Peptides in Musts and Wines. Changes during the Manufacture of Cavas (Sparkling Wines)

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The aim of this work was to increase the knowledge of the peptide fraction in musts and wines, of which there are very few publications in literature. For this purpose, the peptides in four varietal sparkling wines manufactured industrially following the Champenoise method were analyzed by HPLC. Samples of the musts, base wines, and sparkling wines were taken after 9, 12, 15, and 18 months of aging on yeast lees. The peptides of the fractions with molecular weights higher and lower than 700, obtained by fractionation on Sephadex G-10, were separated by reversed-phase HPLC. The chromatographic profiles of the peptides in the wines were similar. During the manufacture of the base wines, there was an increase in the peptides studied. During aging, peptides were released and then degraded by the yeast enzymes.

Keywords: *Peptides; HPLC; must; wine; sparkling wine; cava*

INTRODUCTION

The major compounds of the nitrogenous fraction of wine are peptides and free amino acids. There are numerous studies of the composition of free amino acids in musts and of their changes in the different stages in the manufacture of wine. However, there are few studies of peptides, and most of those that do exist refer to the study of the difference between free amino acids and amino acids released by hydrolysis of the peptide fraction (Poux and Ournac, 1970; Yokotsuka et al., 1975; Usseglio-Tomasset and Di Stefano, 1978; Margheri et al., 1984; Usseglio-Tomasset and Bossia, 1990).

During the first stage of fermentation, peptides are assimilated by the yeast together with free amino acids. At the end of fermentation, there is an excretion of free amino acids and small peptides from the yeast to the wine (Dupuy, 1967; Ough et al., 1991; Dizy and Polo, 1996). This process occurs again during secondary fermentation in the case of sparkling wines. After a period of rest of the cells, estimated by most authors as between 3 and 9 months (Suárez et al., 1979; Colagrande and Silva, 1981; Feuillat and Charpentier, 1982; Marghery et al. 1984; Kelly-Treadwell, 1988), the autolysis of the yeast begins. During autolysis, due to the successive action of different proteases (Kelly-Treadwell, 1988; Lurton et al., 1989; Charpentier and Feuillat, 1993), the yeast proteins are hydrolyzed. First, peptides are formed and then the peptides degrade into amino acids. At this stage, in which the cellular membrane may be intact, peptides with low and medium molecular weights diffuse into the wine. Because peptides in wine may have the same importance as those in other foods, due to their surfactant properties, and because they are responsible for sweet and bitter tastes (Polo et al., 1992) it is necessary to learn more about their characteristics. It is also necessary to increase the knowledge of the transformations that take place during the manufacture of wine and most especially during autolysis that occurs in the manufacture of sparkling wines by the Traditional method. This study was conducted with this object in mind. For this purpose, four cava wines (Spanish sparkling wines made by the Champenoise method) were manufactured industrially and a study made by HPLC of the peptide fraction of the musts, base wines and cavas after 9−18 months of aging in the bottle.

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MATERIALS AND METHODS

Samples. Musts, base wines, and sparkling wines (Champenoise method) were industrially manufactured from white grapes of the Macabeo, Xarel-lo, Parellada, and Chardonnay varieties. The base wines were made from sulfited musts (80 mg SO_2/L) in 100 000-L tanks at 16−18 °C, clarified with 20 g of bentonite/hL and 1 g of gelatin/hL, and tartrate stabilized. Musts were inoculated with a selected winery yeast (*Saccharomyces cerevisiae*). The sparkling wines were obtained by the Champenoise method by inoculation of the base wines with the same culture of yeast from the winery collection (*Saccharomyces bayanus*). Degorging was performed after 9, 12, 15, and 18 months of aging with yeast. All the analyses were conducted on the musts and wines after centrifugation for 15 min at 5000*g*. The musts were frozen and the wines refrigerated until analyzed.

Determination of Nitrogen Compounds. Total nitrogen was determined by the Kjeldahl method with a Buchi 425 digestor and a Buchi 315 distillation unit. Free amino nitrogen was estimated from the sum of proline nitrogen determined by Ough's (1969) method and the nitrogen of each amino acid determined individually by HPLC of the derivatives with *o*-phthalaldehyde (González de Llano et al., 1991). Total amino nitrogen was determined in the same way as free amino nitrogen, after hydrolysis at 110 °C for 24 h, with 6 N HCl in vials sealed under reduced pressure. Protein nitrogen was calculated by dividing the protein content, determined by the Bradford dye-binding assay (Bradford, 1976), by 6.25. Peptide nitrogen was considered as the difference between total amino nitrogen and free amino plus protein nitrogen.

