Capillary Electrophoretic Analysis of Wine Proteins. Modifications during the Manufacture of Sparkling Wines

Consuelo Luguera, Victoria Moreno-Arribas, Encarnación Pueyo, and M. Carmen Polo*

Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

This paper reports a new electrophoretic method for the analysis of wine proteins. The method used for protein isolation and concentration was dialysis followed by lyophilization. Electrophoretic separation was carried out on an uncoated fused silica capillary of 57 cm total length, 50 cm effective length, and 75 μm i.d., using 100 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8.0, at 12 kV. Detection was at 214 nm. The protein fractions of sparkling wines after 3, 6, 9, 12, 15, 18, and 24 months of aging on yeast lees and of the must and the base wine from which they originated were studied. Eight peaks were separated in the must. During the first fermentation, both the intensity and the number of peaks were lower. In the first months of aging with yeast another reduction in the intensity of the peaks was observed, although new peaks appeared, probably corresponding to polypeptide forms released by the yeast. These results were compared with those achieved by native-PAGE.

Keywords: *Capillary electrophoresis; proteins; must; wine; cava; sparkling wine*

INTRODUCTION

Although the concentration of proteins in wine is very low, they can cause a number of technological problems. During vinification, the colloidal nature of proteins makes filtration, clarification, and tartrate stabilization difficult. In addition, proteins may be responsible for the appearance of turbidity in bottled wine. On the other hand, proteins help to retain flavor compounds, as has been demonstrated by Lubbers et al. (1994) in a model wine, to increase the sensation of body in wines, and, thanks to their tensioactive properties, to enhance foam characteristics in sparkling wines.

Studies carried out mainly on beer (Bamforth, 1985; Hii and Herwig, 1982) and base wines used for making sparkling wines (Brissonnet and Maujean, 1991, 1993; Malvy et al., 1994; Andrés-Lacueva et al., 1996) have revealed the existence of a relationship between protein concentration and foam quality. Pueyo et al. (1995) have found a positive relationship between the quantity of protein in still wines, measured by using the Bradford method, and foam height and a negative relationship between the quantity of proteins in "cavas" and foam permanence once formed. These results encouraged us to investigate more closely the characteristics of proteins in wines used for making sparkling wines and the changes occurring during secondary fermentation and aging with yeast.

Most of the studies on wine proteins have been carried out using the conventional electrophoretic methods of native− and SDS−PAGE and isoelectric focusing (Hsu and Heatherbell, 1987a,b; Hsu et al., 1987; González-Lara et al., 1989; Moio and Addeo, 1989; Waters et al., 1991; Brissonnet and Maujean, 1993; Pueyo et al., 1993). High-performance liquid chromatographic techniques in different modes (reversed-phase, ion exchange, size exclusion, etc.) have also been used for the study of wine proteins (Dubordieu et al., 1986; Polo et al., 1989; Waters et al., 1993; Santoro, 1995).

* Author to whom correspondence should be addressed (telephone 34-1-5613481; fax 34-1-5644853; e-mail mcpolo@fresno.csic.es).

Capillary electrophoresis (CE) is an emerging technique widely used in the analysis of food proteins (Lindeberg, 1996; Recio et al., 1997). The advantages of this technique, as compared to conventional electrophoresis, are that it is more efficient, it obtains better resolutions, it is a fully automatic method, and it enables quantitative analysis of proteins. However, to our knowledge, in the literature there are only two studies, carried out in the same laboratory (Ledoux et al., 1992; Ledoux and Dubourdieu, 1994), that demonstrate the possibility of separating wine proteins by CE. The aim of this study was to develop a method of wine protein analysis by CE, to describe it in detail so that it may be used in any laboratory, and to apply it to the study of the changes occurring during the production of sparkling wines made by the Champenoise method. The influence of separation buffer pH, use of additives, and voltage on the resolution was studied. The chosen method was applied to the study of proteins during the manufacture of a Spanish sparkling wine made according to the Champenoise method (cava) with up to 24 months of aging with yeast. The results obtained were compared with those obtained by native−PAGE.

