cosmosil 140 C_{18}-OPN column, were submitted to HPLC for peptide analysis. The equipment consisted of two M-116 Beckman pumps, a Midas automatic injector (Spark Holland, Emmen, The Netherlands), a M-168 detector from Beckman and a personal computer. All separations were performed on a Waters Nova-Pak C_{18} column (150 × 3.9 mm i.d., 60 Å, 4 μm). Eluent A was 0.1% trifluoroacetic acid in water and eluent B, 0.1% trifluoroacetic acid in acetonitrile. Linear gradient elution was from 0% to 40% eluent B during 70 min and flow rate, 1 ml/min. Chromatograms were recorded at 214 and 280 nm and spectral data were obtained from 190 to 340 nm, with a resolution of 2 nm. A Gold Nouveau Chromatography Data System v1.0 (Beckman) was used for chromatographic control and data acquisition and analysis. All samples were analysed under the same conditions and the areas of the peaks were measured by valley to valley integration.

2.6. Peptide hydrolysis

F1 and F2 fractions (500 μl) were submitted to acid hydrolysis with 500 μl of 12 M HCl (final concentration 6 M) and 10 μl of thioglycolic acid under vacuum, for 24 h at 110 °C.

2.7. Amino acid analysis

Amino acid compositions of the F1 and F2 fractions were determined before and after hydrolysis by reversed-phase HPLC, using a liquid chromatograph consisting of a Waters 600 Controller programmable solvent module (Waters, Milford, MA, USA), a WISP 710B autosampler (Waters, Milford, MA, USA), and a HP 1046-A fluorescence detector (Hewlett-Packard). Samples were submitted to automatic precolumn derivatization with OPA in the presence of 2-mercaptoethanol. Solvents and gradient conditions were as described by Moreno-Arribas et al. (1998). Separations were performed on a Waters Nova-Pak C_{18} (150 × 3.9 mm i.d., 60 Å, 4 μm) column and the same type of precolumn. Detection was performed by fluorescence (λ excitation = 340, λ emission = 425) and chromatographic data were collected and analysed with a Millenium32 system (Waters, Milford, MA).

The amino acid composition of the peptides was calculated as the difference between the amino acid content in the fractions before and after hydrolysis. Due to the partial conversion of asparagine and glutamine into aspartic acid and glutamic acids, respectively, during hydrolysis, the data for asparagine plus aspartic acid and glutamine plus glutamic acid are reported as Asx and Glx, respectively, in all the hydrolysed samples. The absence of tryptophan and methionine in the hydrolysed samples may be due to the breakdown of these amino acids during hydrolysis.

2.8. Polysaccharides hydrolysis

One ml aliquots, from fractions F1 and F2 obtained from the Cosmosil 140 C_{18}-OPN column, were submitted to hydrolysis at 100 °C for 24 h, in closed vials containing 1 ml of 2 M trifluoroacetic acid and 0.5 ml *myo*-inositol (0.1% w/v, internal standard) solution.

2.9. Monosaccharides analysis

Monosaccharides were analysed before and after hydrolysis of fractions F1 and F2 by gas chromatography of their corresponding silylated derivatives. Silylation was performed, following the procedure of Troyano, Olano, Fernández-Díaz, Sanz, and Martínez-Castro (1991). Briefly, the dried samples were dissolved in 100 μl of anhydrous pyridine and silylated with 100 μl of trimethylsilylimidazole and 100 μl of trimethylchlorosilane at room temperature shaking at the same time. After derivatization, 0.1 ml of *n*-hexane and 0.2 ml of deionized water were sequentially added, shaken during each step and 2 μl from the organic layer were injected in the GC. Trimethylsilyl derivatives were analysed on a Hewlett-Packard 6890 Chromatograph, equipped with a flame ionization detector (FID) and split/splitless injector. A Carbowax 20 M column (30 m × 0.25 mm) coated with a methyl silicone (0.25 μm film thickness) was used. A temperature programme involving an initial temperature of 175 °C (15 min), a 15 °C/min ramp to 200 °C (13 min), 30 °C/min and a final temperature of 270 °C (10 min), was used. Injector and detector temperatures were 270 °C. The carrier gas was helium (8.9 psi, split 1/15). Response factors were calculated with a series of pure standards at different concentrations using myo-inositol as internal standard. Identification of the compounds present in the samples was carried out by comparing the retention time of the peaks with those of pure standards.

