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White Wine Continuous Protein Stabilization by Packed Column

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Protein stabilization is an important stage in the production of white wine. This paper studies white wine protein stabilization using a continuous process with zirconium oxide (powder and pellets) packed in a column. The results show that the total proteins decrease by 50 and 70% for the pellet and powdered zirconium oxides, respectively. Treatment with all zirconium oxides improves wine stability. The effect of the heat regeneration process on both zirconium oxide forms is to increase the adsorption capacity. The wine treated with powdered zirconium oxide after the regeneration is the most effective for preventing protein haze. The protein profile of wine after treatment shows that the 20−50 kDa and 50−70 kDa fractions are the ones removed preferentially, while the 15 kDa fraction and the ones higher than 70 kDa are removed the least. The results show that the protein fraction with a molecular weight of 15 kDa does not affect the protein instability of the wines studied. The protein fraction with a molecular weight higher than 70 kDa seems to influence protein instability. The physicochemical properties of wine after treatment were not affected, and the values obtained were like those of the standardized range.

KEYWORDS: Protein haze; white wine; zirconium; adsorption; packed column

INTRODUCTION

Protein stabilization is an important stage in the white wine production process and has a direct impact on the commercialization of the product. The traditional stabilization methods used in most cellars consist of discontinuous operations and the addition of adsorbent materials that require considerable manpower. The use of bentonite is the only effective method to stabilize wine (*1*). Some of the drawbacks of these methods are that some products are lost, and solid residues with a high environmental impact are generated.

The review by Ferreira et al. (*1*) shows the state of the art in the wine proteins: origin, characterization, and the effect on wine turbidity and removal of proteins. Proteins in wine vary considerably, and their characteristics are the result of such factors as climate, soil, growth conditions in the vineyard, winemaking conditions, and others. The relationship between the molecular weights and isoelectric points of the wine proteins and their contribution to protein instability in white wines has been studied in different papers (*2*−*5*). However, the results of all this work make it clear how molecular weight and isoelectric points affect protein instability (*6*). The protein level of Spanish wines is in the order of 10−50 mg/L (*7*), and the molecular weights range from 16 to 200 kDa (*8*). The proteins with the highest molecular weights are glycosylated (*8*, *9*). Nowadays, the most widespread procedure for stabilizing white wine is to use bentonite, under a discontinuous process. In the literature, there are several studies on the stabilization capacity of different bentonite types (*3*, *10*, *11*) and the effect of ethanol on the adsorbing capacity of bentonite (*12*). There have been some attempts to carry out wine stabilization using continuous processes. Weetal et al. (*13*) studied the use of immobilized tannic acid. They found that proteins and tannins can be eliminated without affecting the level of the peptides and the acidity of the wine. However, the biggest disadvantage of this method is its cost.

The possibility of stabilizing wine using a continuous mode and materials that can be packed has also been studied. Studies with ion-exchange resins (*5*, *14*) showed that the level of polyphenols and proteins decreased, but the treatment affected the color and the aroma.

Hsu et al. (*15*), and Dumon and Barmier (*16*) reported the use of cross-flow ultrafiltration for protein stabilization in wines. However, the final quality of the wines was low because ultrafiltration removes a considerable number of many key components.

Several studies have dealt with the adsorption of pure proteins on the surface of metal oxides (*17*−*20*).

In a previous study by our group (*20*), the protein adsorption process in a continuous mode with zirconia in a white wine model solution was studied, and the preliminary assays with Chardonnay wine on a laboratory scale were promising.

Thus, the goal of the present paper is to study white wine protein stabilization in a continuous process with zirconium oxide packed in a column and to evaluate how the treatment affects the protein profile and the final quality of the wine.

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Table 1. Adsorbents and their Physical Properties

adsorbents	BET surface[a] (m^2/g)	particle size (mm)	avg pore diameter[a] (nm)	micropore surface[a] (%)	mesopore surface[a] (%)
ZrO_2-(po)	242.8	10^{-3}	5.7	11.80	88.20
ZrO_2-(po)R	169.8	10^{-3}	6.8	1.94	98.06
ZrO_2-(pe)	77.0	3	11.1	5.47	94.53
ZrO_2-(pe)R	73.9	3	11.0	0.02	99.98

[a] Data obtained using the Brunauer–Emmett–Teller model of adsorption with liquid N_2 by a Micromeritics, ASAP 2000 Surface Analyzer.