MATERIALS AND METHODS

Samples. Must, base wine, and sparkling wines were manufactured industrially from white grapes of the Chardonnay variety. The must was sulfited (80 mg of SO_2/L) and fermented in 100 000 L tanks at 16−18 °C, by inoculation of a selected winery yeast (*Saccharomyces cerevisiae*). The resulting wine was clarified with 20 g of bentonite/hL and 1 g of gelatin/hL and tartrate stabilized (−4 °C for 48 h). The sparkling wines were obtained using the Champenoise method, adding 21 g of sucrose/L and 3 g of bentonite/hL to the clarified wine (the base wine) and inoculating it with a yeast culture from the winery's collection (*Saccharomyces bayanus*). Degorging was performed after 3, 6, 9, 12, 15, 18, and 24 months of aging with yeast. Since secondary fermentation of these wines takes place in individual bottles, at least six bottles of every batch were mixed and homogenized before sampling. The must was frozen and the wines were refrigerated until analysis. All of the analyses were conducted in duplicate on the must and wines after being centrifuged for 15 min at 5000*g*.

S0021-8561(97)00141-6 CCC: $14.00 © 1997 American Chemical Society

Table 1. Separation Buffers, Additives, and Voltage Conditions Used in the Different Assays

assay	separation buffer	pH	voltage (kV)
1	100 mM Trizma base-HCl	8	6
2	100 mM Trizma base-HCl	8	8
3	100 mM Trizma base-HCl	8	12
4	100 mM Trizma base-HCl + 30 mM NaCl	8	12
5	100 mM Trizma base-HCl + 30 mM disodium hydrogen phosphate dihydrate	8	12
6	100 mM phosphoric acid	2	6
7	100 mM phosphoric acid	2	8
8	100 mM phosphoric acid	2	12

Preparation of the Sample for Electrophoretic Study. The must and wines were dialyzed against running water on 3500 D Cellu Sep T1 membranes (Membrane Filtration Products, Inc., San Antonio, TX) for 48 h. The retentates were lyophilized, and the lyophilysates were kept at a temperature of −20 °C until analysis. For native−PAGE analysis, 2.5 mg of each lyophilysate was dissolved with 1 mL of pH 8.3 buffer [0.6 g of tris(hydroxymethyl)aminomethane (Tris) (Sigma, St. Louis, MO) and 2.9 g of glycine (Sigma) per liter of water]. For CE analysis, 2.5 mg of each lyophilysate was dissolved in 1 mL of Milli-Q water (Millipore Corp., Bedford, MA). Prior to analysis, samples were filtered through 0.22 μm Fluoropore (Millipore) filters.

Polyacrylamide Gel Electrophoresis (PAGE). In accordance with Hillier (1976), this was carried out on polyacrylamide (80 × 80 × 0.75 mm) containing 9.0 g of acrylamide and 400 mg of *N,N*-methylenebis(acrylamide) in 100 mL of buffer, pH 8.9. The separation buffer used was the pH 8.3 buffer system described previously under Preparation of the Sample for Electrophoretic Study. Electrophoresis was performed at a constant current setting of 12 mA per gel. The gel was stained with Coomassie Brilliant Blue R-250 (Winter et al., 1977).

Capillary Electrophoresis. *Separation Buffers.* Two buffer systems, with different pH values, were used: (A) 100 mM Tris-HCl, pH 8.0; (B) 100 mM H_3PO_4/NaOH, pH 2.0. In the study of the influence of ionic strength on the separation, respective solutions of 30 mM NaCl and $Na_2HPO_4 \cdot 2H_2O$ were added to buffer A. $Na_2HPO_4 \cdot 2H_2O$ was purchased from Merck (Darmstadt, Germany). HCl, H_3PO_4, and NaCl were obtained from Panreac (Barcelona, Spain), and Tris from Sigma. Buffers were filtered using a 0.45 μm Millipore filter.

