Effect of Bentonite Fining on Odor-Active Compounds in Two Different White Wine Styles

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Abstract: Bentonite fining is commonly used in the wine industry as a clarifying technique to remove proteins that are a potential source of haze in wines. Because of mutual flocculation with positively charged hydrocolloids and adsorption, bentonite interacts not only with proteins, but also with other molecules. Aroma depletion during fining is generally observed as a secondary, nonspecific effect of bentonite, but mechanisms and occurrence in white wines are not clear. The effect of fining on odor-active compounds of two white wines was examined using three samples of sodium bentonite applied at three different concentrations. Two Chardonnay wines were produced with different winemaking processes to obtain two wine styles. The period of aging on lees was adjusted to produce two different protein contents. Bentonite dose, bentonite sample, and wine style significantly affected the percent reduction of some odor-active white wine compounds during bentonite fining. Most of these volatiles were indirectly removed via deproteinization, as they can be fixed to macromolecules by weak bonds, and only a few odor-active molecules were directly removed by bentonite through adsorption. Moreover, low adsorbent amounts, useful to stabilize wine, did not significantly affect the concentration of the most odorous substances. Results suggested that the chemical nature, the hydrophobicity, initial concentration of wine odor-active compounds, and the abundance and nature of wine proteins are the "matrix factors" modulating the removal of wine odor-active compounds during bentonite fining.

Key words: bentonite, fining, wine proteins, wine aroma compounds

Bentonite fining is commonly used by the wine industry as a clarifying technique to remove proteins that are a potential source of haze in wines (Ferreira et al. 2002, Ribéreau-Gayon et al. 2000). Bentonite interacts electrostatically with positively charged wine proteins because of its net negative charge at wine pH, which produces flocculation (Hsu and Heatherbell 1987). The adsorption properties of bentonite in wine are chiefly due to cation exchange action. In the structure of montmorillonite (bentonite is mostly composed of this dioctahedral smectite), some Al^{3+} ions in octahedral positions are displaced by Mg^{2+}, Fe^{2+}, and Fe^{3+}, leading to charge imbalances (Brindley 1984). This negative charge is partially balanced by exchangeable cations localized within the interlayer space or on the external surface of the clay particles. These cations are mainly Ca^{2+}, Na^{+}, and Mg^{2+}, but other cations are present to a minor extent.

Bentonite chemistry can be changed through activation, often used on natural Ca bentonites (high Ca^{2+}/Na^{+} ratio). Activation consists of treating wet mud with solid Na_2CO_3 at 80°C to obtain similar properties to natural Na bentonites (high Na^{+}/Ca^{2+} ratio), which bind protein more strongly (Blade and Boulton 1988). Bentonites are classified by the function of exchangeable cations (Na bentonite, Ca bentonite). These exchangeable cations influence the interlayer spacing of the bentonite and its swelling properties, modulating the intercalation of water into the inner layers (Catarino et al. 2008).

Bentonite is not specific to proteins; it also removes other charged species or aggregates. As a result, large amounts of added bentonite can decrease the sensory properties of wines, reducing important aroma and flavor components (Ribéreau-Gayon et al. 2000, Voilley et al. 1990).

It has been estimated that wines contain more than 800 volatile aroma compounds. These components may derive from substances present in the original grapes, either directly or indirectly through chemical, enzymatic, or thermal pathways. Others arise from yeast metabolism or are formed during the complex oxidation/reduction reactions that take place during aging. Wine volatiles include compounds with a wide range of polarity, solubility, and volatility. They include alcohols, esters, aldehydes, ketones, monoterpenes, and sulfurous and phenolic compounds (Ribéreau-Gayon et al. 2000).

Aroma compounds interact with different macromolecules such as proteins or polysaccharides (Guichard 2006, Langourieux and Crouzet 1997), so fining agents may fix substances that act as support for aromatic components (Lubbers et al. 1993). Although the interaction of bentonite on wine proteins is well studied (Achaerandio et al. 2001, Blade and Boulton 1988, de Bruijn et al. 2009, Martinez-Rodriguez and Polo 2003, Puig-Deu et al. 1999, Salazar

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et al. 2006) and some interactions between aromatic substances and macromolecules have been demonstrated in model solutions (Damodaran and Kinsella 1980, Landy et al. 1995, Langourieux and Crouzet 1997, Lubbers et al. 1993, Voilley et al. 1990), little information is available on interactions between odor-active compounds and protein in wine. The effect of clarification/stabilization treatments on the sensory quality (Girard et al. 1997, Martinez-Rodriguez and Polo 2003, Puig-Deu et al. 1999) and aroma (Armada and Falque 2007, Cabaroglu et al. 2003, Moio et al. 2004, Pozo et al. 2003) of wine has been studied, but the origin of this phenomenon has rarely been explained.