MATERIALS AND METHODS

Packed Column. Mel Chemicals, Manchester, England, supplied zirconium oxide in two forms, powder and pellets. We refer to the first type as ZrO_2-(po) and the second type as ZrO_2-(pe). We refer to their regenerated forms as ZrO_2-(po)R and ZrO_2-(pe)R, respectively. Their physical characteristics are presented in **Table 1**. The adsorbent was packed in a column (19 cm high and with an internal diameter of 5.1 cm). The amounts of adsorbent packed were 200 and 250 g for ZrO_2-(po) and ZrO_2-(pe), respectively. The white wine was pumped, up-flow mode, through the column by a Watson Marlow 101 U/R peristaltic pump. The volumetric flow rate was kept at a constant 2 mL/min during the experiments.

Samples. The white wine was produced from Chardonnay variety from Penedès region (Cooperativa de Vila-rodona, Tarragona, Spain) from the 2001 harvest. The wine was elaborated with must clarified by settling. The fermentation was controlled at 18 °C in industrial scale (10000 L). The wine was used after the fermentation without any additional treatment.

Protein Analysis. The molecular weight profiles of wine before and after the treatment were obtained using gel permeation chromatography. Gel filtration was carried out at 25 °C using a liquid chromatograph (Beckman Instruments, Fullerton, CA, Beckman System Gold Programmable Solvent Module 126) equipped with a UV detector (Beckman System Gold Programmable Detector Module 166) at a wavelength of 220 nm. The column system consisted of a TSK−Gel G2000SW column (TosoHaas GmbH, Stuttgart, Germany, 7.5- × 300-mm) with a Guardcolumn SW (TosoHaas GmbH, 7.5- × 75-mm). A 0.2 M phosphate buffer containing 0.1 M sodium chloride (Sigma, St. Louis, MI, cat. no. S-9888) was used as eluent. The phosphate buffer was obtained by mixing dibasic sodium phosphate (Sigma cat. no. S-9390) and monobasic sodium phosphate (Sigma, cat. no. S-9638) and adjusting the pH to 7.0 (Crison Instruments S. A., Alella, Barcelona, Spain, model: micro-pH 2002 pH meter). The injection volume was 20 μL, and the eluent flow rate was 1 mL/min. The sample was not pretreated prior to injection. Bovine serum albumin (BSA, Sigma, cat. no. A-3803, MW 67 kDa), chicken egg albumin (Sigma, cat. no. A-5503, MW 45 kDa) and lysozyme (Sigma, cat. no. L-2879, MW 14.5 kDa) were used as molecular weight standards.

The total protein concentration in the samples was evaluated using Bradford's method (*21*), which consists of adding Coomassie brilliant blue reagent and reading absorbance at 595 nm on the spectrophotometer after 1 h of incubation. BSA was used as the standard to assess macromolecular concentration.

Protein Heat Stability Test. A 50-mL sample of wine filtered through a 45-μm membrane (Millipore, Bedford, Massachusetts, HAWPO1300) was heated for 2 h in a thermostat-controlled bath at 80 °C. It was then incubated for 2 h at 4 °C. Turbidity was measured by nephelometry (Hach Ratio/XR turbidimeter) and expressed in nephelometric turbidity units (NTU). The difference in the turbidity before the wine was heated and after it had cooled was proportional to its protein instability. Wines were considered to be stable if the difference in turbidity did not exceed 2 NTU (*22*).

Physicochemical Properties. All the methods used in the present study to analyze wine have been recommended by the Commission of the European Communities published in the Official Newspaper of the European Communities (*23*). The following properties were evaluated: pH, total acidity, volatile acidity, fixed acidity, reducing sugars, volumetric alcoholic content, total dry extract, ash, chlorides, sulfates, total phenols, and absorbance at 420 nm. All the analyses were carried out in duplicate

Adsorbent Regeneration. Heat regeneration was studied so that metal oxides could be recovered and their physicochemical properties improved, and so that the protein stabilization could be made cheaper. The adsorbent was first treated for 16 h at 500 °C, and it was then saturated in the wine stabilization process.