CE Conditions. CE was performed with a P/ACE 2050 HPCE system equipped with a UV detector (Beckman, Fullerton, CA). Equipment control, data collection, and processing were carried out using the Beckman Gold System program version 8.10. An uncoated fused silica capillary column of 57 cm total length, 50 cm effective length, and 75 μm i.d. (Polymicro Technologies Inc., Phoenix, AZ) was assembled in the P/ACE cartridge with a 100 × 200 μm aperture. Temperature was maintained at 25 °C using a cooling system. The injection was carried out at the anode using N_2 pressure (0.5 psi) for 5 s. On-line detection by UV absorbance was performed at 214 nm. The separations were conducted at 6, 8, and 12 kV. The combinations of separation buffers, additives, and voltage conditions assayed are listed in Table 1. The capillary was reconditioned after each injection by rinsing according to the following routine: 5 min washing with water followed by 5 min washing with 0.1 N NaOH, flushed again with water for 5 min and then separation buffer for 10 min. The separation buffer was changed every two injections.

RESULTS AND DISCUSSION

Development of the CE Method. The complexity of musts and wines and their low protein concentration (2.5−67.5 mg of BSA/L; Moreno-Arribas et al., 1996) make it necessary to use techniques to isolate and concentrate the protein fraction before its analysis. The isolation technique used was dialysis and the concentration technique, lyophilization. In CE, it is also possible to achieve sample concentration inside the capillary, by using sample separation and dissolution buffers of different conductivities (stacking effect). In our case, this effect was achieved by using water as sample solvent.

In protein analysis by CE there are mainly three variables to be considered in the optimization of separation: buffer pH, ionic strength, and voltage. These three variables were taken into consideration when this method was optimized for separating must and wine proteins. To avoid possible interactions between proteins and the inside wall of the capillary, which can lead to loss of efficiency, separation can be carried out at a pH close to the zero charge point of the fused silica or at a pH higher than the p*I* of the proteins in the sample. In this study, separation buffers with pH 2, close to the zero charge point of the fused silica, and pH 8, higher than the p*I* values of the major must and wine proteins, which range from 3.0 to 5.6 (González-Lara et al., 1989; Pueyo et al., 1993; Santoro, 1995), were used. The use of a high ionic strength buffer reduces the adsorption of proteins on the capillary wall, thus improving their separation. In this sense, alkali metal salts are one of the additives often used (Green and Jorgerson, 1989; Bullock et al., 1995). In this work, two different buffer additives were tested, NaCl and Na_2HPO_4. On the other hand, the use of high potential differences leads to greater efficiency but also has the disadvantage of generating high levels of heat produced by the Joule effect. To discover the effect of these factors on the separation of proteins in this type of sample, the voltages applied were 6, 8, and 12 kV. Table 1 shows the electrophoretic conditions used in each of the assays. In these methods optimization assays the sample used was the Chardonnay must.

Figure 1 shows the electrophoretic profiles of the proteins in the Chardonnay must in assays 1−5 and 8 from Table 1. Under the conditions of assay 1, Tris-HCl buffer, pH 8, and 6 kV (Figure 1a), eight major peaks were separated in a reasonable period of time, <25 min. The use of higher voltages, 8 and 12 kV, parts b and c, respectively, of Figure 1, does not substantially decrease the resolution, while separation is obtained in a shorter time, 17 min in the assay carried out at 8 kV and 13 min in the assay carried out at 12 kV. Parts d and e of Figure 1 show the electrophoretic profiles obtained at 12 kV using additives in the separation buffer (assays 5 and 6 in Table 1). It can be observed that no advantage is gained by the use of these additives.

Ledoux et al. (1992) and Ledoux and Dubourdieu (1994) used 20 mM citric acid, pH 2.5, for the electrophoretic separation of wine proteins. Due to the lack of buffer effect of this solution and because of the fact that its pH (2.5) is very close to the p*I* of wine proteins, it was considered preferable to use as separation buffer a solution of 20 mM phosphoric acid, pH 2.0, in the assays carried out at acidic pH (assays 6−8). At this pH, analysis times were much greater than that when the separation was carried out at pH 8.0, even when high voltages were used (data not shown). Figure 1f shows the electrophoretic profile obtained in assay 8, in which a phosphoric buffer, pH 2, was used at a separation voltage of 12 kV. It can be observed that not all of the proteins had eluted after 30 min, so the use of acidic pH was rejected for the separation of wine proteins.