3. Results and discussion

3.1. Nitrogenated composition of the initial wine and changes taking place during malolactic fermentation and ageing

The nitrogenated fraction is formed mainly of free amino acids, peptides and proteins, comprising the proteic

nitrogen, generally less than 1% of the total nitrogen (Moreno-Arribas et al., 1996). Therefore, in the absence of reliable methods to determine the peptide and proteic nitrogen of red wines, the peptide nitrogen can be estimated from the difference between the total nitrogen and the free amino acids.

Fig. 1a–c shows the concentrations of the different nitrogen fractions in the initial wine (Iw), in the wines after malolactic fermentation (MLF-T and MLF-B) and in the wines after 12 months ageing in the barrel (T-C-12, T-L-12 and B-L-12). The initial wine had a relatively small total nitrogen content, of 227 mg/l that decreased slightly, and there was approximately the same amount in the wine in which malolactic fermentation took place in the tank (MLF-T) and that in which it was carried out in the barrel (MLF-B) (Fig. 1a). However, the nitrogen contents in free amino acids increased in this step (Fig. 1b), so the decrease in total nitrogen was mainly due to the drop in total peptide nitrogen (Fig. 1c). The proteasic activity of the lactic acid bacteria, clearly revealed when this takes place in nitrogen-deficient media, results in the release of amino acids and peptides to the wine (Manca de Nadra, Farias, Moreno-Arribas, Pueyo, & Polo, 1999) and sometimes, as occurred in this wine, a greater amount of amino acids is released than is consumed. During ageing in the barrel, the contents of all the nitrogenated fractions in the barrel increased with ageing (Fig. 1a–c), and this was greater in wines aged on lees.

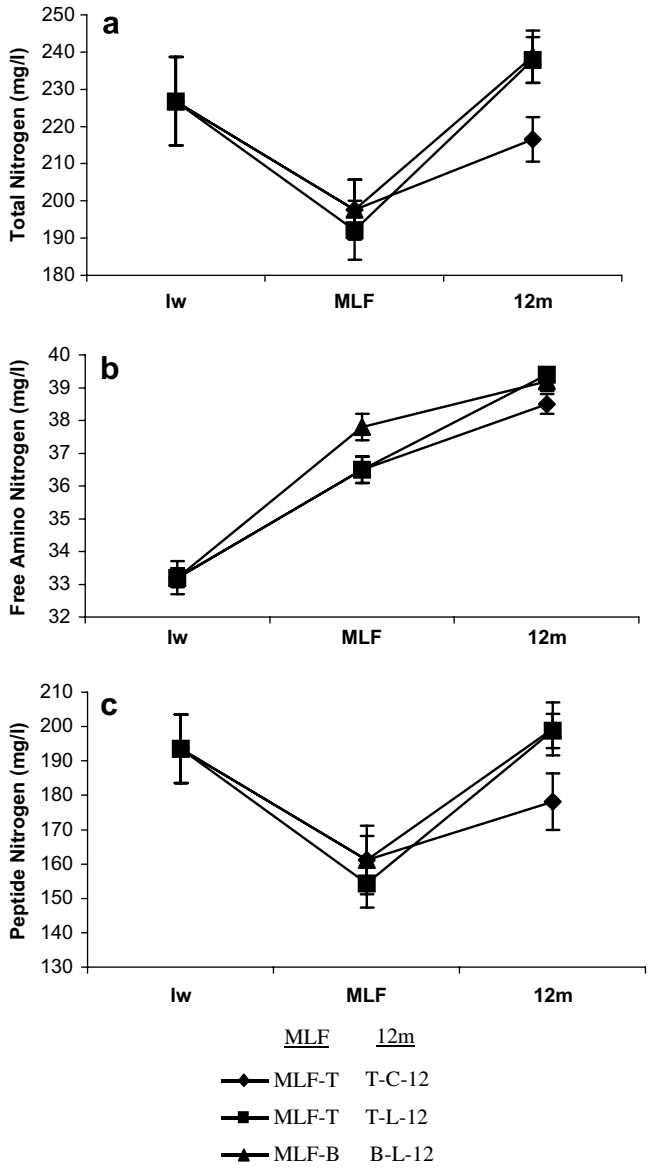

Fig. 1. Concentration of total nitrogen (a), amine nitrogen (b) and peptide nitrogen (c) in the initial wine (Iw), in wine peptides after malolactic fermentation (MLF) and in wines after 12 months ageing in barrel (12 m).