Analysis of Peptides. Elimination of the Compounds with a High Molecular Weight. The compounds with a high molecular weight (mainly proteins and polysaccharides) in 200 mL of must and wine concentrated under reduced pressure to 10 mL were eliminated by precipitation with five volumes of 95% ethanol in an acid medium (Usseglio-Tomasset and Castino, 1975) and centrifugation at 10000*g*, 30 min.

Fractionation of the Ethanol-Soluble Fraction by Low-Pressure Column Chromatography. The supernatant liquid obtained in the previous centrifugation was concentrated under reduced pressure and fractionated by

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Table 1. Nitrogen Content (mg/L) in Musts and Wines

	total N	free amino N	peptide N	protein N
		Macabeo Variety		
must	—	—	—	7.2
base wine	129.0	42.7	20.2	1.3
sparkling wines				
9 months	123.2	41.4	48.8	0.4
12 months	117.6	39.0	63.3	1.2
15 months	112.0	51.1	65.3	1.1
18 months	117.6	57.8	24.5	1.3
		Xarel-lo Variety		
must	252.0	118.9	118.2	10.8
base wine	100.8	58.5	57.6	1.7
sparkling wines				
9 months	112.0	53.9	16.9	0.7
12 months	123.2	56.1	61.1	1.3
15 months	128.8	63.4	90.2	1.6
18 months	128.8	62.5	40.8	1.6
		Parellada Variety		
must	238.0	137.5	108.9	9.7
base wine	126.0	57.9	14.5	1.4
sparkling wines				
9 months	123.2	50.8	17.9	0.6
12 months	117.6	60.7	44.8	1.4
15 months	128.8	59.5	44.5	1.3
18 months	117.6	68.6	19.9	1.4
		Chardonnay Variety		
must	336.0	283.3	162.2	10.5
base wine	347.0	234.3	41.5	2.6
sparkling wines				
9 months	313.6	231.6	58.3	1.5
12 months	313.6	204.7	35.8	2.0
15 months	319.2	215.2	61.1	1.9
18 months	313.6	223.7	67.7	2.0

chromatography on a Sephadex G-10 column (Pharmacia Fine Chemical, Uppsala, Sweden); 92 cm long × 2.5 cm i.d.) that was swollen and packed according to the supplier's instructions. The eluent was 3% acetic acid, the flow rate was 2.5 mL/min, and the absorbance at 280 nm was measured with a model 2138 Uvicord S, LKB detector and a model 2210 LKB recorder. The eluate was collected in 2.5-mL fractions (model 2112 Redirac LKB fraction collector). The void volume was calculated with dextran blue. The exclusion limit of Sephadex G-10 is, according to the supplier's information, ~700 Da. Free and total amino nitrogen were determined in the eluted fractions by the method (method C) described by Doi et al. (1981), which is based on the reaction of amino groups in an acid medium with an ethanolic solution of ninhydrin with $CdCl_2$ and measurement of absorbance at 507 nm. Phenolic compounds were determined by the method described by Singleton and Rossi (1965).

Separation of Peptides by HPLC. A liquid chromatograph consisting of two Beckman M116 pumps, a Beckman System Organizer, a Waters M 717Plus automatic injector, and a Beckman M168 diode array detector were used. Equipment control, obtention, and processing of data were carried out with the Beckman Gold System program. All separations were performed on a 150 × 3.9-mm i.d. Waters Nova-Pak C18, 60 Å, 4-μm column. Eluent A was 0.1% trifluoroacetic acid in water, and eluent B was 0.1% trifluoroacetic acid in acetonitrile. The gradient of B increased from 0 to 40% over 70 min. The flow rate was 1 mL/min.

Twenty microliters of the fractions eluted from the Sephadex G-10 column, previously filtered through a 0.45 μm membrane filter, were injected onto the HPLC column.