Taking into account these results, the conditions selected for the analyses that followed were those used in assay 3 in Table 1, 100 mM Tris-HCl, pH 8.0, as separation buffer and a 12 kV voltage. Relative standard deviations ($n = 4$, two different capillaries) ranged

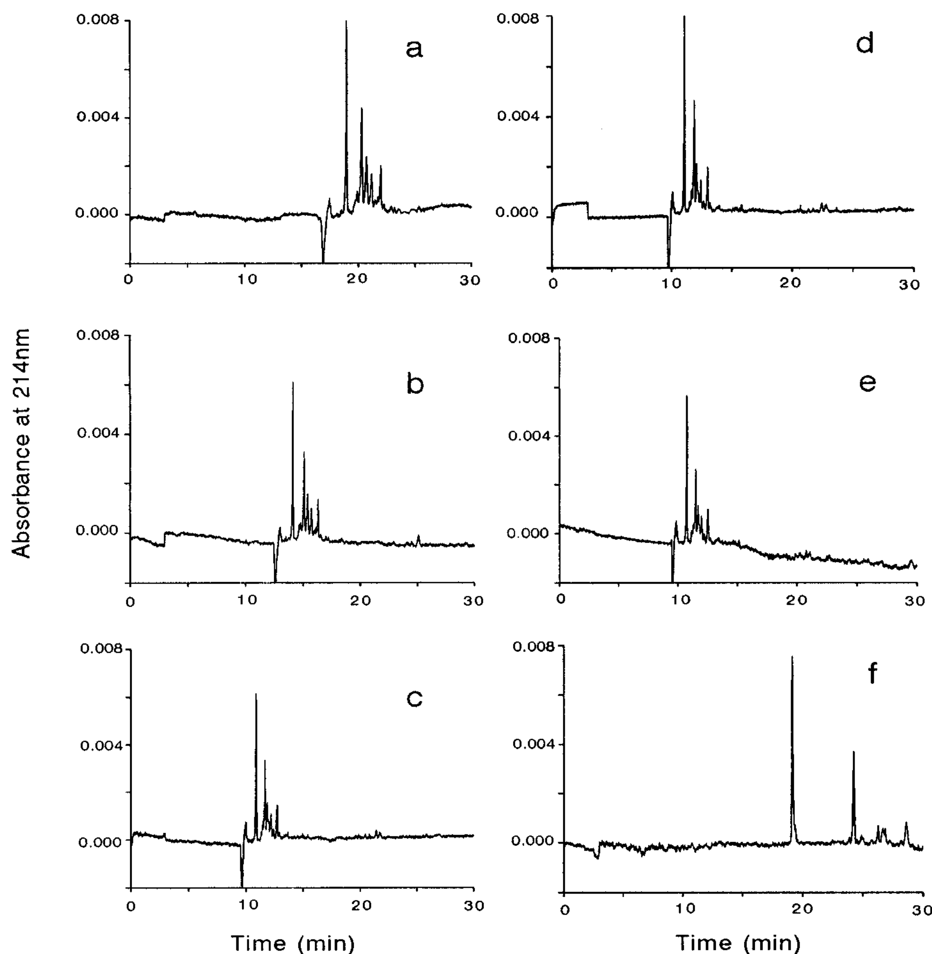

Figure 1. Capillary electrophoregrams obtained for proteins from the Chardonnay must: (a−e) assays 1−5 and (f) assay 8 from Table 1.

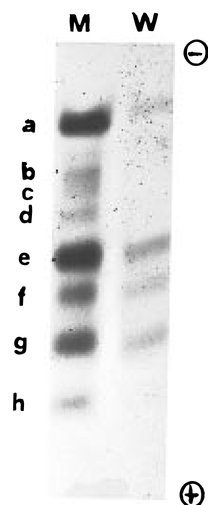

Figure 2. PAGE profiles obtained for proteins from the Chardonnay must (M) and base wine (W).

from 2.6 to 3.3% for migration times and from 4.6 to 10.6% for areas of major peaks. There was no appreciable degradation of the capillary after several hundred injections.

