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Fractionation and partial characterization of protein fractions present at different stages of the production of sparkling wines

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Soluble proteins from sparkling wines (var. Chardonnay) manufactured industrially following the Champenoise method were analysed by analytical anion-exchange FPLC (fast protein liquid chromatography). Samples of the must, the unfined wine, the fined wine and the sparkling wines after 3, 6, 9, 12, 15 and 18 months of ageing with yeast lees were taken. Eight main must protein fractions were collected and characterized by isoelectric focusing and capillary electrophoresis. Six fractions consisted of proteins with isoelectric points (pIs) from 4.27 to 3.04. Two fractions, according to their UV spectra, seemed to contain phenolic compounds. A comparison of FPLC profiles of the must, the unfined wine and the fined wine indicated that both fermentation and stabilization processes decrease the concentration of those proteins with less affinity for the anion-exchange column and with a higher pI. No changes in the protein profiles of sparkling wines were observed during the first 18 months of ageing with yeast.

INTRODUCTION

Proteins are minor constituents of wine, but they certainly contribute to wine quality. Proteins are partly responsible for the sensation of 'body' in wines. They can also bind volatile compounds so that the aroma of the wine is retained (Lubbers *et al.*, 1994), and they have a positive effect on foam stability in sparkling wines (Brissonnet and Maujean, 1991, 1993; Malvy *et al.*, 1994; Pueyo *et al.*, 1995; Andrés-Lacueva *et al.*, 1996). However, proteins can cause turbidity in finished wines, which damages the commercial image of the product and, therefore, lessen its preference by consumers.

Proteins with molecular weight between 20 and 30 kDa and an isoelectric point (pI) higher than the pH of the wine are the most important fractions contributing to wine instability (Hsu and Heatherbell, 1987b; Paetzold *et al.*, 1990; Waters *et al.*, 1992; Ledoux *et al.*, 1992). Most of the studies about the characterization of wine proteins are based on analysis of the whole protein fraction by native electrophoresis, sodium or lithium dodecyl sulfate–polyacrylamide gel electrophoresis (LDS or SDS–PAGE) and isoelectric focusing (IEF) (Hsu and Heatherbell, 1987a; Pueyo *et al.*, 1993), and on analysis of protein fractions previously isolated by other techniques (Dawes *et al.*, 1994; Waters *et al.*, 1995; Canals *et al.*, 1996; Marchal *et al.*, 1996). Chromatographic profiles obtained by cation-exchange chromatography have also been used for protein characterization purposes (Dorrestein *et al.*, 1995; Canals *et al.*, 1996). However, since wine proteins are mainly acidic (Görg *et al.*, 1982; Pueyo *et al.*, 1993; Santoro, 1995; Marchal *et al.*, 1996) and the differences among them are based more on their pI rather than on their molecular weight, anion-exchange chromatography is likely to be a more suitable technique for wine protein studies. To our knowledge, there are only a few references in the literature about the application of this technique to wine proteins. Lagace and Bisson (1990) studied the proteolytic activity of several yeasts on white wine proteins by using anion-exchange chromatography. Waters *et al.* (1995) successfully fractionated soluble wine (var. Muscat gordo Blanco) proteins by anion-exchange chromatography and the corresponding fractions were characterized by SDS–PAGE and were subjected to amino acid and sugar analysis. Anion-exchange chromatography has also been used to compare the protein profiles of four Portuguese white wines (Dorrestein *et al.*, 1995).

In this paper, anion-exchange FPLC (fast protein liquid chromatography) has been used to monitor the

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changes that occur in proteins during industrial manufacture of sparkling wines. Proteins from the must (var. Chardonnay), the unfined wine, the fined wine, and the sparkling wines (Champenoise method) with different ageing times, have been fractionated by analytical anion-exchange FPLC. In addition, the main must protein fractions have been collected and characterized by other analytical techniques, such as IEF and novel capillary electrophoresis (CE).