Statistical Analysis. Statistical significance was checked by using a two-sample *t*-test, assuming equal variances. One-way analysis of variance (ANOVA) and multiple-range least significant difference (LSD) tests for sensory profile analysis were carried out by using a statistical program (SPSS ver. 10.0) for $p < 0.05$ significance level.

RESULTS AND DISCUSSION

Protein Fractions and Total Proteins. The protein fractions identified by HPLC were 15, 20−30, 50−70, and >70 kDa. An example of the wine protein fractions identified and the pure proteins used as standard is shown in **Figure 1**. According to Polo et al. (*24*), this technique quickly gives information on the approximate molecular weight of proteins. The total protein concentration obtained using the Bradford method was 30.0 ± 0.5 mg/L.

Breakthrough Curves in White Wine. Figure 2 shows the saturation curves of the total protein for the two forms of zirconium oxide. The C/C_0 values are plotted on the ordinate axis, where C is the total protein concentration at the outlet to the column and C_0 is the concentration at the inlet with reference to the bed volume (BV) expressed in mL of the wine per gram of adsorbent. Protein removal for ZrO_2-(po) is about 50% during the first 60 BV, and decreases progressively until the end of the treatment (100 BV). The protein removal obtained in the present study is similar to that obtained in a previous one (*20*), even though the total volume of wine treated in the latter was higher and the initial protein content was lower (12 mg/L). When ZrO_2-(pe) was used in the present study, protein reduction in the packed column was lower, although the profile is very similar for the two zirconium oxide forms. The difference in the protein adsorption capacity of the two zirconium forms used in the present study is due to the difference in their surface area, which is three times larger for ZrO_2-(po) than for ZrO_2-(pe) (**Table 1**).

Figure 3 shows how the adsorption process affects the different protein fractions identified in the Chardonnay wine. The C/C_0 data in **Figure 3** has been calculated by relating the areas of each fraction in the chromatogram of wine at the outlet (C) and inlet (C_0) flow of the column. **Figure 3a** shows that relatively few proteins with a molecular weight higher than 70 kDa are removed and that this removal is independent of the size of the adsorbent particles, even though there is a big difference between the surface areas. The average pore diameter for ZrO_2-(pe) is 11.1 nm, which may compensate for the fact that its surface area is lower, because the bigger pore diameter may enhance the access of bigger proteins to the active adsorption sites. The removal of proteins between 50 and 70 kDa depends on the type of adsorbent (**Figure 3b**). Unlike the previous situation, in this case the average pore diameter does not prevent the proteins from reaching the adsorbent active sites. The greater surface area of ZrO_2-(po) plays a more important role in protein removal. **Figure 3c** plots data on the removal of proteins with a molecular weight between 20 and 30 kDa. The behavior of the two adsorbents tested is very similar to the behavior of the 50−70 kDa protein fraction. However, it is worth mentioning that in this case there is slightly less protein removal than for the 50−70 kDa fraction. This is thought to be