Analysis of Must and Wine Proteins. *Native−PAGE.* Figure 2 shows the electrophoretic patterns of the Chardonnay must and the base wine after staining with Coomassie Brilliant Blue R-250. Eight bands (a−h) were detected in the must. In the base wine only bands a and e−g were detected. In samples taken after secondary fermentation and aging with yeast, using Coomassie Brilliant Blue R-250 as staining, no electrophoretic bands were detected. When the more sensitive method of silver staining (Blum et al., 1987) was applied to these samples (data not shown), only slightly stained bands were detected. This indicates that during secondary fermentation there is a reduction in the protein content of the wines and that no proteins are detected originating from the autolytic process of the yeasts during aging with the lees.

CE. Figure 3 shows the electrophoretic profiles obtained from the CE analysis of the must, base wine, and sparkling wines. In the must a total of eight major peaks were separated, numbered 1−8. The peaks in the base wine are fewer and less intense than in the must, and some of the peaks present in the electrophoregram of the must are not even detected in the base wine (peaks 2 and 6). The sum of peak areas detected in the base wine is 41.6% of those in the must. This reduction in the protein fraction of the must is mainly due to the treatment with bentonite carried out on the wine in the winery (see Materials and Methods) for protein haze prevention (Blade and Boulton, 1988; Marchal et al., 1995) and partly due to the insolubilization of the proteins as the alcoholic content increased during fermentation (Zoecklein et al., 1990). A longer migration peak, which was not detected in the must (peak 9), is observed in the electrophoretic profile of the base wine. In the wine that has been in contact with the yeast for 3 months, we again observe a reduction in the absorbance of most of the peaks.

Secondary fermentation took place in the presence of a small quantity of bentonite (3 g/hL, see Materials and Methods) to facilitate flocculation and the later elimination of the yeast, which is a regular practice in the