3.2. Peptide analysis

Owing to the complexity of wine, successive fractionation steps must be used to study peptides in order to eliminate, as far as possible, the high molecular weight compounds. As a first step in studying the peptides, the wine was passed through a Sephadex LH-20 column. This stationary phase is a beaded cross-linked hydroxypropylated dextran and acts as an exclusion and a partition gel. Under the conditions applied, peptides larger than 4000 Da are excluded and most phenolic compounds are retained in the column (Amersham Biosciences, Application note 18-1107-22). However, some amino acids elute with the peptides (Moreno-Arribas et al., 1996). To separate these compounds from the peptides of interest, the eluted fraction after the void volume of the Sephadex LH-20 column, after being concentrated, was passed through a Cosmosil 140 C_{18}-OPN column to separate the more polar compounds that elute with water (F1), from the less polar compounds that elute with 10% ethanol (F2) and most of the phenolic compounds remaining after passing the sample through the Sephadex LH-20 column were retained. Both fractions, F1 and F2, were chromatographed by HPLC.

3.3. More polar peptides

Fig. 2 shows, as an example, the chromatogram obtained with the F1 fraction of one of the wines analysed. From the analysis of free and total amino acids, together with the analysis of the ultraviolet spectra obtained during the chromatogram, we can see that the free amino acids elute in the first five minutes of the chromatogram (Fig. 2, zone I). Most of the peptides were eluted in the interval from minutes 5 to 14 (Fig. 2, zone II), and after 14 min (Fig. 2, zone III) some phenolic compounds were eluted. Therefore, to study the peptides, only the compounds eluting in Zone II of the chromatograms are considered.

Table 2 shows the areas under the peaks detected in Zone II of fraction F1, for each of the samples studied and the sum of the areas of all the peaks. Some of the peaks (#3 and #4, #8 and #9, #11 and #12, and #18 and #19)