MATERIALS AND METHODS

Manufacture of must and wines

White grapes (var. Chardonnay) were processed industrially into must and wine. Must was sulfited (80 mg SO_2 litre^{-1}) and inoculated with a selected winery yeast (*Saccharomyces cerevisiae*). The wine was fermented to dryness in a 100 000 litre tank at 16–18°C, fined with 20 g bentonite and 1 g gelatine hl^{-1} and tartrate-stabilized. Sparkling wines were obtained using the Champenoise method. Sucrose (21 g litre^{-1}) was added to the base wine (fined wine), and then inoculated with a yeast (*Saccharomyces bayanus*) culture from the winery's collection. Degorging was performed after 3, 6, 9, 12, 15 and 18 months of ageing with yeast. Since secondary fermentation of sparkling wine takes place in individual bottles, at least six bottles of every degorging time were mixed and homogenized before sampling. All the analyses were conducted on must and wines after being centrifuged at 5000***g*** for 15 min.

Determination of total soluble protein

The Bradford (1976) method was used to determine the total soluble protein.

Isolation of soluble proteins

Must and wines (200 ml) were dialysed against running water in 3500 D Cellu Sep T1 membranes (Membrane Filtration Products Inc., San Antonio, TX, USA) for 48 h. The retentates were lyophilized and the lyophilizates were stored at −20°C until analysed.

Anion-exchange chromatography

A FPLC system (Pharmacia-LKB Biotechnology AB, Uppsala, Sweden) consisting of two P-500 pumps, a LCC-501 Plus controller and a UV detector with a 280 nm filter was used. The FPLC instrument was controlled by the FPLC Director ver. 1.03 software (Pharmacia-LKB). The separation of must and wine proteins was achieved by using an anion-exchange Mono Q® HR 515 column (Pharmacia-LKB). Eluent A was 20 mM Tris (hydroxymethyl)aminomethane-HCl buffer, pH 8.0, and eluent B was 1 M NaCl in eluent A. The eluents were filtered through Durapore (Millipore Corp., Bedford, MA, USA) 0.45 μm filters, before analysis. The flow rate was 0.5 ml min^{-1}. The gradient was as follows: 0–10 min, 5% B linear; 10–30 min, 10% B linear; 30–50 min, 30% B linear; 50–70 min, 45% B linear. After each run, the column was regenerated by washing with 100% B for 20 min, and equilibrated with 100% A for 20 min. Every tenth run, the column was inverted and subjected to a cleaning procedure that consisted of sequential injections of 2 M NaCl (500 μl), 2 M NaOH (500 μl) and 75% acetic acid (500 μl) using Milli-Q (Millipore) water as the eluent. Samples were dissolved in eluent A (2.5 mg lyophilizate ml^{-1}) and 500 μl was injected on to the column. Prior to analysis, samples were filtered through Fluoropore (Millipore Corp.) 0.22 μm filters. At least two replicate analyses were performed. For isolation of the main must proteins, fractions (0.5 ml) were collected by a fraction FRAC-100 (Pharmacia-LKB) collector. These fractions were gathered in eight pooled fractions: A, B, C, D, E, F, G and H (see Fig. 1). In order to obtain a sufficient amount of each protein fraction for IEF and CE analysis, five consecutive chromatographic separations were performed and corresponding fractions were pooled. They were concentrated and desalted by ultrafiltration with a Centricon-3 concentrator (Amicon, Beverly, MA, USA) until the volume was 400 μl.

Isoelectric focusing

PhastSystem™ (Pharmacia-LKB) electrophoresis equipment was used. Separation was achieved by using PhastGel dry IEF gels (T = 5% and C = 3%, Pharmacia-LKB), rehydrated for 30 min with an aqueous solution containing 2.1% (v/v) of each of three ampholytes with pH ranges of 2.5–5.0 (Pharmacia-LKB), 4.0–4.5 (Serva, Heidelberg, Germany) and 4.5–5.4 (Pharmacia-LKB). Samples (3 μl) of each concentrated and desalted fraction A–H, and of must proteins (2.5 mg lyophilizate ml^{-1} Milli-Q water) were directly placed on each hole of the Sample Applicator 6/4 (Pharmacia-LKB) with a