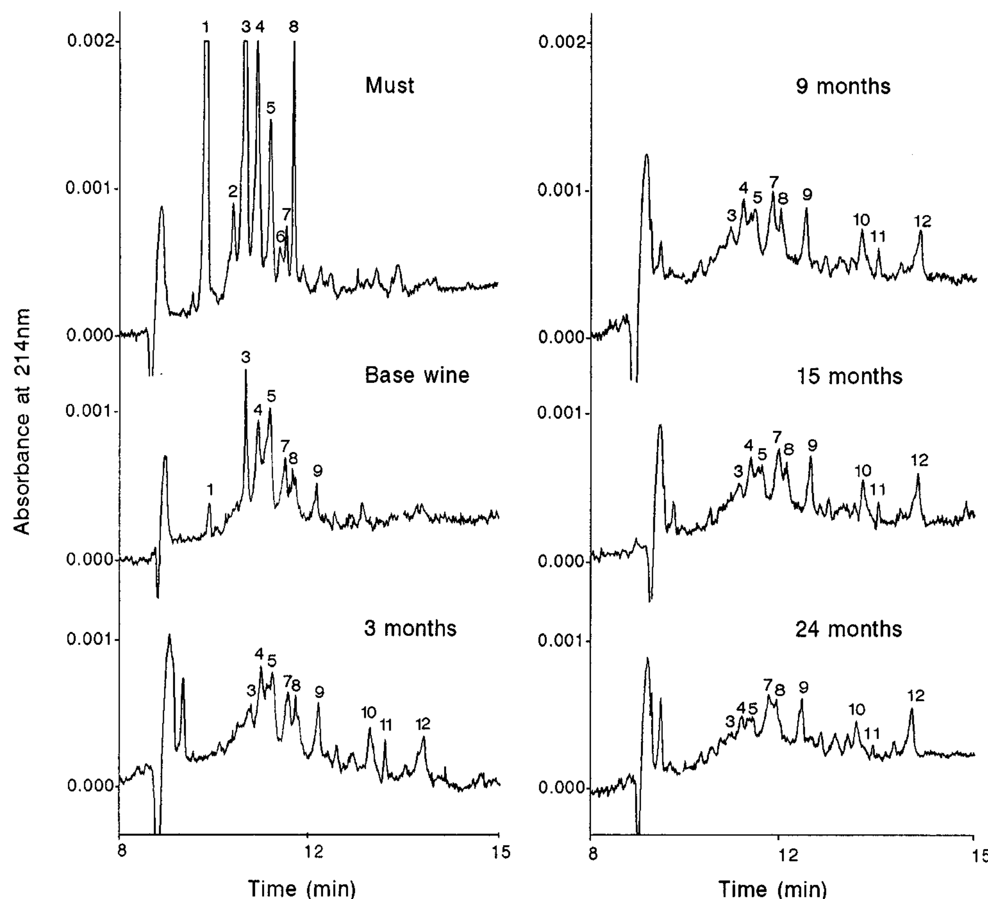

Figure 3. Capillary electrophoretic profiles obtained from proteins of the Chardonnay must, base wine, and sparkling wines after 3, 9, 15, and 24 months of aging on yeast lees.

manufacture of wines by the Champenoise method. This quantity of bentonite is much lower than that used in treatments to eliminate proteins in wines. However, it cannot be ruled out that the reduction in proteins observed between the base wine and the wine after 3 months of aging with yeast may be due not only to their insolubilization as a result of the increase in alcoholic strength but also to their adsorption by the bentonite included in the bottle.

Three new peaks, identified in Figure 3 as numbers 10−12, appear in the wine that has been in contact with the yeast for 3 months. These peaks remain during the aging with yeast and might correspond to polypeptide forms released by the yeast. In the study carried out on the peptidic fraction of these same wines (Moreno-Arribas et al., 1996), it was observed that yeast released peptidic material. The electrophoretic profiles obtained from the analysis of the wines after more than 3 months of aging are very similar. As an example, Figure 3 shows the electrophoretic profiles of the sparkling wines after 9, 15, and 24 months in contact with the yeast. Total peak area decreases as aging progresses, so that total peak area in the wine after 24 months of aging represents 27.3% of that in the must. In the determination of proteins by Bradford's colorimetric method carried out on these same wines (Moreno-Arribas et al., 1996), no reduction in the concentration of proteins was observed during aging with yeast. This discrepancy between these findings and those obtained in this work is probably due to the fact that the colorimetric method measures not only proteins but also peptides with a molecular mass of over 3000 Da that also react with Coomassie Blue (Sedmak and Grossberg, 1977).

Colagrande et al. (1987) and Feuillat et al. (1988) observed that during the aging of wine with yeast, colloids of parietal origin are released and that as aging time increases the protein content of these decreases. It is probably due to their low proportion of proteins that these colloids were not detected in this work.

According to the principles governing free-zone CE, the separation of macromolecules occurs as a function of their q/m ratio. Under the separation conditions chosen, pH 8.0, the proteins are negatively charged and thus tend to move toward the anode. Since the electro-osmotic flow toward the cathode is greater than the electrophoretic flow, all of the proteins in the wine migrate toward the cathode, and it is those with the highest q/m ratio that reach the detector at the cathode last. Since there is no electro-osmotic flow in native−PAGE separation, it is those with the highest q/m ratio that advance most quickly toward the anode. Thus, the proteins migrate in the same order, but in the opposite direction, under each of these electrophoretic techniques, and the results are comparable.

The electrophoretic profiles of the must and of the base wine obtained by these two electrophoretic techniques (Figures 2 and 3) are similar in terms of the number of bands separated and their relative intensities. The lower sensitivity of PAGE techniques meant that proteins were not detected with this technique in any of the sparkling wines analyzed.

This work demonstrates the advantages of CE over other electrophoretic techniques. The use of CE to study proteins in sparkling wines during their production has made it possible for the first time to present the protein profiles of these wines, which would not have been possible using other electrophoretic techniques.

ABBREVIATIONS USED

PAGE, polyacrylamide gel electrophoresis; SDS−PAGE, sodium dodecyl sulfate−PAGE; CE, capillary electrophoresis; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet; BSA, bovine serum albumin.

ACKNOWLEDGMENT

We are grateful to Castellblanch S.A. (Sant Sadurní d'Anoia, Spain) for manufacturing the cava wines especially for the purpose of this research.