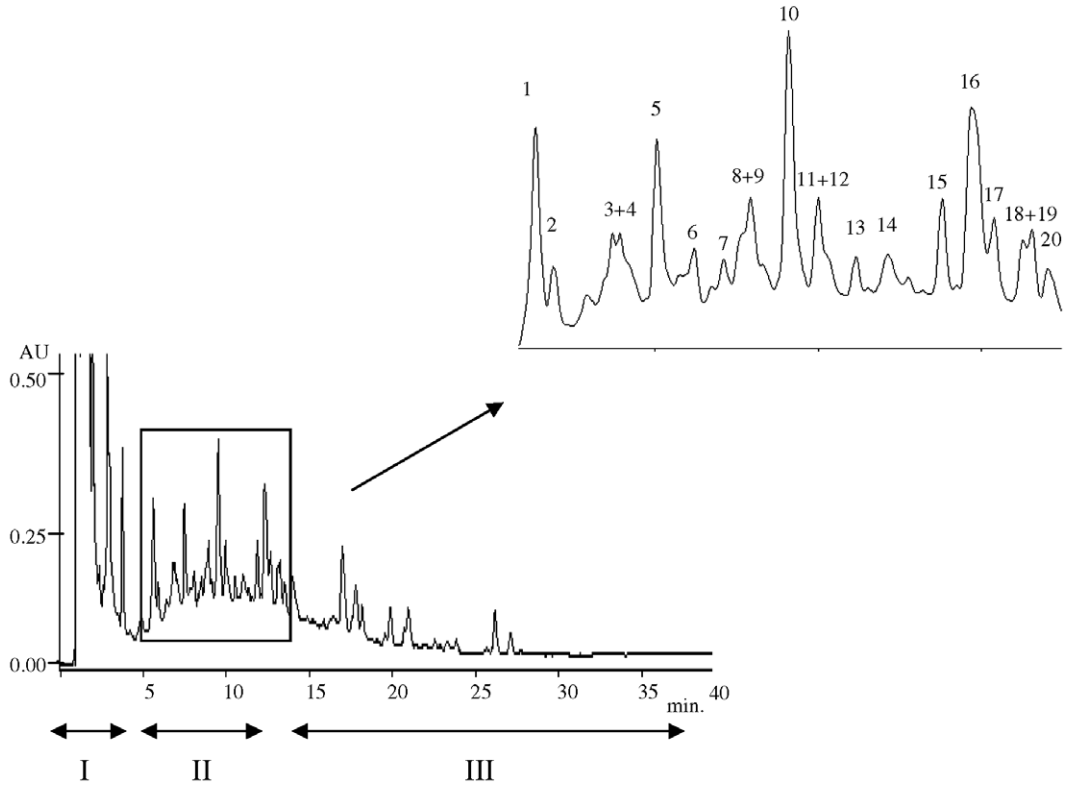

Fig. 2. Chromatogram obtained by HPLC, of the F1 fraction also obtained by Cosmosil-140 C_{18} OPN, of one of the wines analysed. Zone I: amino acids + peptides, zone II: peptides and zone III: phenols + peptides.

Table 2
Areas arbitrary units of the chromatogram peaks of fraction F1 (zone II) obtained from the column of Cosmosil-140 C_{18} OPN, of the initial wine (Iw), of the wines after malolactic fermentation (MLF-T and MLF-B) and of the wines after ageing for 12 months in barrel (T-C-12, T-L-12 and B-L-12)

Peak numbers[a]	Initial wine	Wines after malolactic fermentation		Wines after 12 months of ageing in barrels		
	Iw	MLF-T	MLF-B	T-C-12	T-L-12	B-L-12
1	236	154	146	587	882	1419
2	nd	nd	nd	109	nd	nd
3 + 4	213	120	172	465	683	640
5	nd	39	nd	421	664	755
6	119	42	nd	170	129	254
7	nd	nd	nd	95	92	58
8 + 9	592	404	462	447	180	227
10	838	326	414	806	798	954
11 + 12	264	167	183	320	384	368
13	nd	nd	nd	97	76	70
14	414	115	299	125	252	110
15	nd	nd	nd	227	161	163
16	903	500	609	690	768	831
17	nd	nd	nd	118	60	110
18 + 19	214	44	nd	319	217	131
20	129	122	nd	119	134	186
Sum of areas	3921	2034	2285	5116	5481	6276

[a] For peak identification, see Fig. 2; nd = not detected.

overlap in some chromatograms so the measurement of their area has been expressed by the sum. In total, 20 peaks were detected. Peaks #1, #(3 + 4), #(8 + 9), #10, #(11 + 12), #14 and #16, were in all the wines. In the initial wine, there were 14 peaks and the predominant ones were #16, #10 and #(8 + 9). In the F1 fractions of the wine undergoing malolactic fermentation in the tank and in the barrel, the sum of the areas, 2034 and 2285, respectively, was smaller than in the initial wine, 3921. These results indicate that there was a reduction in peptides during malolactic fermentation (a reduction in the sum of their areas). This agrees with data obtained from peptide nitrogen determination for these wines (Fig. 1c). During ageing in the barrel, both the number of peaks and also their intensity increased in all the wines, and the highest values were obtained in the wine in which malolactic fermentation took place in the barrel, aged for 12 months on lees. These results indicate that while the wine was ageing, with or without lees, there was a residual proteasic activity in which peptides were released into the wine, which is greatest in wines aged on lees (T-L-12 and B-L-12, Table 1).