RESULTS

Amino, Peptide, and Protein Nitrogen. The values found for amino, peptide, and protein nitrogen in the musts and wines studied are shown in Table 1. The must that contains the highest nitrogenous content was the Chardonnay. During the transformation from must to wine, there was a decrease in amino and peptide nitrogen to 58 and 29%, respectively. The peptide nitrogen content of these wines, 33.5 mg/L mean value, is close to the 41.2 mg/L found by Poux and Ournac (1970). Protein nitrogen content decreases by over >80% on average during vinification. This decrease is due in part to the insolubilization of protein nitrogen during fermentation and in part to the treatments to which the wine has been submitted.

During secondary fermentation, amino nitrogen content decreased again, so that after secondary fermentation and aging with yeast for 3 months, the wines had a lower free amino acid content than the base wines. Between 3 and 9 months after tirage, no differences were evident in the concentration of free amino acids in any of the wines (data not shown). The increase in amino nitrogen content from 9 months onward in the Xarel-lo and Parellada variety wines and from 12 months onward in the Macabeo and Chardonnay varieties indicates the beginning of autolysis. In addition, from 9 months onward there was an increase in the concentration of protein nitrogen. The observed fluctuations in the values of peptide nitrogen content during aging are probably due to an increase caused by autolysis of the yeast and its subsequent degradation to free amino acids.

Analysis of Peptides. The complexity of the wine made it necessary to perform a fractionation prior to chromatography study. To this end, the higher molecular mass compounds, mainly proteins, polysaccharides, and polymerized phenolic compounds, were precipitated with ethanol in an acid medium as described under Materials and Methods. Because the ethanol-soluble fraction contained free amino acids and nonpolymerized phenolic compounds, a refractionation proved necessary for the analysis of peptides. In a previous studied carried out on the peptides in wine after their fractionation by Sephadex LH 20 (exclusion volume 5000), we observed that peptides were mainly of low molecular weight (Acedo et al., 1994); thus, the sample was fractionated on Sephadex G-10 (exclusion limit 700) in the present study. The chromatographic profile at 280 nm for a sparkling wine after 15 months of aging with yeast and the content of free and total amino nitrogen and phenolic compounds (such as gallic acid) of the eluate are shown in Figure 1. The determination of amino nitrogen before and after hydrolysis revealed that fractions 1 and 2 contained peptides but did not contain free amino acids, whereas fraction 3 contained some peptides but mainly free amino acids. In the fractions eluted between 150 and 300 mL, there are free amino acids. Fractions between 150 and 400 mL and between 730 and 850 mL contain phenolic compounds. Fraction 1 corresponded to the exclusion volume of the column, and according to the manufacturer's information, compounds with a molecular mass of >700 elute in this volume. Fraction 2 contained the peptides with molecular mass of <700. Both fractions were studied by HPLC. The data of the sum of the HPLC peak areas of the peptides of fraction 1 and 2, total area and ratio of area of hydrophobic to the area of hydrophilic peptides are shown in Table 2. The relative standard deviations obtained from triplicate analysis of a wine were 15% for the area of peptides of fraction 1, 7% for the area of peptides of fraction 2, 10% for the total area, and 2% for the ratio of area of hydrophobic peptides to the area of hydrophilic peptides.

The chromatographic profiles obtained at 214 nm from the HPLC analysis of fraction 1 of the Parellada