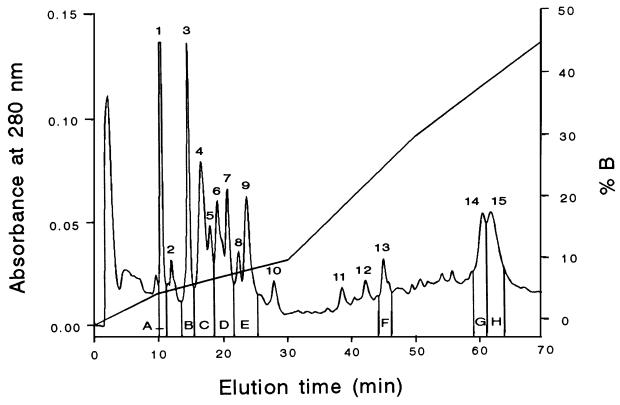

Fig. 1. Separation of soluble must (var. Chardonnay) proteins by anion-exchange fast protein liquid chromatography (FPLC).

micropipette. Running conditions were as described in EEC (1992). The IEF silver staining procedure was as described in the PhastGel® Silver Kit Instruction Manual (Cat. No. 17-0617-01, Pharmacia-LKB). A Pharmacia Fine Chemicals (Pharmacia-LKB) IEF calibration kit was used as a marker to determine the pIs of the proteins. Protein standards were: pepsinogen (pI 2.80), amyloglucosidase (pI 3.50), glucose oxidase (pI 4.15), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85) and human carbonic anhydrase B (pI 6.55).

CE

A P/ACE 2050 HPCE system (Beckman, Fullerton, CA, USA) equipped with a UV detector was used. Equipment control, data collection and processing were carried out by using the Beckman Gold System program version 8.10. Electrophoretic separation was carried out on a 57 cm long uncoated fused silica capillary using Tris–HCl buffer (pH 8.0). Running conditions were previously described by Luguera *et al.* (1997). Must protein fractions were analysed in duplicate.

Spectrophotometry

Because of the small amount of sample available, spectra of must protein fractions A–H were obtained by a Beckman M168 Photodiode Array detector coupled with a liquid chromatograph consisting of a Beckman M116 pump, a Beckman System Organizer, and a Waters M717Plus automatic injector (Waters Corp., Mildford, MA, USA). Equipment control, acquisition and processing of data were carried out by using the Beckman Gold System program (Beckman, San Ramon, CA, USA). The outlet from the injector was directly connected to the inlet of the detector. Milli-Q water (Millipore) was used as the mobile phase. The flow rate increased from 0.01 to 1 ml min^{-1} in 1 min and these conditions were maintained until peaks were detected. Detection was performed by scanning from 190 to 340 nm with a resolution of 1 nm. Five microlitres of each concentrated and desalted fraction A to H was injected.

RESULTS AND DISCUSSION

Characterization of must proteins

A FPLC chromatogram of must (var. Chardonnay) proteins is shown in Fig. 1. This chromatogram is similar to the one obtained by Waters *et al.* (1995) for the separation of wine (var. Muscat gordo Blanco) proteins on a preparative anion-exchange column. Some material eluted at the void volume of the column. Eight major (nos 1, 3, 4, 6, 7, 9, 14, 15) and seven minor (nos 2, 5, 8, 10, 11, 12, 13) peaks were resolved. The majority of the compounds eluted between 10 and 30 min, when the NaCl concentration of the eluent was relatively low. Therefore, according to the principles governing anion-exchange chromatography, these compounds exhibit few negative charges. Eluates from five consecutive separations of must were collected as described in Materials and Methods. Fractions were pooled into eight fractions (A, B, C, D, E, F, G and H; see Fig. 1) for further analysis by IEF and CE.