This study examined the interactions between bentonite and odor-active compounds in wine, where grape and yeast proteins were present. Although the simultaneous presence of many compounds complicated the investigation, working in real conditions allowed the entire wine colloidal matrix to be considered. The goal was to provide practical information about the role played by the wine matrix and, consequently, by the wine style during bentonite fining. A hypothesis describing the mechanism for interactions among proteins, aromatics, and bentonite interactions in wine is developed.

Materials and Methods

Chemicals. Standards of odor-active compounds ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl pyruvate, ethyl lactate, diethyl malonate, diethyl maleate, diethyl succinate, diethyl oxalate, isoamyl lactate, isoamyl acetate, phenylethyl acetate, β-phenylethanol, benzyl alcohol, n-butanol, 1-hexanol, *trans*-3-hexenol, *cis*-3-hexenol, *trans*-2-hexenol, *cis*-2-hexenol, n-octanol, benzaldehyde, γ-butyrolactone, isovaleric acid, hexanoic acid, octanoic acid, and 1-heptanol were purchased (Fluka, Sigma-Aldrich, Switzerland). Absolute ethanol 99.8% v/v, pentane, and dichloromethane were from Carlo Erba reagents (Milan, Italy).

Wines. Two heat-unstable Chardonnay wines were used. Chardonnay A was processed without any aging on lees after the end of alcoholic fermentation (AF) and with no malolactic fermentation (MLF). Chardonnay B was aged for six months on yeast lees after the end of AF. During this time, MLF occurred. To increase the wine protein concentration, contact with humid lees was forced to a 18/100 (v/v) ratio.

Bentonites. Three samples of activated sodium bentonite were purchased: Superbenton, Top Gran, and an experimental clay (Dal Cin Gildo S.p.A., Sesto S. Giovanni, Milan, Italy). Superbenton and Top Gran contained 85 to 89% montmorillonite. Superbenton was a powder and Top Gran was granular, with average diameters of 63 µm and 1 mm, respectively. The experimental clay was not used for commercial wine clarification. It was a powder of 90 to 95% montmorillonite, with an average diameter <180 µm.

Bentonite analysis. Bentonite samples were analyzed in triplicate. Elemental analysis of the inorganic content was determined with a Genesis energy dispersive X-ray detector (EDAX, Inc., Mahwah, NJ) attached to a scanning electron microscope (Philips XL30 ESEM). Surface charge density was measured as described elsewhere (Ferrarini et al. 1996). Swell index was determined using standard methods (OIV 2003). External specific surface area (SSA) was detected using the BET method (Brunauer et al. 1938). Methylene blue titration was also performed to measure the internal SSA as described in Resolution Oeno 11/2003 (OIV 2003).

Bentonite fining. Laboratory-scale trials were carried out on Chardonnays A and B. Each bentonite was added to each wine at three different concentrations: 20, 50, and 100 g/hL. Samples were prepared in duplicate. Untreated samples of each wine were kept as a control. The bentonite slurries were prepared in deionized water at a concentration of 10% (w/w). After 90 min rehydration, the gels were stirred. Each solution was added to 1 L wine and thoroughly mixed. All samples were put into 4-L demijohns and kept for 5 days at 16 to 18°C at 60% relative humidity. The limpid liquid phases were separated and filtrated through folded filters (595 ½, Whatman GmbH, Germany). The untreated controls were filtered under the same conditions.

Wine chemical analysis. Wine pH, alcohol, and total nitrogen were determined in triplicate according to methods reported in E.U. Regulation 2676/90 (1990).

Wine total protein concentration. Total protein was determined by a previously reported method (Schacterle and Pollak 1973) with bovine serum albumin (BSA) as a standard. The analysis was carried out after protein purification as follows: 400 mL absolute ethanol was added to 100 mL wine. After 72 hr precipitation, the samples were centrifuged (20 min at 5000 rpm). The precipitate was suspended in water and then dialyzed in tubes with a 3500 Da molecular weight cut-off (Membrane Filtration Products, San Antonio, TX). The dialyzed samples were lyophilized and protein concentration was determined after resuspension in 10 mL water.

Wine odor-active compounds. Odor-active compounds were recovered as described (Silva et al. 1988) by continuous liquid-liquid extraction with pentane:dichloromethane (2:1 v/v). A total of 0.5 mL internal standard (1-heptanol 1% v/v in absolute ethanol) was added to 500 mL wine sample, previously filtered through a 0.8-µm membrane (Waters-Millipore, Milford, MA). The sample was then put into the extraction tube with 100 mL pentane:dichloromethane (2:1 v/v). After 6 hr the organic layer was collected, dried with sodium sulfate, and concentrated to 1 mL at 50°C with a reflux condenser. Next, 0.5 µL of this extract was injected using splitless mode into a GC Autosystem XL chromatograph with a flame ionization detector (FID) (PerkinElmer, Shelton, CT) and using a Supelcowax 10 fused silica capillary column (30 m x 0.32 mm i.d. and 1.0 µm film thickness) (Supelco, Bellefonte, PA). Chromatographic conditions were the following: He (purity 99.000%) as carrier gas at 30 mL/min, 210°C injector temperature, and 250°C FID temperature. The compounds were separated using an initial oven temperature of 50°C for 10 min, a temperature gradient of 2°C/min to a final temperature of 250°C, maintained for 40 min. The standards were prepared at concentrations between

50 and 500 mg/L. Three chromatographic analyses of each sample were made.