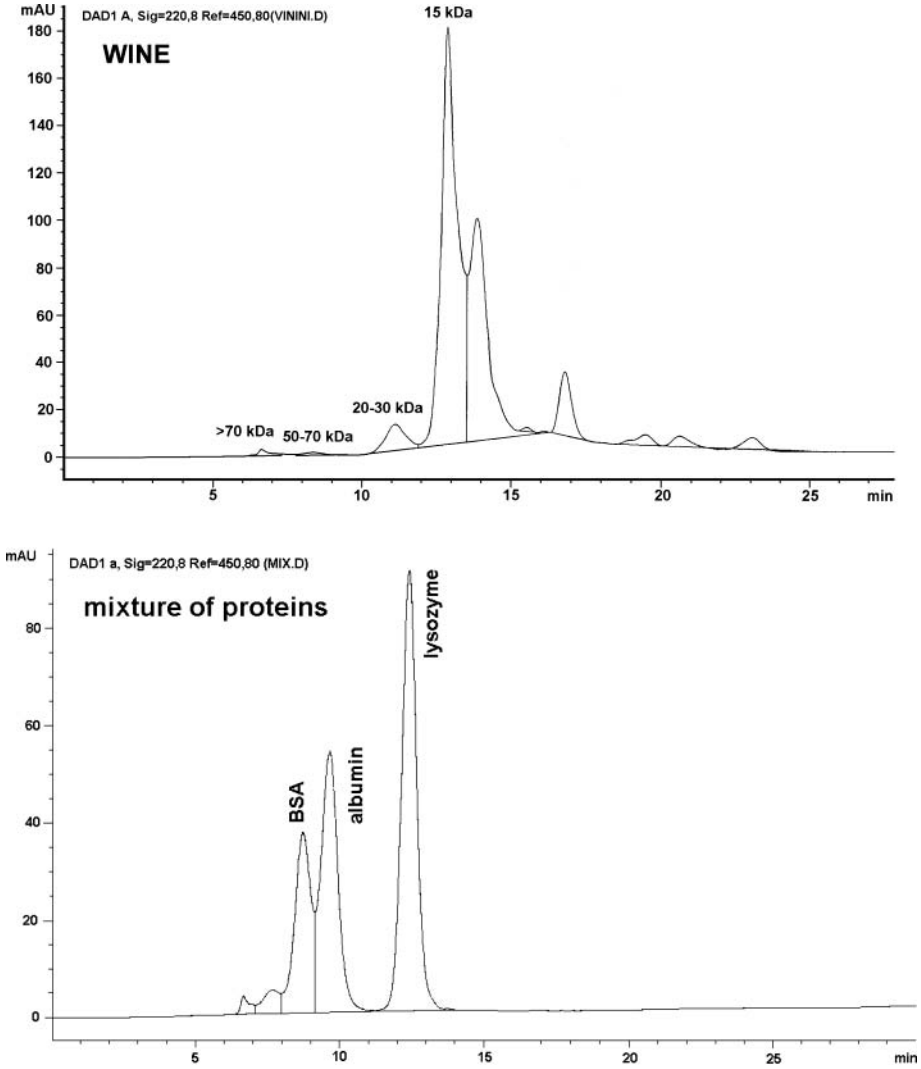

Figure 1. Chromatogram of a Chardonnay wine and mixture of standard proteins.

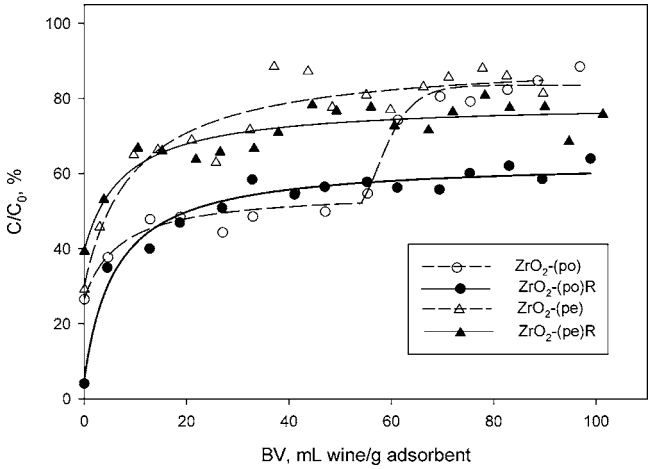

Figure 2. Behavior of total proteins in white wine.

due to the smaller proteins, which do not favor the retention of the proteins in the pores by occlusion, since they can easily exit. The protein fraction with a molecular weight of 15 kDa presents the lowest removal (**Figure 3d**). However, for ZrO_2-(po) the removal is bigger than for ZrO_2-(pe) since the surface area is higher and the average pore diameter is lower. These two factors favor the higher adsorption capacity and retention of this protein fraction. Nevertheless, the average pore diameter of the adsorbent is bigger than the equivalent diameter of 15 kDa molecules, and the protein adsorption observed is probably due to the fact that there are more active sites, because the surface area is higher (*25*).

Regeneration and Its Effect On Adsorption Capacity. Once both forms of zirconium oxide had been saturated, they were regenerated using the method described above. The thermal regeneration method decreases the surface area, increases the mesopore area, and increases the average pore diameter, except for ZrO_2-(pe), which presents a slight decrease in this parameter (**Table 1**). From a structural point of view, thermal regeneration affects the adsorption capacity of zirconium oxide.