Preliminary IEF separations of the whole must protein fraction were carried out on commercial gels with an ampholyte pH range of 3.0–9.0. The results from these analyses indicated that the pI values of major must proteins ranged from 3.0 to 4.5. In order to improve protein resolution, later separations were performed on dry gels rehydrated with an ampholyte mixture of a final pH range of 2.5–5.4 (see Materials and Methods). Gels with this pH range are not commercially available. Figure 2 shows the IEF patterns of the whole must protein fraction and the must protein fractions A–H. Seven major protein bands with pIs from 3.04 to 4.27 and a minor band with a pI of 4.4, were found in the whole must protein fraction. The stains observed at about pI 4.55 (Fig. 2) corresponded to sample application points. Marchal *et al.* (1996) obtained similar results for a Chardonnay wine. These authors isolated several wine protein fractions with pIs from 2.53 to 4.27 by using preparative IEF. The major must protein bands were present in the fractions A–F isolated by FPLC. The minor protein band (pI 4.4) was not found in any of the fractions. Fraction A showed one band, whereas fractions D and F showed two bands, and fractions B, C and E showed three bands. No proteins were observed in fractions G and H. Table 1 illustrates estimated pI values for the protein bands observed in fractions A–F. Notice that the proteins from fractions A–F eluted through the anionic exchange column in order of their pI. The protein band with pI 4.27 was the only exception, which could be due to a

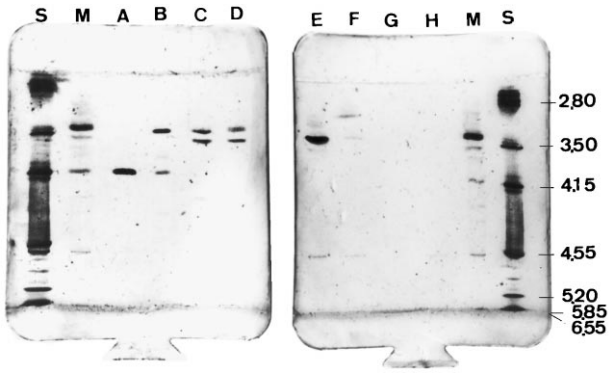

Fig. 2. Isoelectric focusing of the whole must (var. Chardonnay) protein fraction (M) and of must protein fractions obtained by anion-exchange fast protein liquid chromatography (FPLC; A, B, C, D, E, F, G and H; see Fig. 1). The position of the pI markers (S) are shown on the right side of the gel.

Table 1. Estimated isoelectric points of protein bands separated by isoelectric focusing (IEF) of the must protein fractions A–F (see Fig. 1) isolated by fast protein liquid chromatography (FPLC)

	Isoelectric point						
Fraction	4.27	4.00	3.78	3.50	3.31	3.18	3.04
A		+					
B		+	+		+		
C	+			+	+		
D				+	+		
E				+	+	+	
F					+		+

different protein tertiary structure with a high percentage of charged groups on its surface, or to a greater glycosylation. Many authors have demonstrated the glycosylate nature of wine proteins (Waters *et al.*, 1993; Marchal *et al.*, 1996; Yokotstuka and Singleton, 1997).

Figure 3 shows the capillary electrophoregrams of must protein fractions A–E. Fraction A exhibited only one peak, whereas two peaks were detected in fractions D and E, three peaks in fraction B, and four peaks in fraction C. No peaks were detected in fraction F, which might be due to the lower amount of protein contained in fraction F compared with the other fractions. Similar to IEF, no response was observed by CE for fractions G and H. Disagreement between the number of protein bands observed by IEF and the number of peaks detected by CE for fractions C, E, and F could be attributed to the different separation mechanisms of these two techniques.

UV spectra of fractions A–H were obtained by the photodiode array detector. Fractions A–F showed protein-type UV spectra (data not shown), with a main band at 195–200 nm due to the peptidic bonds of the molecule. A minor band was observed at 276–282 nm due to the phenyl groups of the aromatic amino acids of the proteins. The relative intensity of this latter band, regarding the peptidic band, was slightly different among fractions. UV spectra of fractions G and H consisted of a band at 205–225 nm and a wider one at 260–320 nm (Fig. 4). The former band was prevalent in the spectrum of fraction G, whereas the second band was relatively higher in the spectrum of fraction H. Phenolic compound spectra consist of two (at 219–235 and 266–358 nm) or three bands (an additional one at 236–298 nm when substituents are conjugated with the