LITERATURE CITED

Andrés-Lacueva, C.; López-Tamames, E.; Lamuela-Raventós, R. M.; Buxaderas, S.; Torre-Boronat de la, M. C. Characteristics of sparkling base wines affecting foam behavior. *J. Agric. Food Chem.* **1996**, *44*, 989−995.

Bamforth, C. W. The foaming properties of beer. *J. Inst. Brew.* **1985**, *91*, 370−380.

Blade, W. H.; Boulton, R. Adsorption of proteins by bentonite in a model wine solution. *Am. J. Enol. Vitic.* **1988**, *39*, 193−199.

Blum, H.; Beier, H.; Gross, H. J. Improved silver staining of plant proteins RNA and DNA in polyacrilamide gels. *Electrophoresis* **1987**, *8*, 93−99.

Brissonet, F.; Maujean, A. Identification of some foam-active compounds in champagne base wines. *Am. J. Enol. Vitic.* **1991**, *42*, 97−102 .

Brissonet, F.; Maujean, A. Characterization of foaming proteins in a champagne base wine. *Am. J. Enol. Vitic.* **1993**, *44*, 297−301.

Bullock, J.; Strasters, J.; Snider, J. Effect of multiple electrolyte buffers on peak symmetry, resolution, and sensitivity in capillary electrophoresis. *Anal. Chem.* **1995**, *67*, 3246−3252.

Colagrande, O.; Silva, A.; Montesissa, G. Evolution des proteines et des polysaccharides au cours de la preparation des vins mousseux (Changes in proteins and polysaccharides during the manufacture of sparkling wines). *Assise Internazionali della Vite e del Vino*; OIV: Roma, Oct 25−31, 1987.

Dubordieu, D.; Llauberes, R. M.; Ollivier, C. Estimation rapide des constituants macromoleculaires des mouts et des vins par chromatographie liquide haute pression (CLHP) de tamisage moleculaire (Fast estimation of macromolecular compounds of must and wines by molecular exclusion high performance liquid chromatography). *Connais. Vigne Vin* **1986**, *20*, 119−123.

Feuillat, M.; Charpentier, C.; Picca, G.; Bernard, P. Production des colloides par les levures dans les vins mousseux elabores selon la methode champenoise (Production of colloids by the yeast in sparkling wines manufactured by the champenoise method). *Rev. Fr. Oenol.* **1988**, *111*, 36−45.

González-Lara, R.; Correa, I.; Polo, M. C.; Martín-Alvarez, P. J. Classification of variety musts by statistical analysis of their electrophoretic protein pattern. *Food Chem.* **1989**, *34*, 103−110.

Green, J. S.; Jorgenson, J. W. Minimizing adsorption of proteins on fused silica in capillary zone electrophoresis by the addition of alkali metal salt to the buffers. *J. Chromatogr.* **1989**, *478*, 63−70.

Hii, V.; Herwig, W. C. Determination of high molecular weight proteins in beer using Coomassie blue. *J. Am. Soc. Brew. Chem.* **1982**, *40*, 46−50.

Hillier, R. M. The quantitative measurement of whey proteins using polyacrylamide gel electrophoresis. *J. Dairy Res.* **1976**, *43*, 259−265.

Hsu, J. C.; Heatherbell, D. A. Isolation and characterization of soluble proteins in grapes, grape juice and wine. *Am. J. Enol. Vitic.* **1987a**, *38*, 6−10.

Hsu, J. C.; Heatherbell, D. A. Heat-unstable proteins in wine I. Characterization and removal by bentonite fining and heat treatment. *Am. J. Enol. Vitic.* **1987b**, *38*, 11−16.

Hsu, J. C.; Heatherbell, D. A.; Flores, J. H.; Watson, B. T. Heat-unstable proteins in grape juice and wine II. Characterization and removal of ultrafiltration. *Am. J. Enol. Vitic.* **1987**, *38*, 17−22.

Ledoux, V.; Dubordieu, D. Stabilisation proteique des vins blancs (Protein stabilization of white wines). *Rev. Oenol.* **1994**, *73*, 41−42.