Statistical analysis. There were two independent replications of each treatment and each analysis was performed in triplicate; therefore the data referred to each odor-active compound as the mean of six values (n = 6). Results presented in tables and graphs are reported as means ± standard deviation (SD). Statistically significant differences between samples were tested using a post-hoc comparison test (Tukey's test) at $\alpha = 0.05$. Effects of bentonite dose, bentonite sample, and wine on aroma reduction were assessed by factorial ANOVA. Statistics were carried out by SPSS software ver. 15.0 (SPSS, Inc., Chicago, IL).

Results

Physicochemical analysis of bentonite. Experimental clay had significantly more Mg (2.34 ± 0.14 % w/w) and less K (0.39 ± 0.11 % w/w), Fe (0.54 ± 0.10 % w/w), Al (5.74 ± 0.30 % w/w), and Si (25.96 ± 1.59 % w/w) than Superbenton and Top Gran. Superbenton had the most Na (0.85 ± 0.05 % w/w) and K (0.86 ± 0.18 % w/w). Top Gran had significantly more Fe (1.50 ± 0.59 % w/w), Al (7.53 ± 0.50 % w/w), and Si (32.77 ± 3.32 % w/w) than the other bentonite samples. The surface charge density was 102 meq/100 g for Top Gran and experimental clay and 97 meq/100 g for Superbenton (Table 1). These differences were not significant.

External specific surface area (SSA) measured by the BET method (Brunauer et al. 1938) was 38.68, 46.40, and 47.54 m^2/g for experimental clay, Superbenton, and Top Gran, respectively. The experimental clay had significantly less external SSA than Superbenton or Top Gran. SSA determined using the methylene blue titration method (OIV 2003) was 86.73 m^2/g for experimental clay, 103.46 m^2/g for Superbenton, and 402.47 m^2/g for Top Gran. These three values were significantly different from each other.

Swell index was similar for Superbenton and Top Gran at 11.7 and 13.5 mL/2 g, respectively. These bentonites showed significantly more swelling than experimental clay (8.5 mL/2 g), although the clay had greater montmorillonite.

Wine chemical analysis. Chardonnay A was more acidic and had less alcohol than B (Table 2). Nitrogen concentration was 340 mg/L for Chardonnay A and 2.7 g/L for Chardonnay B. The total protein was 41.5 mg/L in A and 318.6 mg/L in B, consistent with the different nitrogen concentrations.

Effect of bentonite fining on wine protein. Reductions in total protein, expressed as absolute concentration (mg/L) and as a percentage of the initial protein concentration are reported (Table 3). There was a higher percentage reduction in wine A than in wine B, but more milligrams of proteins were removed from wine B than wine A. Three different doses of bentonite in both wines (20, 50, and 100 g/hL) were tested to determine the minimum dose that stabilized the wine proteins. In Chardonnay A, the minimum dose was 20 g/hL and in Chardonnay B, the minimum dose was 50 g/hL. In wine A, Top Gran removed the most protein at each dose used. Clarification with 50 g/hL absorbed more protein than 100 g/hL of the other bentonites. At this dosage experimental clay and Superbenton showed the same efficacy, while at lower concentrations (20 and 50 g/hL) experimental clay reduced the protein more than Superbenton. In wine B, experimental clay removed the most protein, especially at 100 g/hL. Superbenton and Top Gran gave similar results at 20 and 50 g/hL.

Effect of bentonite fining on white wine odor-active compounds. Twenty-six aromatic compounds were identified in both wines. The most significant substances were ethyl butyrate, ethyl hexanoate, ethyl octanoate, isoamyl acetate, phenylethyl acetate, β-phenylethanol, 1-hexanol, hexanoic acid, and octanoic acid. Aromatic compounds were analyzed in the untreated control samples and after bentonite treatment. Results are reported separately for wine A (Table 4) and wine B (Table 5). Bentonite fining produced losses in some odor-active compounds of white wine. The significance of dose, type of bentonite, and wine style on white wine aroma loss was analyzed with factorial analysis of variance (Table 6).

Ethyl butyrate was depleted in all wines treated with bentonite, but the residual concentration was always higher

Table 1 Physicochemical analysis of bentonite samples.