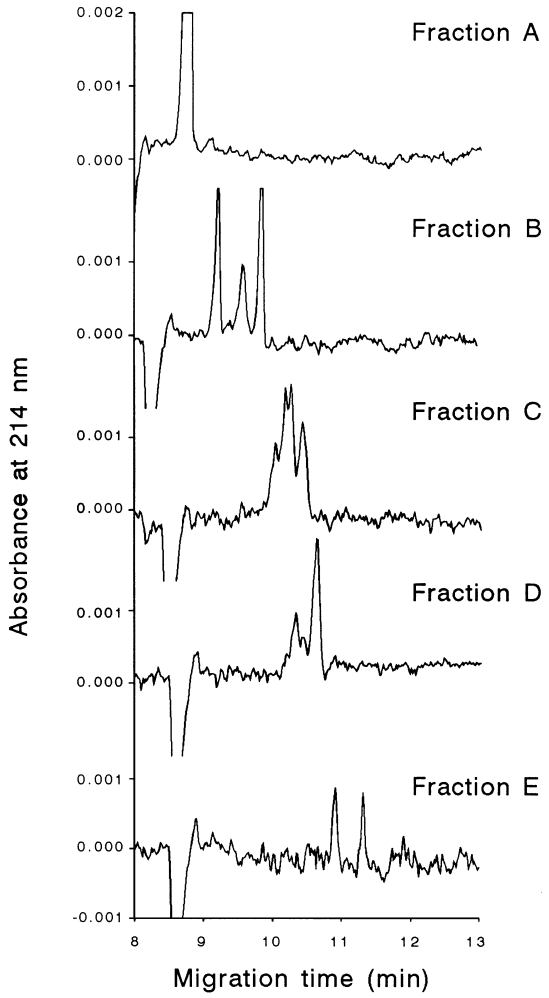

Fig. 3. Capillary electrophoregrams at 214 nm of must (var. Chardonnay) protein fractions A, B, C, D and E obtained by anion-exchange fast protein liquid chromatography (FPLC; see Fig. 1).

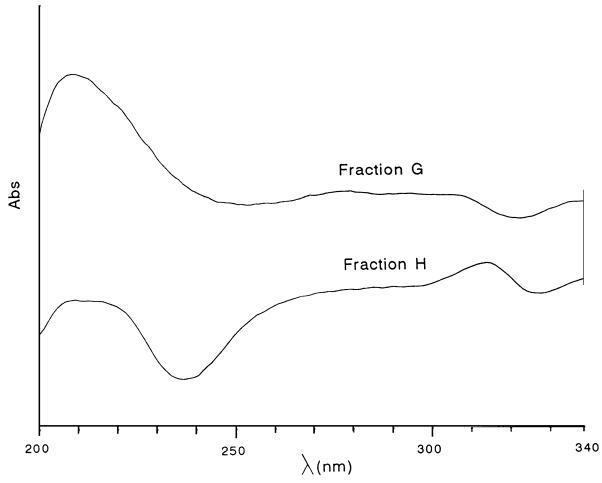

Fig. 4. UV spectra of must (var. Chardonnay) fractions G and H obtained by anion-exchange fast protein liquid chromatography (FPLC; see Fig. 1). The same absorbance scale was used for both spectra.

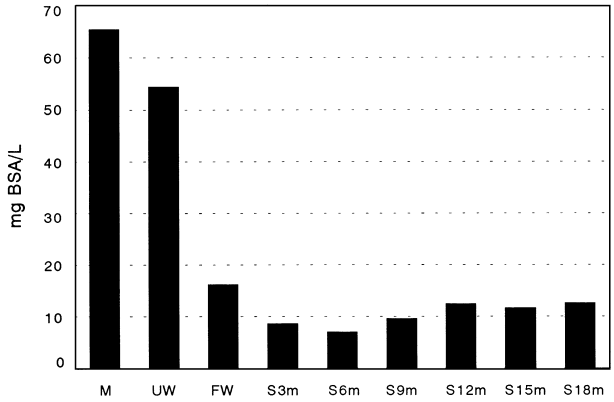

Fig. 5. Bradford data for must (M), unfined wine (UW), fined wine (FW), and sparkling wines at different ageing times: 3 (S3m), 6 (S6m), 9 (S9m), 12 (S12m), 15 (S15m) and 18 (S18m) months.