Ledoux, V.; Dulau, L.; Dobordieu, D. Intérpretation de l'amélioration de la stabilité protéique des vins au cours de l'elevage sur lies (Improvement of protein stability during the storage of white wines on its lees). *J. Int. Sci. Vigne Vin* **1992**, *26*, 239−251.

Lindeberg, J. Capillary electrophoresis in food analysis. *Food Chem.* **1996**, *55*, 73−94.

Lubbers, S.; Voilley, A.; Feuillat, M.; Charpentier, C. Influence of mannoproteins from yeast on the aroma intensity of a model wine. *Lebensm. Wiss. Technol.* **1994**, *27*, 108−114.

Malvy, J.; Robillard, B.; Duteurtre, B. Influence des protéines sur le comportement de la mousse des vins de Champagne (Influence of proteins on the foam behavior of Champagne wines). *Sci. Aliments* **1994**, *14*, 87−98.

Marchal, R.; Barret, J.; Maujean, A. Relations entre les caracteristiques physico-chimiques d'une bentonite et son pouvoir d'adsorption (Relationship between physicochemical characteristics of a bentonite and its adsorptive capacity). *J. Int. Sci. Vigne Vin* **1995**, *29*, 27−42.

Moio, L.; Addeo, F. Focalizzatione isoelettrica delle proteine dei mosti e dei vini (Isoelectric focusing of must and wine proteins). *Vignevini* **1989**, *4*, 53−57.

Moreno-Arribas, V.; Pueyo, E.; Polo, M. C. Peptides in musts and wines. Changes during the manufacture of Cavas (sparkling wines). *J. Agric. Food Chem.* **1996**, *44*, 3783−3788.

Polo, M. C.; Cáceres, I.; Palop, L.; Dizy, M.; Pueyo, E.; Martín-Álvarez, P. J. Study of the proteic fraction of grape must by high performance liquid chromatography and electrophoretical techniques. Variety differentiation. In *Flavor Science and Technology*; Charalambous, G., Ed.; Elservier Science Publishers: Amsterdam, 1989; pp 87−101.

Pueyo, E.; Dizy, M.; Polo, M. C. Varietal differentiation of must and wines by means of protein fraction. *Am. J. Enol. Vitic.* **1993**, *44*, 255−260.

Pueyo, E.; Martín-Alvarez, P. J.; Polo, M. C. Relationship between foam characteristics and chemical composition in wines and cavas (sparkling wines). *Am. J. Enol. Vitic.* **1995**, *46*, 518−524.

Recio, I.; Amigo, L.; López-Fandiño, R. Assessment of the quality of dairy products by capillary electrophoresis in milk proteins. *J. Chromatogr. B* **1997**, in press.

Santoro, M. Fractionation and characterization of must and wine proteins. *Am. J. Enol. Vitic.* **1995**, *46*, 250−254.

Sedmak, I. I.; Grossberg, S. E. A rapid, sensitive, and versatile assay protein using Coomassie Brilliant Blue G-250. *Anal. Biochem.* **1977**, *79*, 544−552.

Waters, E. J.; Wallace, W.; Williams P. J. Heat haze characteristics of fractionated wine proteins. *Am. J. Enol. Vitic.* **1991**, *42*, 123−127.

Waters, E. J.; Wallace, W.; Tate, M. E.; Williams, P. J. Isolation and partial characterization of a natural haze protective factor from wine. *J. Agric. Food Chem.* **1993**, *41*, 724−730.

Winter, A.; Ek, K.; Anderson, V. B. Analytical electrofocusing in thin layers of polyacrilamide gels. *LKB Appl. Note* **1977**, 250.

Zoecklein, B. W.; Fugelsang, K. C.; Gump, B. H.; Nury, F. S. Nitrogenous compounds. In *Production Wine Analysis*; Van Nostrand Reinhold: New York, 1990; Section III, Chapter 16, pp 544−552.

Received for review February 20, 1997. Accepted June 17, 1997.[⊗] This work has been supported by the Spanish Comisión Interministerial de Ciencia y Tecnología, Project ALI94-0217-CO2-02, and the Comunidad Autónoma de Madrid, Project 1-COR-035/94.

JF970141T

[⊗] Abstract published in *Advance ACS Abstracts,* September 15, 1997.