Figure 2 shows the total protein saturation curves for ZrO_2-(po) and ZrO_2-(pe) before and after the regeneration step. The effect of the regeneration process on both zirconium oxide forms is to increase adsorption capacity. The total protein saturation curves for the wine treated with original and regenerated ZrO_2-(pe) are equal, and the values obtained for C/C_0 are very similar. Therefore, the adsorption mechanism onto this material was not changed after the high-temperature treatment. The total protein reduction with ZrO_2-(po) notably increased when the regenerated form was used (**Figure 2**). The total protein adsorption is close to 50% throughout the treatment. The higher adsorption capacity of ZrO_2-(po)R can be explained by its larger average pore diameter (**Table 1**). The slight increase in the adsorption capacity of ZrO_2-(pe)R can be explained by the increase in the

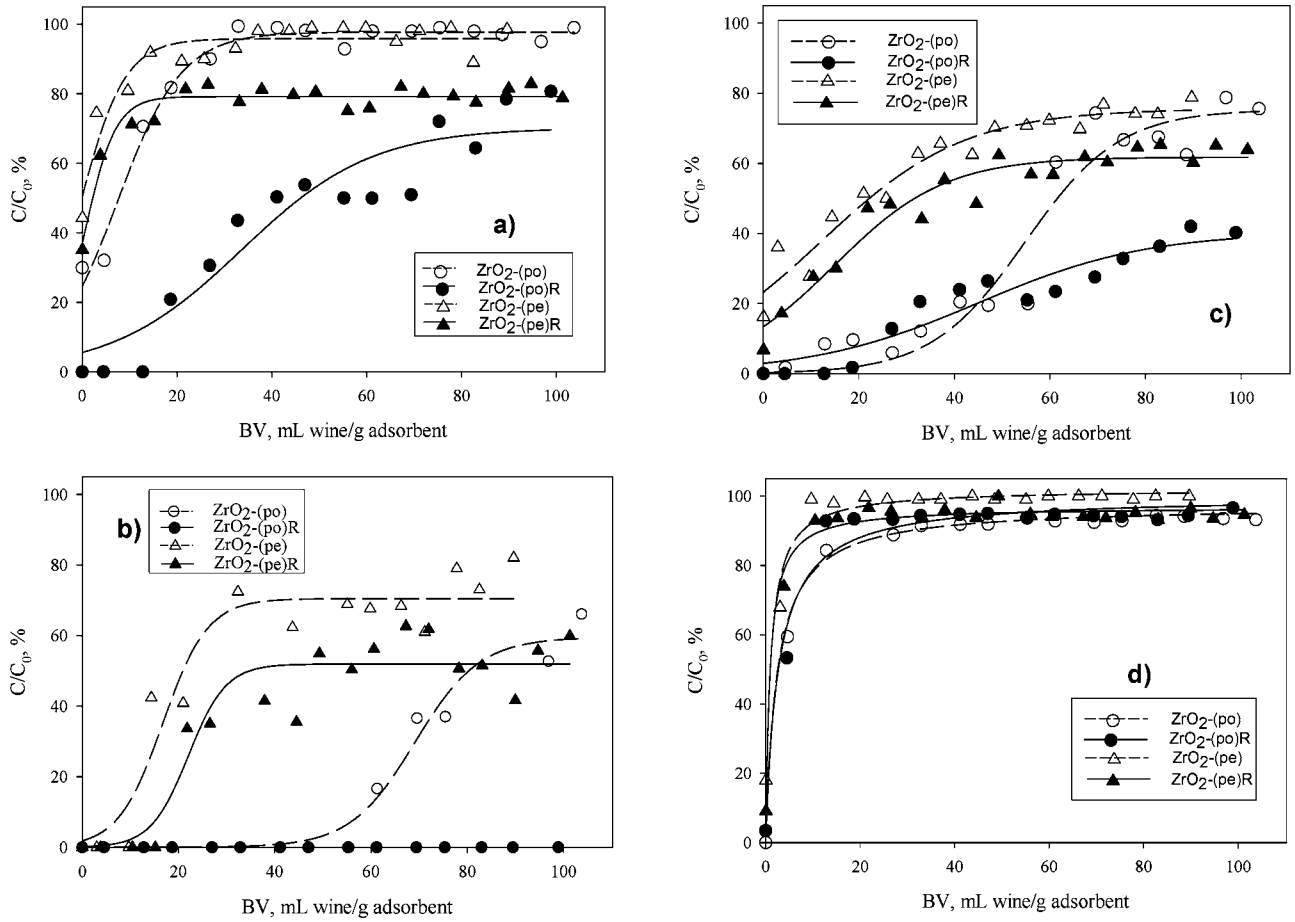

Figure 3. Behavior of the white wine protein fraction. (**a**) molecular weight > 70 kDa, (**b**) molecular weight 50–70 kDa, (**c**) molecular weight 20–30 kDa, (**d**) molecular weight 15 kDa.

Table 2. Heat Stability Test Results of Chardonnay Wine Treated with Zirconium Oxides

wine samples	turbidity (NTU)		
	before testing	after testing	difference
without treatment	0.83	115.80	114.98
wine collected up to 30BV, treated with ZrO_2-(po)	0.57	18.13	17.67
wine collected up to 60BV, treated with ZrO_2-(po)	0.46	26.94	26.48
wine collected up to 100BV, treated with ZrO_2-(po)	0.31	61.30	60.99
wine collected up to 30BV, treated with ZrO_2-(po)R	0.38	0.75	0.37
wine collected up to 60BV, treated with ZrO_2-(po)R	0.22	1.14	0.92
wine collected up to 100BV, treated with ZrO_2-(po)R	0.51	2.58	2.07
wine collected up to 30BV, treated with ZrO_2-(pe)	1.13	5.31	4.18