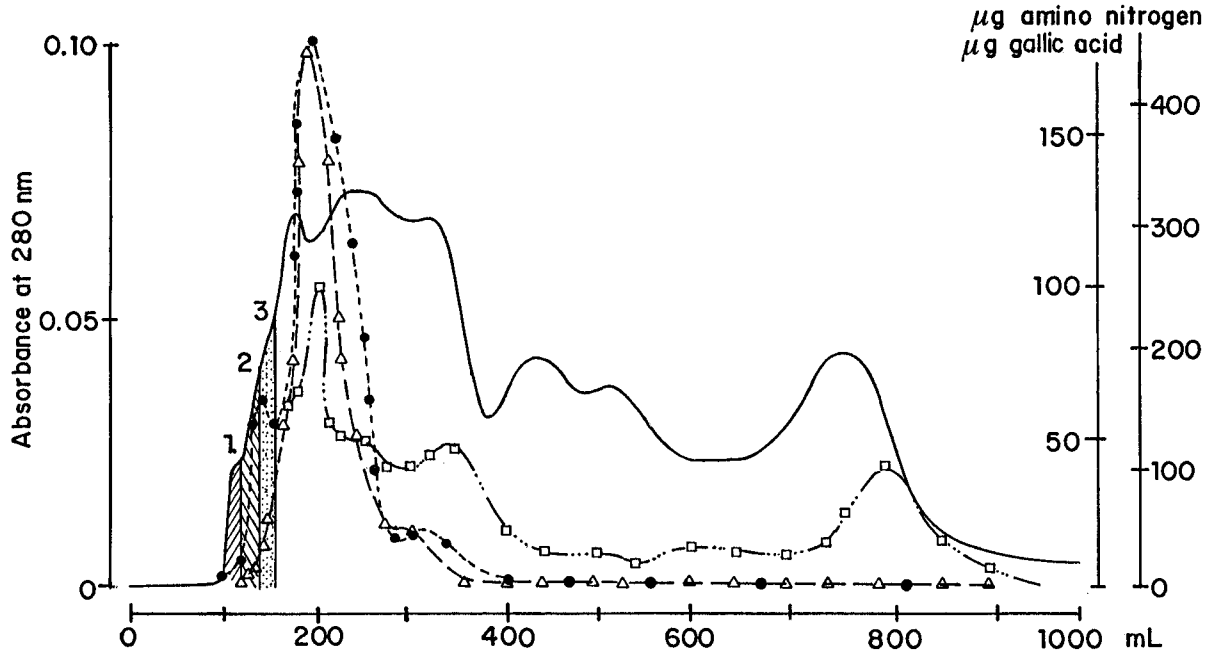

Figure 1. Elution profile of the sparkling wine from the Parellada variety, after 15 months of aging on yeast lees, on the Sephadex G-10 column (92 cm long × 2.5 cm i.d.), with 3% acetic acid as eluent at flow rate of 2.5 mL/min: (—) absorbance at 280 nm; (□···□) phenolic compounds; (●- -●) total amino nitrogen; (△- -△) free amino nitrogen; (1), (2), and (3) fractions 1, 2, and 3, respectively.

Table 2. Sum of the Areas of Peptides Eluted by HPLC of Fractions 1 and 2 from Sephadex G-10 Column, and Ratio of the Area of Hydrophobic to the Area of Hydrophilic Peptides in Fractions 1 and 2

	sum of the areas			
	fraction 1	fraction 2	total	ratio hydrophobic/ hydrophilic peptides
Macabeo Variety				
must	3.84	0	3.84	—
base wine	149.99	29.99	179.98	6.18
sparkling wines				
9 months	78.05	82.82	160.87	1.17
12 months	105.85	104.24	210.09	0.71
15 months	26.23	148.17	174.40	0.76
18 months	62.09	15.00	77.09	5.87
Xarel-lo Variety				
must	13.16	0	13.16	—
base wine	70.38	110.02	180.40	1.70
sparkling wines				
9 months	30.93	21.68	52.61	1.84
12 months	54.33	70.57	124.90	1.54
15 months	24.30	163.62	187.92	0.46
18 months	57.58	19.69	72.27	7.58
Parellada Variety				
must	5.60	0.09	5.69	—
base wine	109.75	60.85	170.60	1.42
sparkling wines				
9 months	47.17	41.85	89.02	1.67
12 months	48.34	176.33	224.67	2.74
15 months	101.55	208.37	309.92	0.86
18 months	155.77	50.31	206.04	3.34
Chardonnay Variety				
must	8.07	0	8.07	—
base wine	67.76	91.77	159.53	0.92
sparkling wines				
9 months	38.66	82.34	121.00	0.75
12 months	25.14	130.41	155.55	0.56
15 months	93.24	138.57	231.81	0.46
18 months	71.00	67.80	138.80	0.90

variety must and wines are shown in Figure 2. The chromatograms obtained for the musts and wines of the Macabeo, Xarel-lo, and Chardonnay varieties are similar. During the first minutes, compounds elute that are not chromatographically well resolved, together with the dead volume of the column. To obtain comparable data in all the chromatograms, peak area integration was carried out after 10 min (González de Llano et al., 1995). The chromatograms are complex, and peptides with a wide range of polarity were separated. The number and concentration of peptides in the musts were very low but increased during alcoholic fermentation, probably because release from the yeast (Poux and Ournac, 1970). The majority of peptides in the Macabeo, Xarel-lo, and Parellada base wines eluted between 20 and 40 min. Acedo et al. (1994), in a still white wine analyzed by HPLC under conditions similar to those of this study, found only peptides that had a low molecular weight and were highly polar.