aromatic ring) (Bartolomé *et al.*, 1993). This suggest that phenolics could be present in fractions G and H and form complex phenolic polymers associated or not with proteins and peptides. Phenolics in fraction G would more likely have simple phenol-type structures, whereas phenolics in fraction H would have more cinnamic or flavonoid-type structures (Bartolomé *et al.*, 1993). Because of their hydroxyl groups, it was expected that phenolic polymers would elute late through an anion-exchange column at the pH used in this study, which agrees with the fact that fractions G and H were the final fractions obtained in the FPLC system.

In summary, fraction A appears to consist of a protein with pI 4.00; fraction B, three proteins with pIs 4.00, 3.78 and 3.31, respectively; fraction C, four proteins (two of them with the same pI) with pIs 4.27, 3.50 and 3.31; fraction D, two proteins with pIs 3.50 and 3.31, respectively; fraction E, three proteins with pIs 3.50, 3.31 and 3.18, respectively, and fraction F, two proteins with pIs 3.31 and 3.04, respectively. Fractions G and H seem to contain phenolic compounds.

Changes in the protein fraction during industrial manufacture of base and sparkling wines

Figure 5 depicts the Bradford data for all the samples studied in this paper. These values correspond to protein and polypeptide content, since it is known that Coomassie Blue (Bradford's reagent) reacts with both proteins and peptides with molecular weights higher than 3.0 kDa (Sedmak and Grossberg, 1977). The protein plus polypeptide concentration in must (var. Chardonnay) was 65.4 mg BSA (bovine serum albumin) litre^{-1} and decreased slightly with fermentation. After stabilization treatments (see Materials and Methods), only 25% of the initial protein and peptides remained in the wine. An initial decrease in protein and peptide content was observed during secondary fermentation, followed by an