wine collected up to 60BV, treated with Zr O_2-(pe)	0.76	7.56	6.80
wine collected up to 100BV, treated with ZrO_2-(pe)	0.73	19.46	18.73
wine collected up to 30BV, treated with ZrO_2-(pe)R	0.50	25.28	24.78
wine collected up to 60BV, treated with ZrO_2-(pe)R	0.56	38.89	38.33
wine collected up to 100BV, treated with ZrO_2-(pe)R	0.45	56.67	56.22

active sites (mainly acid centers) on the metal oxide surface after treatment at high temperatures (*25*).

Figure 3a shows the saturation curves corresponding to the protein fraction with a molecular weight higher than 70 kDa. The regeneration treatment increased the removal of this fraction for both ZrO_2, powder and pellets. The increase in the adsorption capacity of the ZrO_2-(po)R may be due to the fact that the regeneration treatment affects the average pore diameter and mesopore distribution (**Table 1**) and favors the diffusion of large molecules in the material. The diffusion phenomenon is not so important in ZrO_2-(pe)R, and the increase in the adsorption capacity may be due to the fact that the thermal treatment increases the number of active centers (see above). The adsorption capacity of both regenerated zirconium oxides increased notably for protein fractions with molecular weights between 50 and 70 and 20−30 kDa, as can be seen in **Figure 3**, parts **b** and **c**, respectively. Finally, **Figure 3d** shows that thermal treatment of a 15 kDa protein fraction hardly affects the adsorption capacity of any oxide.

Protein Heat Stability. Table 2 shows how the protein stability improves after the treatment with zirconium oxides. The white wine treated with ZrO_2-(po)R is stable according to

Table 3. Physicochemical Properties of the Chardonnay Wine Samples after Treatment with ZrO_2-(po)R[a]