The chromatograms obtained for peptides with a molecular weight of >700 from the sparkling wines at 9 months of aging with yeast were similar to those of the base wines, but the sum of the area of the peptides in these wines (Table 2) is lower than that of the base wines. In the following months, an increase in the content of these peptides was observed, which in some cases (Parellada wines at 18 months and Chardonnay at 15 and 18 months) reached higher concentrations than in the base wines from which they originated.

The chromatographic profiles of the HPLC analysis of the compounds eluted in fraction 2 are shown in Figure 3. The data of the sums of the HPLC peak areas of the peptides of fraction 2 eluted from 10 to 50 min are shown in Table 2. As in the case of the peptides from fraction 1, the number of peaks and their intensity in the chromatograms of the musts are also low. During fermentation, the content of peptides with a low molecular weight increased, so that their number and concentration were much higher in the base wine than in the must. During secondary fermentation of the wine, there was a decrease in peptide content in the majority of samples analyzed. Between 9 and 15 months of aging with yeast another increase in the number and concentration of peptides separated by HPLC was observed.

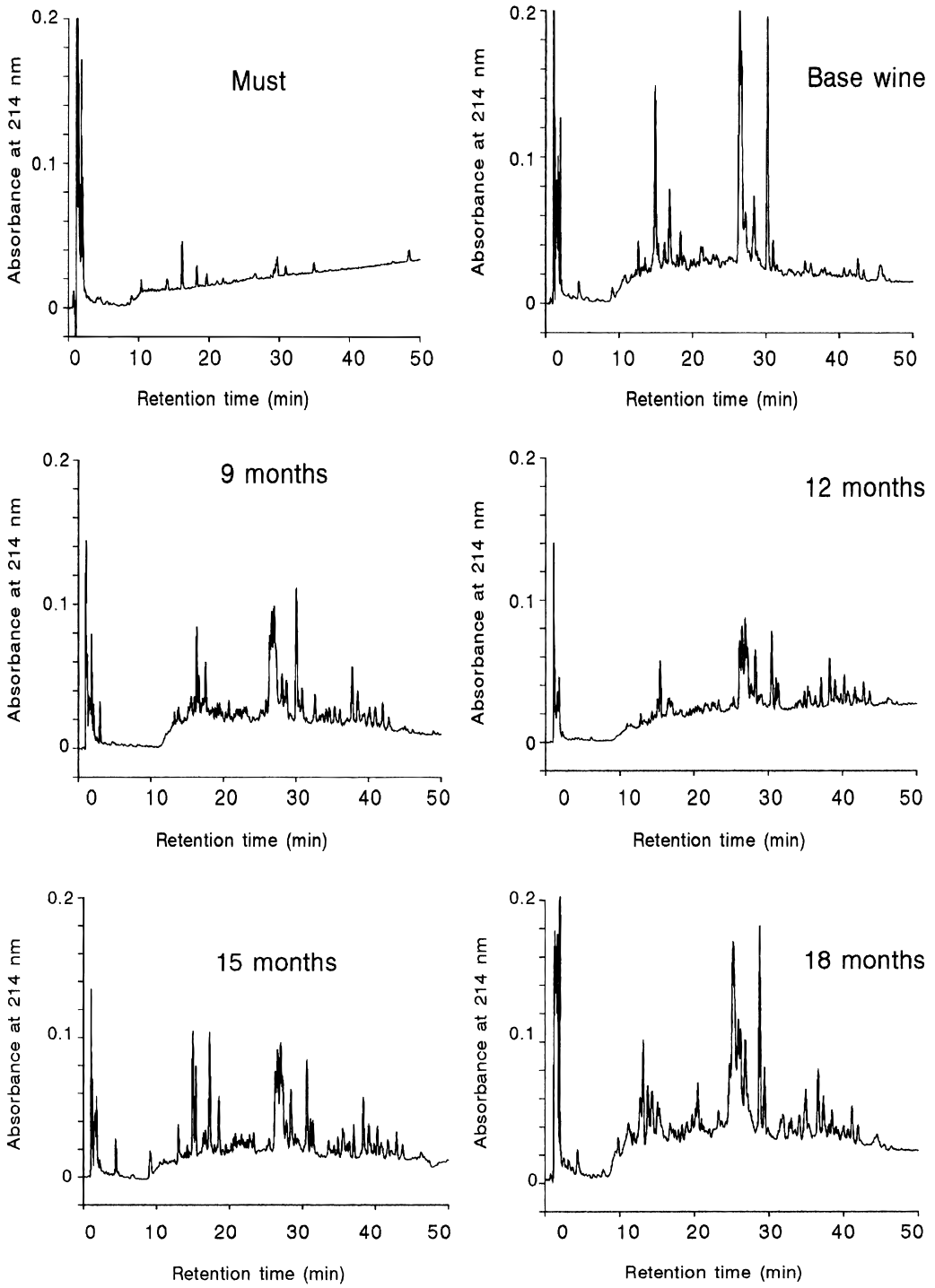

Figure 2. HPLC profiles of fraction 1 from the Sephadex G-10 column (Figure 1) of must and wines from Parellada variety.