In order to establish the amino acid composition of the more polar peptides (fraction F1), free and total amino acids of this fraction were determined. The peptide amino acids were estimated as the difference between the results of these two determinations. Table 3 shows the molar distribution of the peptide amino acids of this fraction. The amino acids of the most polar peptides of the initial wine were mostly aspartic acid and/or asparagine, glutamic acid and/or glutamine, serine, glycine, α-alanine and tyrosine. These amino acids constitute approximately 80% of the total amino acids of the F1 fraction. These were also the

Table 3
Molar distribution (%) of the amino acids of the peptides of fraction F1 obtained from the Cosmosil-140 C_{18} OPN column, of the initial wine (Iw), of the wines after malolactic fermentation (MLF-T and MLF-B) and of the wines after ageing for 12 months in barrel (T-C-12, T-L-12 and B-L-12)

	Initial wine	Wines after malolactic fermentation		Wines after 12 months of ageing in barrels		
	Iw	MLF-T	MLF-B	T-C-12	T-L-12	B-L-12
Asx	12.30	18.60	3.71	12.60	4.39	10.10
Glx	17.40	20.60	8.01	22.10	10.40	12.50
Ser	9.21	14.40	7.69	9.23	6.69	8.29
His	nd	nd	5.95	nd	nd	nd
Gly	12.20	7.32	34.10	16.30	26.50	9.83
Thr	5.24	8.84	3.38	nd	9.75	5.80
Arg	4.07	5.76	4.37	nd	2.70	2.80
β-Ala	nd	nd	nd	nd	nd	nd
α-Ala	17.20	15.60	9.36	17.10	10.30	16.50
Gaba[a]	nd	nd	nd	nd	2.97	2.30
Tyr	11.00	nd	3.75	5.92	6.03	16.20
Val	5.02	4.77	3.06	6.24	3.65	4.46
Phe	nd	nd	nd	nd	nd	nd
Ile	2.26	nd	nd	4.16	nd	2.91
Leu	4.06	4.14	2.71	6.42	3.73	5.88
Orn	nd	nd	4.66	nd	nd	nd
Lys	nd	nd	9.27	nd	12.9	2.38

nd = not detected.
[a] Gaba = gamma aminobutyric acid.

predominant amino acids detected in wine peptides by Moreno-Arribas et al. (1998) and Desportes et al. (2000). Threonine, arginine, valine, isoleucine and leucine were also detected in this fraction. These amino acids are described as constituents of the wine peptides by different authors (Doi et al., 1981; Usseglio-Tomasset & Bosia, 1990; Yokotsuka, Aihara, Umehara, & Kushida, 1975). The distribution of the peptide amino acids of wines that have undergone malolactic fermentation in a tank (MLF-T) was similar to that of the initial wine peptides (Iw), except for the tyrosine, one of the predominant amino acids of the peptides of the initial wine. In a study of the metabolism of free amino acids and the formation of biogenic amines in the same wines (Alcaide-Hidalgo et al., 2007), the tyrosine content was shown to decrease during malolactic fermentation, from 9.3 to 1.5 mg/l and 3.4 mg/l of tyramine, the corresponding biogenic amine, were formed. Since tyrosine is an essential amino acid for *O. oeni* (Remize et al., 2006), this could explain why tyrosine was not detected in these peptides. In the amino acids that comprise the wine peptides that undergo malolactic fermentation in the barrel (MLF-B), it is interesting to note the increase in glycine and the detection of histidine, ornithine and lysine, amino acids that do not form part of the most polar peptides of the initial wine. These amino acids could derive from the hydrolysis of larger proteins or peptides, performed by lactic acid bacteria or/and yeasts.

The distribution of the most polar peptides in the wine aged without lees (T-C-12), is analogous to the peptides of the wine from which it is derived (MLF-T), with the exception of threonine and arginine that have not been detected in the peptides of this wine and the appearance of tyrosine and isoleucine. The distribution of the amino