	wine/samples[c]			
determination[b]	initial	0–30 BV	0–60 BV	0–100 BV
pH	3.44(0.04)a	3.47(0.06)a	3.45(0.03)a	3.43(0.03)a
total acidity, g/L tartaric acid	5.0(0.3)a	4.1(1.0)a	4.5(0.2)a	5.0(0.3)a
fixed acidity, g/L tartaric acid	4.6(0.3)a	3.1(0.2)b	3.5(0.3)ab	4.4(0.3)ab
volatile acidity, g/L acetic acid	0.4(0.1)a	0.8(0.5)a	0.8(0.2)a	0.5(0.3)a
volumetric alcoholic degree, %vol	13.9(0.3)a	13.7(0.4)a	13.2(0.7)a	14.1(0.3)a
total dry extract, g/L	20.6(2.9)a	20.6(3.2)a	18.0(3.8)a	20.6(1.6)a
ash, g/L	2.42(0.35)a	1.88(0.23)a	2.00(0.25)a	1.82(0.34)a
sugar reducers, g/L	1.10(0.49)a	0.90(0.31)a	0.85(0.24)a	0.80(0.17)a
total polyphenol index, IPT	5.98(0.43)a	5.20(0.66)a	5.82(0.74)a	5.84(0.47)a
total polyphenol, mg/L gallic acid	296.6(15.3)a	256.5(9.8)a	280.3(18.1)a	281.1(6.5)a
chlorides, g/L NaCl	2.4(0.4)a	1.5(0.2)a	1.5(0.1)a	3.1(0.8)a
sulfates, g/L K_2SO_4	< 0.7	0.7	< 0.7	< 0.7
absorbance at 420 nm	0.185(0.008)a	0.140(0.013)b	0.145(0.012)ab	0.149(0.013)ab

[a] All reported data are the mean of duplicate determinations. [b] Standard deviations are given in parentheses. [c] Different letters mean significant differences ($p < 0.05$).

the standard definition by Moine-Ledouxt and Dubourdieu (*22*) mentioned above. Even though the difference in turbidity before and after the stability tests for wine treated with ZrO_2-(po) is higher than 2 NTU, the stability of the treated wine was greater than that of the untreated wine. Although the zirconium oxide in pellets does not stabilize the protein of the treated wine, the instability decreases notably, mainly after treatment with ZrO_2-(pe).

The relationship between the turbidity and the reduction in the protein fractions was analyzed. The protein fraction corresponding to 15 kDa seems not to affect the difference in turbidity, because in all the tests it remained practically constant, although the protein stability varied (**Figure 3d**). In all cases, the reduction is around 5−10% of initial value.

The concentration levels of the protein fractions of 20−30 and 50−70 kDa depend on the adsorbent used (see **Figure 3**, parts **b** and **c**). There is no clear link between these concentration levels and protein stability, so it is quite possible that this turbidity difference is influenced by the fractions interacting among themselves or proteins interacting with other components in wine (*3*, *6*). Nevertheless, the protein fraction that has a molecular weight above 70 kDa seems to have the greatest effect on whether the white wine is thermally stable or not. If this protein fraction is below 40% (**Figure 3b**) only when ZrO_2-(po)R is used as adsorbent, the wines produced are thermally stable (see **Table 1**). Even though the levels of the 50−70 kDa fraction were below 20% during the first 30BV (**Figure 3c**), for adsorbents ZrO_2-(po) and ZrO_2-(po)R, only the wine treated with the second adsorbent was stable (**Table 1**). Therefore, it cannot be assumed that the 50−70 kDa fraction plays an important role in the stabilization as the fraction above 70 kDa.

Physicochemical Properties. Once the sample of protein-stabilized white wine had been obtained, it was important to analyze how the physicochemical properties had changed as a result of the treatment. **Table 3** summarizes the physicochemical properties of the white wine samples, which were measured after the treatment with ZrO_2-(po)R. None of the characteristics studied were statistically affected by the treatment, except the fixed acidity and absorbance at 420 nm in the first 30 BV treated. This may be because the initial wine treated with the zirconia presented a maximum retention of organic acids and color matter. Next the retention of these kinds of compounds diminishes and the values of total wine treated are similar to those of initial wine. The other parameters have a similar tendency with an initial reduction in the first 30 BV, but the values do not show any significant differences ($p < 0.05$).

The protein of a Chardonnay white wine was stabilized using zirconium oxide as adsorbent. Tests were carried out with the adsorbents (ZrO_2-(po) and ZrO_2-(pe)) on a Chardonnay white wine with a view to applying them in the enology industry, and it was found that neither of them significantly affected the physicochemical properties of the final product. The selectivity that the two forms of adsorbents showed for the protein fractions depended on their molecular weight. The protein fraction with a molecular weight of 15 kDa does not affect the protein instability of the wines studied. The protein fraction with a molecular weight higher than 70 kDa is the one that seems to affect the protein instability of the wine studied. Heat treatment completely regenerated both adsorbents, although in the future, the influence of the temperature and the time of treatment on the regeneration of the material will be studied in greater detail.

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