Bentonite	Surface charge density (meq/100 g)	Specific surface area (m^2/g)		Swell index (mL/2 g)
		BET method	Methylene blue titration	
Experimental clay	102 ± 5.1 a[a]	38.68 ± 4.08 a	86.73 ± 2.48 a	8.5 ± 1.2 a
Superbenton	97 ± 4.9 a	46.40 ± 4.90 b	103.46 ± 2.43 b	11.7 ± 1.2 b
Top Gran	102 ± 8.9 a	47.54 ± 5.02 b	402.57 ± 6.43 c	13.5 ± 1.3 b

[a]Values are means ± SD (n = 3). Within each column, different letters indicate statistically different values according to post-hoc comparison (Tukey's test) at $\alpha = 0.05$.

Table 2 Wine chemical analysis.

	pH	Alcohol (% v/v)	Total nitrogen (mg/L)	Total proteins (mg/L)
Chardonnay A	3.30 ± 0.02[a]	11.00 ± 0.10	340 ± 18.7	41.5 ± 8.3
Chardonnay B	3.60 ± 0.03	14.20 ± 0.20	2700 ± 176	318.6 ± 70.1

[a]Values are means ± SD (n = 3).

Table 3 Protein reductions in wine samples treated with bentonite. Protein removal is expressed as absolute concentration (mg/L) and as percentages (%) of the initial protein content (41.5 ± 8.3 mg/L for Chardonnay A and 318.6 ± 70.1 mg/L for Chardonnay B).

	Protein removal[a]			
	Chardonnay A		Chardonnay B	
Bentonite (dose)	(mg/L)	(%)	(mg/L)	(%)
Experimental clay, 20 g/hL	14.2 ± 2.7 bc	34.2 ± 6.5 bc	20.4 ± 5.4 c	6.4 ± 1.7 c
Experimental clay, 50 g/hL	16.7 ± 4.0 ab	40.3 ± 9.6 ab	43.9 ± 12.1 b	13.8 ± 3.8 b
Experimental clay, 100 g/hL	17.1 ± 2.7 ab	41.1 ± 6.4 ab	79.0 ± 7.6 a	24.8 ± 2.4 a
Superbenton, 20 g/hL	10.9 ± 2.7 c	26.3 ± 6.4 c	16.6 ± 6.4 c	5.2 ± 2.0 c
Superbenton, 50 g/hL	13.5 ± 4.3 bc	32.6 ± 10.4 bc	37.3 ± 15.0 bc	11.7 ± 4.7 bc
Superbenton, 100 g/hL	17.1 ± 4.0 ab	41.1 ± 9.7 ab	43.0 ± 5.7 b	13.5 ± 1.8 b
Top Gran DC, 20 g/hL	16.1 ± 2.4 b	38.8 ± 5.7 b	18.5 ± 11.2 c	5.8 ± 3.5 c
Top Gran DC, 50 g/hL	19.3 ± 4.6 ab	46.6 ± 11.1 ab	33.5 ± 8.6 bc	10.5 ± 2.7 bc
Top Gran DC, 100 g/hL	22.7 ± 3.6 a	54.6 ± 8.8 a	42.4 ± 3.5 b	13.3 ± 1.1 b

[a]Values are means ± SD (n = 6). Within each column, different letters indicate statistically different values according to post-hoc comparison (Tukey's test) at $\alpha = 0.05$.

than threshold level (20 µg/L). Percentage reductions varied significantly according to bentonite dose and wine (Table 6; Figure 1). In wine A, ethyl butyrate was reduced similarly by 20 and 50 g/hL, but 100 g/hL gave rise to significantly more aroma loss. In wine B, depletion increased significantly from 20 to 50 g/hL with no additional variation at 100g/hL. Percentage reductions of ethyl butyrate were higher in wine A (from 82 to 89%) than in B (from 15 to 75%). Differences between wines were greatest at 20 g/hL (82 and 15% for A and B, respectively).

Ethyl hexanoate removal was significantly affected by bentonite sample and wine style (Table 6). The effect of bentonites changed significantly according to wine style (Figure 2). Experimental clay lowered ethyl hexanoate less (28%) than Superbenton and Top Gran (74 and 83%, respectively) in wine A. In wine B, Superbenton and Top Gran produced significant differences in aroma loss (27% and 43%, respectively). The lowest amounts of Superbenton did not reduce ethyl hexanoate, but 100 g/hL reduced it by 68% (Table 5).

Ethyl octanoate removal was significantly affected by bentonite dose, but in a similar way for all the adsorbent samples and for the two wines (Table 6). Reductions of this aroma compound varied from 13.8% to 32.1% when the adsorbent amount increased from 20 to 100 g/hL (Figure 3).

Removal of isoamyl acetate was not related either to dose or to type of bentonite and did not significantly vary according to wine style (Table 6). Nevertheless, there was a significant interaction ($p < 0.01$) between bentonite sample and wine style. In Chardonnay A (Table 4), Superbenton and Top Gran reduced the initial aroma concentration by 32% and 60%, respectively, while experimental clay did not significantly reduce isoamyl acetate. Conversely, in Chardonnay B (Table 5), Superbenton did not reduce isoamyl acetate, Top Gran reduced it only slightly, and the highest loss (76%) was observed with 100 g/hL experimental clay.