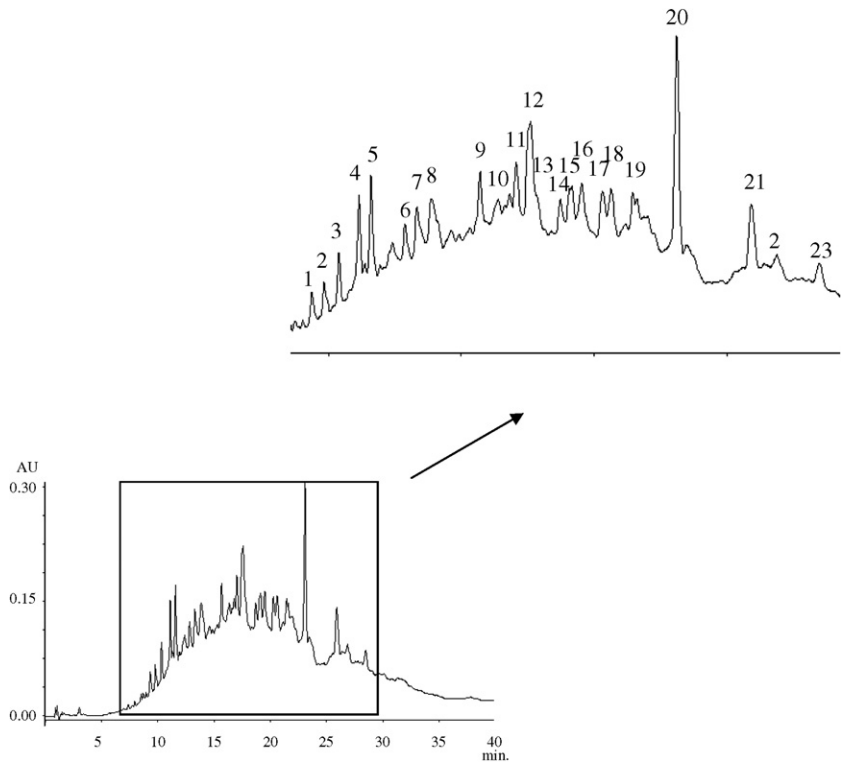

Fig. 3. Chromatogram obtained by HPLC, of the F2 fraction also obtained by Cosmosil-140 C_{18} OPN, of one of the wines studied.

acids in peptides of wines aged with lees (T-L-12 and B-L-12) is different from that of the control wine (T-C-12) and is also different from those of the wines from which these proceed (MLF-T and MLF-B); the increase in glycine and lysine in wine T-L-12 is noteworthy, as also are the increases in aspartic acid and/or asparagine, glutamic acid and/or glutamine, alanine and tyrosine in the wine B-L-12. Important increases have not been detected in threonine and serine, predominant amino acids of yeast mannoproteins (Frevert & Ballou, 1985), which indicates that they are not produced by yeast autolysis. These peptides could proceed from the hydrolysis of polypeptides or of grape proteins.

3.4. Less polar peptides

HPLC analysis was also carried out for the less polar peptides that elute with ethanol at 10% (F2 fraction). Fig. 3 shows the chromatogram obtained from this fraction for one of the wines studied. In total, 23 peaks were detected, almost the same as the number in the F1 fraction (Fig. 2). Table 4 shows the areas of the peaks detected in fraction F2 of all the wines. In the initial wine, 6 more peaks were detected than in fraction F1 (Table 1), but with a lower intensity. The predominant peaks, peaks #20–22, appear in the final part of the chromatogram, in other words, corresponding to the most hydrophobic peptides. The chromatographic profile of the peptides of fraction F2 of the wine, in which malolactic fermentation took place in tanks (MLF-T wine) was different from that of the initial wine (Iw). Peak #1 was not detected and there were three peaks that were not in the initial wine, peaks #2, #4 and #7. However, the sum of its areas was almost 30% of the sum of the areas of the peaks of fraction F2 of the initial wine. The intensity of the peaks of the wine that underwent malolactic fermentation in the barrel (MLF-B) was much greater than that of the peaks of wine that underwent malolactic fermentation in the tank (MLF-T) and even than that of the initial wine (Iw). The chromatographic profiles of the F2 fraction of wines after ageing in barrel were very different from those of wines at the end of malolactic fermentation. The wine T-C-12, control wine, that was subject to clarification and stabilisation processes at the end of malolactic fermentation, had a much smaller number of peaks than had the wine from which it was derived (MLF-T), but a greater intensity. This demonstrates the existence of residual protease activity in the wine, even in the absence of yeasts or bacteria. During the ageing of wines in barrel on lees, the intensity of the peaks increased greatly, and it was interesting to observe the increase in peaks in the wine undergoing malolactic fermentation in the barrel and aged on lees (wine B-L-12).