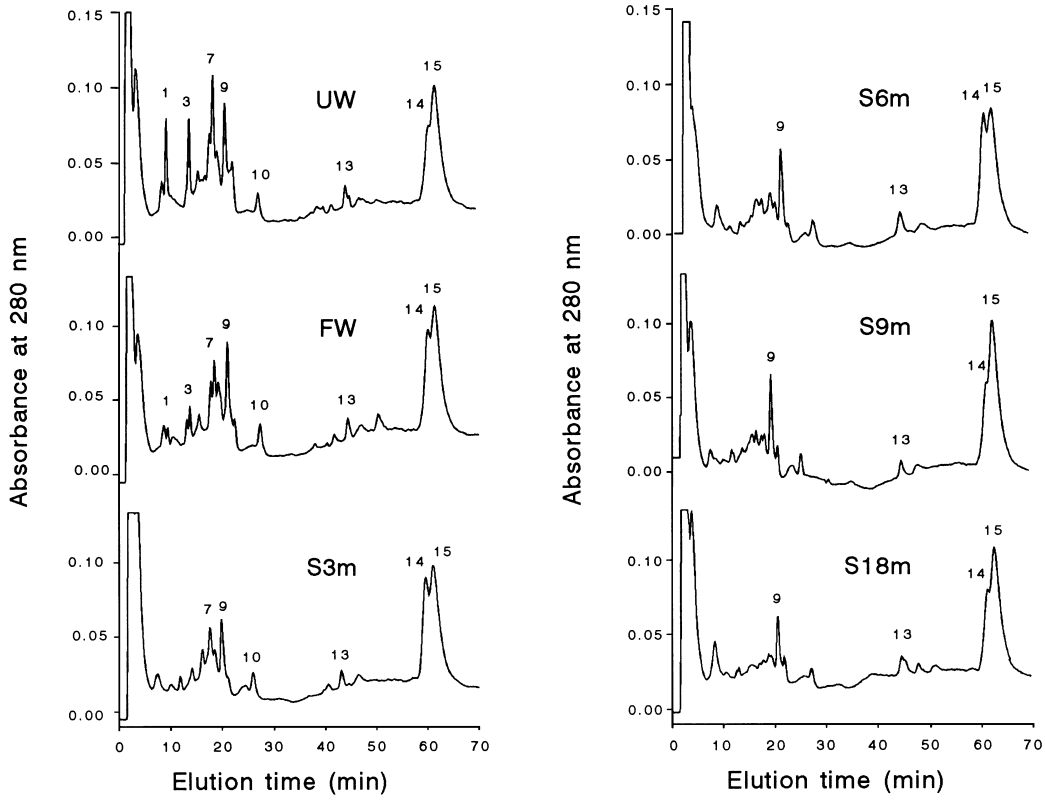

Fig. 6. Anion-exchange fast protein liquid chromatography (FPLC) profiles of proteins from unfined wine (UW), fined wine (FW), and sparkling wines at different ageing times: 3 (S3m), 6 (S6m), 9 (S9m) and 18 (S18m) months.

increase from 6 to 12 months, and a subsequent plateau until 18 months of ageing.

The chromatographic profiles of the FPLC analysis of proteins from unfined wine, fined wine and sparkling wines at different ageing times are shown in Fig. 6. Peaks that exhibited the same retention times as those from must (Fig. 1) were given the same number. Fewer and lower peaks were found in the chromatogram corresponding to unfined wine compared with the one corresponding to must (Fig. 1). This effect was greater for compounds with low retention times (peak nos 1, 2, 3 and 4) that, according to the results reported above, correspond to proteins with high pI. Only peak nos 14 and 15 were higher in the unfined wine than in the must samples. The latter agrees with our tentative identification of fractions G and H (peak nos 14 and 15, respectively) as phenolic polymers, since fermentation causes complexations among phenolic compounds and between them and various macromolecules, such as proteins and polysaccharides (Macheix *et al*., 1990). Trace peak nos 1 and 3, that correspond to less acid proteins, were found in FPLC chromatograms of the fined wine (Fig. 6). However, slight changes were found in peaks corresponding to more acid proteins. Since, after fining treatments, the wine became stable (as checked in the winery), it can be inferred that the first eluting proteins, the less acidic ones, could be responsible for turbidity, which is in agreement with previous studies where other techniques or technique combinations for wine protein analysis have been used (Hsu and Heatherbell, 1987b; Murphey *et al*., 1989; Dawes *et al*., 1994). Figure 6 also shows the FPLC chromatograms corresponding to sparkling wines from 3 to 18 months' ageing (the ones corresponding to 12 and 15 months are not included). The FPLC chromatogram corresponding to 3 month-aged sparkling wine showed lower peaks than the one corresponding to fined wine. This agrees with the Bradford data reported above that indicated a decrease in the content of protein and peptides during the first months of ageing with yeast. Chromatograms corresponding to sparkling wines with a longer ageing time (from 6 to 18 months) were very similar, which indicated there were no significant changes in the wine proteins during this time. Therefore, the increase in the Bradford data (Fig. 5) from 6 to 12 months might be due to polypeptide release by yeast. This is in agreement with previous studies that have shown an increase in the amount of wine peptides during sparkling wine manufacture (Moreno-Arribas *et al*., 1996).

CONCLUSIONS

These results confirm that analytical anion-exchange FPLC is a very suitable technique for the study of must and wine proteins. FPLC chromatographic profiles of must and wine proteins, obtained under the conditions reported in this paper, were well resolved and can be used to evaluate changes that occur in the protein fraction during wine manufacture. The main fractions separated by anion-exchange FPLC can be easily isolated and characterized by other techniques, such as IEF and CE, which give complementary information about must protein properties. During industrial wine manufacture, both fermentation and stabilization processes decrease the concentration of those wine proteins with less affinity for the anion-exchange column and with higher pI, which agrees with previous studies where other techniques or technique combinations for wine protein analysis have been used. Protein release by yeast did not seem to occur during wine ageing under industrial conditions in the first 18 months.

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