Phenylethyl acetate reductions varied significantly by bentonite dose and wine (Table 6). More phenylethyl acetate was removed in wine B than in A with a similar dose effect in the two wines: 20 g/hL adsorbent had the least impact, while 50 and 100 g/hL removed more.

β-Phenylethanol removal was affected significantly by bentonite dose, but not by bentonite type or wine (Table 6). β-Phenylethanol was reduced by 15.5%, 21.9%, and 40.5% for 20, 50, and 100 g/hL bentonite, respectively (Figure 3).

The other alcohol, 1-hexanol, had significantly different initial concentrations in the two wines: ~3 mg/L in wine A and only a few µg/L in wine B. Final 1-hexanol concentrations were significantly affected by bentonite dose and type (Table 6) and statistics showed a significant ($p < 0.05$) interaction between factors (bentonite sample x wine style). Experimental clay had a similar dose effect in both wines: reductions were significantly greater from 20–50 g/hL to 100 g/hL (Figure 4). A greater percentage was removed in wine B (from 23% to 45%) than in wine A (from 10% to 25%). Fining with Superbenton produced similar losses of 1-hexanol in the two wines, especially at 20 and 100 g/hL (2–5% and 50–58%, respectively). At 50 g/hL, Superbenton reduced 1-hexanol by 8% and 20% in wines A and B, respectively. In wine A, 1-hexanol loss was very high (from 65 to 70%) at all doses of Top Gran. In wine B, the loss was significantly lower (20%) at 20 g/hL Top Gran and increased to 44% and 46% at 50 and 100 g/hL.

Among fatty acids, bentonite dose and wine style significantly affected the reduction of hexanoic acid (Table 6). This compound was reduced significantly more in wine B than in wine A, but the dose effect was similar in the two wines: 20 g/hL of adsorbent had a negligible impact, while 50 and 100 g/hL removed more (Figure 1). Conversely, octanoic acid reductions did not vary significantly with dose or type of bentonite or with wine (Table 6). Octanoic acid concentrations were strongly reduced by 100 g/hL adsorbent (89% and 94% in wines A and B, respectively). In these wines, the concentration fell below the threshold level for perception (500 µg/L). At doses of 20 and 50 g/hL, there was negligible depletion of octanoic acid.

Discussion

Physicochemical analysis of bentonites. Each clay mineral presents different characteristics depending on origin, type, and label. Elemental analysis showed statistically significant but probably unimportant differences among the bentonite types. We tested three natural calcium bentonites. To increase efficiency, these bentonites undergo a commercial activation process, which is achieved by heating calcium-rich bentonite in water at 80°C with sodium carbonate, enriching the natural calcium-dominant bentonite with sodium and thereby increasing its capacity for protein removal. The efficacy of this process can vary depending on the bentonite used.

Superbenton and Top Gran had a very similar elemental composition: they only differed significantly in Na concentration. Superbenton bentonite had the highest Na concentration and Na^+/Ca^{2+} ratio. A Na^+-rich bentonite (high Na^+/Ca^{2+} ratio) is more efficient for protein removal than bentonites with a higher concentration of other interlayer cations such as calcium (Blade and Boulton 1988). As a consequence, Superbenton would remove the most protein. Si, Al, and Fe concentrations were higher in Top Gran bentonite and differed significantly from those in experimental clay. Bentonite is mainly composed of montmorillonite, a mineral consisting of two tetrahedral silicon oxide sheets and one octahedral aluminum hydroxide sheet, combined as a crystalline structural layer unit (Catarino et al. 2008).

The base structure of bentonite is silicon, which does not transfer to the wine. However, Al and Fe can be extracted during fining (Catarino et al. 2008). The Organisation Internationale de la Vigne et du Vin (OIV) defines acceptable limits for extractable elements such as Al and Fe. Occasionally in a montmorillonite structure, Al is replaced by a different metal such

Table 4 Odor-active compounds (µg/L) in Chardonnay A wine samples treated with bentonite (20, 50, and 100 g/hL) and in untreated control wine A.