However, peptide number and concentration decreased once more between 15 and 18 months in all samples, probably because of their degradation into smaller peptides or free amino acids. The total nitrogen content remained constant during aging.

Hydrophobic and Hydrophilic Peptides in Wines. Reversed-phase HPLC is a useful technique for separating hydrophobic and hydrophilic peptides. One criterion to evaluate relative hydrophobicity used in dairy products (Lau et al., 1991; McSweeney et al., 1993; González de Llano et al., 1995) is that of the ratio of the areas of the peptides that elute with retention times higher than that of tryptophan (hydrophobic peptides) and those that elute between the retention time of tyrosine and that of tryptophan (hydrophilic peptides). This is the criterion used in this study, and the results are shown in Table 2. The retention time of tyrosine under the chromatographic conditions used in this study is 10 min and that of tryptophan is 20 min. The ratio of hydrophobic to hydrophilic peptides in sparkling wines after 9, 12, and 15 months of aging with yeast ranged from 0.46 to 2.74. Between 15 and 18 months, an increase of the ratio of the area of hydrophobic to the area of hydrophilic peptides was observed in all wines. The hydrophobicity of peptides may be related to the quality of wine foam. Therefore, if these results were confirmed in other samples, the increase of hydrophobicity may be one of the causes that could explain why the quality of the foam in sparkling wine improves with aging time.