The peptide amino acids of fraction F2 were also analysed and the results are shown in Table 5. As expected, these peptides had a smaller proportion of the more polar amino acids, aspartic acid and/or asparagine and glutamic acid and/or glutamine and a greater proportion of the less polar amino acids, isoleucine and leucine than had those of fraction F1 (Table 4).

Table 4
Areas arbitrary units under the chromatogram peaks of fraction F2 obtained from the Cosmosil-140 C_{18} OPN column, of the initial wine (Iw), of the wines after malolactic fermentation (MLF-T and MLF-B) and of the wines after ageing for 12 months in the barrel (T-C-12, T-L-12 and B-L-12)

Peak numbers[a]	Initial wine	Wines after malolactic fermentation		Wines after 12 months of ageing in barrels		
	Iw	MLF-T	MLF-B	T-C-12	T-L-12	B-L-12
1	109	nd	57	nd	nd	nd
2	nd	25	64	nd	nd	nd
3	41	28	86	nd	nd	nd
4	nd	62	145	nd	nd	151
5	74	48	164	nd	53	208
6	48	23	71	nd	nd	nd
7	nd	27	117	nd	113	190
8	62	67	185	nd	57	114
9	193	47	131	130	235	782
10	70	25	56	66	122	413
11	63	32	93	nd	nd	nd
12	75	58	416	62	125	433
13	37	20	25	nd	41	142
14	72	25	54	nd	27	64
15	96	29	95	71	88	177
16	32	24	113	nd	251	1836
17	43	37	79	nd	nd	175
18	126	45	83	93	399	917
19	49	31	112	217	291	653
20	472	142	525	640	959	3848
21	482	30	189	362	106	1285
22	246	24	62	nd	nd	126
23	105	12	77	154	25	274
Sum of areas	2496	860	2998	1795	2894	11787

[a] For peak identification, see Fig. 3; nd = not detected.

In the initial wine, there was a larger proportion of glycine and a smaller proportion of tyrosine than in the F1 fraction of this same wine. The amino acids β-alanine, phenylalanine and lysine that had not been detected in the fraction F1, were also detected in this fraction. Important changes were not observed in the distribution of amino acids that constitute the peptides, either due to the effect of malolactic fermentation or due to ageing, with or without lees.

3.5. Monosaccharide composition of the polysaccharides

Wine peptides can proceed from grape proteins or from yeast proteins. In both cases, these are glycoproteins (Marchal, Bouquelet, & Maujean, 1996; Guilloux-Benatier, Remize, Gal, Guzzo, & Alexandre, 2006; Núñez, Carrascosa, Gonzalez, Polo, & Martinez-Rodriguez, 2006). It is, therefore, reasonable to presume that the wine peptides are glycopeptides. This was verified by determining the composition of free monosaccharides following hydrolysis of fractions F1 and F2. Free monosaccharides were not

Table 5
Molar distribution (%) of the amino acids of the peptides of the F2 fraction obtained in the Cosmosil-140 C_{18} OPN column, of the initial wine (Iw), of the wines after malolactic fermentation (MLF-T and MLF-B) and of the wines after ageing for 12 months in the barrel (T-C-12, T-L-12 and B-L-12)

	Initial wine	Wine after malolactic fermentation		Wines after 12 months of ageing in barrels		
	Iw	MLF-T	MLF-B	T-C-12	T-L-12	B-L-12
Asx	6.75	7.55	7.10	8.68	8.82	3.45
Glx	10.40	8.60	10.50	10.60	7.51	2.09
Ser	5.98	4.61	6.99	9.09	7.49	1.16
His	nd	nd	nd	nd	6.37	0.59
Gly	20.40	18.70	18.80	17.40	9.98	22.90
Thr	6.44	5.81	6.34	6.95	7.37	2.57
Arg	nd	nd	nd	nd	6.20	1.74
β-Ala	0.55	0.69	0.56	0.28	nd	1.21
α-Ala	12.90	15.30	12.00	9.79	7.69	18.90
Gaba[a]	nd	nd	nd	nd	nd	nd
Tyr	2.88	2.43	2.87	3.11	6.69	4.60
Val	7.22	10.10	8.70	8.42	7.37	9.93
Phe	3.04	3.30	3.34	3.64	4.10	3.80
Ile	6.04	7.13	7.14	8.29	6.96	8.95
Leu	10.10	10.30	10.50	10.60	8.14	11.00
Orn	nd	nd	nd	nd	nd	nd
Lys	4.87	5.36	5.24	3.36	7.71	4.67

nd = not detected.
[a] Gaba = gamma aminobutyric acid.