		Experimental clay dose			Superbenton dose			Top Gran dose		
Compound	**Control A**	**20 g/hL**	**50 g/hL**	**100 g/hL**	**20 g/hL**	**50 g/hL**	**100 g/hL**	**20 g/hL**	**50 g/hL**	**100 g/hL**
Ethyl butyrate (µg/L)	3798 ± 228 a[a]	553 ± 21 cd	522 ± 16 d	411 ± 33 e	728 ± 44 b	677 ± 13 bc	591 ± 24 c	784 ± 63 b	614 ± 55 c	222 ± 18 f
Ethyl hexanoate (µg/L)	777 ± 62 a	726 ± 28 a	552 ± 34 b	406 ± 28 c	229 ± 16 d	209 ± 12 de	180 ± 14 e	189 ± 26 e	138 ± 14 f	133 ± 12 f
Ethyl octanoate (µg/L)	114 ± 6 a	100 ± 11 ab	97 ± 11 ab	87 ± 10 ab	111 ± 12 a	97 ± 11 ab	96 ± 21 ab	94 ± 23 ab	81 ± 15 b	81 ± 12 b
Isoamyl acetate (µg/L)	2975 ± 208 a	2910 ± 224 a	2168 ± 239 b	1285 ± 141 c	1999 ± 100 b	1421 ± 185 bc	1176 ± 164 c	2027 ± 203 b	1796 ± 214 b	1699 ± 203 bc
Phenylethyl acetate (µg/L)	812 ± 41 a	699 ± 25 b	629 ± 31 bc	571 ± 17 c	793 ± 42 a	672 ± 34 bc	545 ± 65 c	621 ± 49 bc	589 ± 53 c	588 ± 58 c
β-Phenylethanol (µg/L)	9595 ± 576 a	9417 ± 485 ab	8032 ± 802 b	7288 ± 802 bc	7218 ± 519 bc	7051 ± 704 bc	6923 ± 692 cd	6987 ± 699 cd	6479 ± 778 d	6353 ± 670 d
1-Hexanol (µg/L)	3362 ± 168 a	3040 ± 303 ab	2885 ± 375 ab	2513 ± 219 b	3299 ± 160 a	3089 ± 133 a	1664 ± 98 c	1163 ± 58 d	1101 ± 76 d	1019 ± 79 d
Hexanoic acid (µg/L)	92 ± 8 a	70 ± 6 b	53 ± 4 c	53 ± 5 c	86 ± 14 a	42 ± 8 cd	35 ± 4 d	55 ± 5 c	31 ± 5 d	29 ± 5 d
Octanoic acid (µg/L)	1547 ± 155 a	1413 ± 285 a	222 ± 47 cd	163 ± 29 d	339 ± 34 bc	291 ± 26 c	286 ± 29 c	478 ± 24 b	452 ± 28 b	419 ± 33 b

[a]Values are means ± SD (n = 6). Within each column, different letters indicate statistically different values according to post-hoc comparison (Tukey's test) at $\alpha = 0.05$.

Table 5 Odor-active compounds (µg/L) in Chardonnay B wine samples treated with bentonite (20, 50, and 100 g/hL) and in untreated control wine B.

		Experimental clay dose			Superbenton dose			Top Gran DC dose		
Compound	**Control B**	**20 g/hL**	**50 g/hL**	**100 g/hL**	**20 g/hL**	**50 g/hL**	**100 g/hL**	**20 g/hL**	**50 g/hL**	**100 g/hL**
Ethyl butyrate (µg/L)	4145 ± 497 a[a]	4103 ± 586 a	2204 ± 265 b	1910 ± 278 b	3238 ± 143 a	586 ± 47 d	508 ± 46 d	3208 ± 257 a	821 ± 99 c	703 ± 77 c
Ethyl hexanoate (µg/L)	1199 ± 96 a	838 ± 84 b	731 ± 95 bc	683 ± 82 c	1194 ± 138 a	1060 ± 92 a	384 ± 31 e	825 ± 57 b	658 ± 59 bcd	583 ± 61 d
Ethyl octanoate (µg/L)	232 ± 23 a	222 ± 28 a	207 ± 25 a	48 ± 6 c	228 ± 29 a	220 ± 28 a	163 ± 14 b	217 ± 28 a	213 ± 24 a	197 ± 16 a
Isoamyl acetate (µg/L)	4428 ± 399 a	2620 ± 340 b	2301 ± 297 b	1073 ± 106 c	4353 ± 522 a	4096 ± 210 a	4055 ± 283 a	4213 ± 254 a	2466 ± 197 b	2367 ± 189 b
Phenylethyl acetate (µg/L)	157 ± 14 a	57 ± 8 b	40 ± 4 c	15 ± 2 e	129 ± 28 a	26 ± 4 d	25 ± 3 d	30 ± 7 d	22 ± 6 de	17 ± 5 de
β-Phenylethanol (µg/L)	2082 ± 187 a	1658 ± 199 b	1612 ± 209 b	453 ± 54 d	2037 ± 272 a	1856 ± 99 ab	1066 ± 104 c	1740 ± 193 ab	1613 ± 183 b	1452 ± 159 bc
1-Hexanol (µg/L)	77 ± 6 a	59 ± 7 b	55 ± 10 b	42 ± 5 c	73 ± 15 ab	62 ± 12 ab	32 ± 4 d	62 ± 6 b	43 ± 4 c	42 ± 5 c
Hexanoic acid (µg/L)	33 ± 3 a	8 ± 1 b	6 ± 1 b	4 ± 1 b	27 ± 5 a	9 ± 1 b	7 ± 1 b	8 ± 1 b	7 ± 2 b	5 ± 2 b
Octanoic acid (µg/L)	730 ± 73 a	650 ± 81 ab	75 ± 10 e	44 ± 5 f	388 ± 29 c	122 ± 15 d	52 ± 10 f	722 ± 90 a	676 ± 75 ab	568 ± 67 b

[a]Values are means ± SD (n = 6). Within each column, different letters indicate statistically different values according to post-hoc comparison (Tukey's test) at $\alpha = 0.05$.