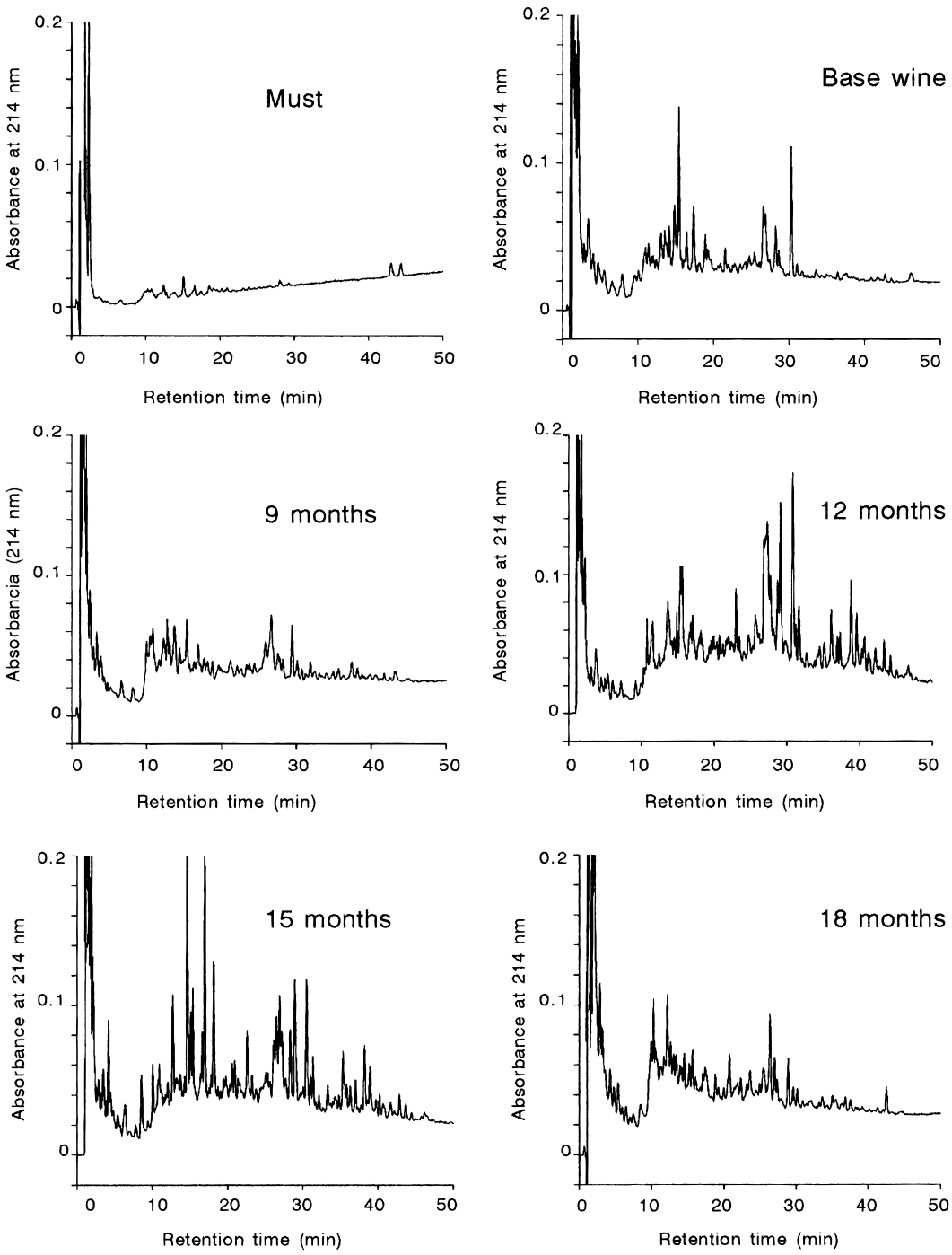

Figure 3. HPLC profiles of fraction 2 from the Sephadex G-10 column (Figure 1) of must and wines from Parellada variety.

DISCUSSION

Although each wine of those studied in this work originates from musts of a different grape variety and each sparkling wine was fermented and aged with yeast in individual bottles, there is a great similarity in the chromatographic profiles of all the wines.

The sum of the areas of the peptides separated by HPLC in fractions 1 and 2 (Table 2) in the base wines is higher in all cases than that in the musts. According to the data of Table 1, the peptide content of the musts is higher than that of the base wine; therefore, it may be assumed that it is oligopeptides that elute in the fraction 3 of the Sephadex G-10 column (Figure 1) together with the free amino acids. The fact that we have not detected peptides in fractions 1 and 2 in the musts may indicate that peptides of wines come from the yeast.

The sum of the areas of the peptides in the wines after secondary fermentation and 9 months of aging with yeast is lower, in all cases, than in their respective base wines (Table 2). Later, there is an increase in the concentration of peptides, which reaches its maximum after 12 or 15 months of aging with yeast, but then decreases once more. This decrease was also observed in the data on peptide nitrogen in Table 1. The reduction in the peak areas of the fraction 1 (molecular weight >700) corresponds in some cases (between 12 and 15 months in Macabeo and Xarel-lo and between 9 and 12 months in Chardonnay) to an increase in the fraction with a molecular weight of <700, which may

confirm that a successive degradation of peptides from larger to smaller sizes is occurring.

The results obtained throughout this study indicate that the release and degradation of the peptides in wine through the action of yeast enzymes occur simultaneously. To interpret this result it is necessary to assume that not only are peptides of different sizes released into the wine by the action of endocellular proteases, but that simultaneously there occurs a release of proteases into the wine that hydrolyze the peptides. The existence of exocellular proteases was also observed in still wines by Feuillat et al. (1980) and during yeast autolysis by Miguel-Gordillo et al. (1990) and Hernawan et al. (1995).

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