detected in any of the fractions, so there were only polysaccharides. The molar distribution of the polysaccharides of fractions F1 and F2 is shown in Tables 6 and 7, respectively.

Arabinose, rhamnose, galactose, xylose and glucose were detected in polysaccharides of fraction F1 (Table 6). Both in the initial wine and in the wines after malolactic fermentation, the predominant sugar of the polysaccharides was glucose, from 53.6% to 67.9%, followed by arabinose with a ratio of glucose/arabinose of 2.27–5.25. After ageing in the barrel, the content of arabinose rose and that of the glucose fell, resulting in a mean ratio of 0.41. The fact that mannose was not detected in these polysaccharides indicates that these are not derived from yeast mannoproteins which agrees with the results obtained from the peptide amino acid determinations.

Table 6
Molar distribution (%) of the monosaccharides of the polysaccharides of the F1 fraction obtained in the Cosmosil-140 C_{18} OPN column, of the initial wine (Iw), of the wines after malolactic fermentation (MLF-T and MLF-B) and of the wines after ageing for 12 months in the barrel (T-L-12 and B-L-12)

	Initial Wine	Wines after malolactic fermentation		Wines after 12 months of ageing in barrels	
	Iw	MLF-T	MLF-B	T-L-12	B-L-12
Arabinose	12.90	14.20	23.60	55.10	42.10
Mannose	nd	nd	nd	nd	nd
Rhamnose	5.85	12.80	13.90	14.30	19.30
Galactose	9.68	5.95	8.95	6.56	16.00
Xylose	3.63	4.73	nd	nd	6.41
Glucose	67.90	62.20	53.60	24.10	16.20

nd = not detected.

Table 7
Molar distribution (%) of the monosaccharides of the polysaccharides of the F2 fraction obtained in the Cosmosil-140 C_{18} OPN column, of the initial wine (Iw), of the wines after malolactic fermentation (MLF-T and MLF-B) and of the wines after ageing for 12 months in the barrel (T-C-12, T-L-12 and B-L-12)

	Initial wine	Wines after malolactic fermentation		Wines after 12 months of ageing in barrels		
	Iw	MLF-T	MLF-B	T-C-12	T-L-12	B-L-12
Arabinose	44.00	21.80	29.60	34.00	29.60	12.00
Mannose	5.64	12.40	9.60	6.35	9.60	9.55
Rhamnose	4.27	10.90	5.30	2.84	5.30	4.57
Galactose	2.73	8.35	7.04	3.07	7.04	3.52
Xylose	1.50	3.12	4.04	3.11	4.04	3.66
Glucose	41.90	43.40	44.50	50.70	44.50	66.70

In the polysaccharide of fraction F2 (Table 7), the same monosaccharides as in the F1 fraction were detected, as well as mannose. Moreover, the predominant monosaccharides of this fraction, in all the wines, were glucose and arabinose, with a ratio lower than 2 in all the wines except for the wine that underwent malolactic fermentation in the barrel aged for 12 months on lees (wine B-L-12), in which the ratio of glucose/arabinose was 5.56. The presence of mannose in these polysaccharides indicates that, at least in part, these are derived from the yeasts.

From the results obtained in this study, it can be concluded that the peptide fraction of the red wines is complex and is comprised, at least partly, of glycopeptides from grapes and yeasts. Important changes occur during malolactic fermentation and ageing in barrels, which are greatest in the wines aged on lees. Owing to the sensorial importance of peptides, it is necessary to take this consideration into account because of the repercussions that this technology may have on the sensorial quality of the wines. To the best of our knowledge, although peptide profiles and composition have been determined in white wine, this is the first study carried out on the peptide fraction from red wines.

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