Table 6 Effect of bentonite on odor-active compounds in wine: significance of percentage reduction of aromatic compound versus bentonite dose, bentonite type, and wine style.

	Bentonite dose	Bentonite type	Wine style
Ethyl butyrate	***[a]	ns	***
Ethyl hexanoate	ns	*	**
Ethyl octanoate	*	ns	ns
Isoamyl acetate	ns	ns	ns
Phenylethyl acetate	*	ns	***
β-Phenylethanol	**	ns	ns
1-Hexanol	*	*	ns
Hexanoic acid	**	ns	**
Octanoic acid	ns	ns	ns

[a]*, **, ***, and ns indicate significant differences at $p < 0.05$, 0.01, 0.001, and not significant, respectively.

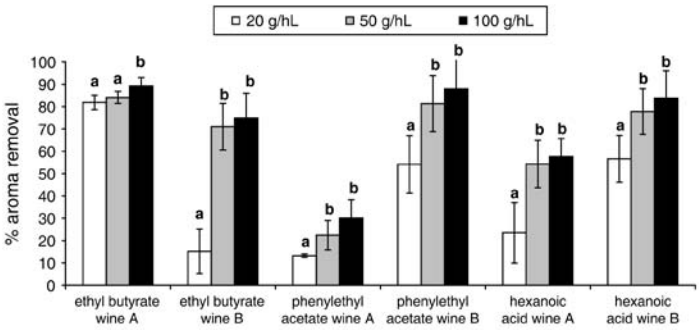

Figure 1 Effect of bentonite dose on percentage reductions of ethyl butyrate, phenylethyl acetate, and hexanoic acid in wines A and B. Each bar represents the mean, n = 18; error bars denote standard error. Different letters at each bar indicate statistically different values according to post-hoc comparison (Tukey's test) at α = 0.05.

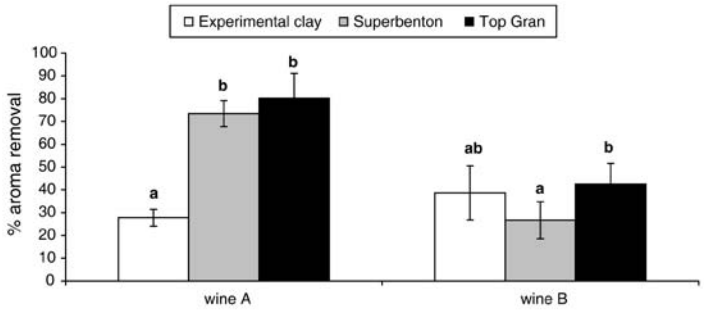

Figure 2 Effect of bentonite sample on percentage reductions of ethyl hexanoate in wines A and B. Each bar represents the mean, n = 18; error bars denote standard error. Different letters at each bar indicate statistically different values according to post-hoc comparison (Tukey's test) at α = 0.05.

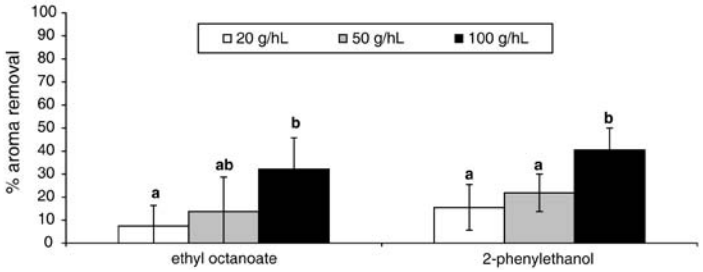

Figure 3 Effect of bentonite dose on percentage reductions of ethyl octanoate and β-phenylethanol, expressed as the means of the percentage reductions observed in wines A and B). Each bar represents the mean, n = 36; error bars denote standard error. Different letters at each bar indicate statistically different values according to post-hoc comparison (Tukey's test) at α = 0.05.

as Fe or Mg. Experimental clay had significantly more Mg than the other bentonites, possibly because it contained magnesium smectites.

The electric charge of bentonites is responsible for their ability to remove proteins and to adsorb other cationic compounds in wine (Ferrarini et al. 1996, Xifang et al. 2007). The differences in surface charge density of the tested bentonites (Table 1) were not significant, but all three were high compared to reported surface charge densities ranging from 57.0 to 80.4 cmol/kg (Catarino et al. 2008). Nevertheless, previous works reported that granular and powder-activated sodium bentonites can reach 150 and 130 meq/100 g, respectively (Ferrarini et al. 1996).

The external specific surface areas (SSAs) measured by the BET method (Table 1) were in the range for montmorillonitic soils, from 11.2 to 56.7 m^2/g (Yukselen and Kaya 2008), but were higher than the 13.4 to 38.3 m^2/g reported elsewhere (Catarino et al. 2008). Even if the external SSA of experimental clay was significantly different from that of the other bentonites, such differences could not explain the different mineral release capacity by itself. Experimental clay had the highest montmorillonite content and the lowest SSA, suggesting that clay fraction characteristics are more important than its content. Similar results have been reported (Catarino et al. 2008). These authors studied the physical and chemical characteristics of several bentonites and reported the highest cation exchange capacity in the sample having the lowest external SSA. Our results are probably related to montmorillonite structural particularities and they confirm that montmorillonite content is not a satisfactory indicator of bentonite reactivity.

The methylene blue titration method provides information on the mineral SSA and results in much higher values than those measured by the BET method (Yukselen and Kaya 2008). The methylene blue method is applied under wet conditions in which ions or water intercalate into inner montmorillonite layers, so it measures both external and internal surface areas. Our results showed highly significant differences among samples (Table 1), which could explain their distinct activity. The highest external + internal surface was in Top Gran, which had the strongest potential adsorption activity among the samples examined. Higher SSA indicates more active adsorption sites on the bentonite surface. This augments the contact opportunity with proteins and consequently increases protein adsorbtion onto bentonite (Xifang et al. 2007).

Swell index reflects the water volume adsorbed by bentonite pores. Higher values indicate an increased adsorption capacity of wine colloidal particles. Our results (Table 1) stayed in a restricted range (from 8.5 to 13.5 mL/2 g),

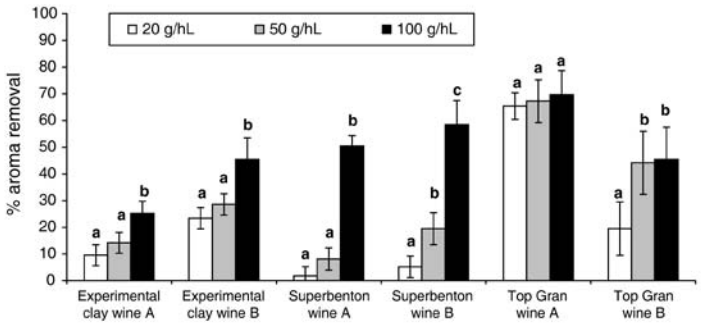

Figure 4 Effect of bentonite dose and sample in wines A and B on percentage reductions of 1-hexanol. Each bar represents the mean, n = 6; error bars denote standard error. Different letters at each bar indicate statistically different values according to post-hoc comparison (Tukey's test) at $\alpha = 0.05$.

although a greater variability for montmorillonitic soils was observed (Yukselen and Kaya 2008). Swell index is dependent on intrinsic soil properties such as SSA and surface charge density. An exponential correlation between the amount of adsorbed methylene blue and SSA is evidenced by the correlation between swell index and adsorbed methylene blue values (Yukselen and Kaya 2008). In experimental clay and Superbenton, a greater swell index was observed where more methylene blue was adsorbed (in Superbenton), but in Top Gran, the increase in swell index was not as high as the corresponding increase in SSAs measured with methylene blue titration. This could be due to difficulties in rehydration for a granular bentonite like Top Gran. Rehydration must be performed under static conditions (OIV 2003) and the deposit volume must be measured after 24 hours of contact. It is probable that for a granular bentonite more time in contact with water is necessary to permit the water molecules to intercalate into the clay layers. As with SSA, the swell index of experimental clay confirmed that a greater clay content is not linked to increased swelling.

Wine nitrogen and protein concentration. Differences in nitrogen compound concentration betweens wine A and B resulted from the contact with lees after alcoholic fermentation and on the lees/wine ratio during aging of wine B. During aging on yeast cells, there is an increase of proteins and peptides in wine that may be associated with autolysis (Martinez-Rodriguez et al. 2003), in which the yeast releases intracellular compounds into the wine (Pérez-Serradilla and Luque de Castro 2008). Wine A was not aged onto the lees. Its protein concentration was at the lower limit of the reported range for concentration of proteins in wine: from 15 to 230 mg/L (Ferreira et al. 2002). The vast majority of proteins present in wine A are of grape origin, while in wine B proteins and peptides released from yeasts were also present.

Effect of bentonite fining on wine protein concentration. The different initial protein concentration modified the efficacy of bentonite clarification (Table 3): wine with less initial protein had a higher percentage of protein removal as previously reported (Achaerandio et al. 2001). The adsorption isotherm of the protein-bentonite system shows an increased adsorption at low solute concentration (Achaerandio et al. 2001, Blade and Boulton 1988, de Bruijn et al. 2009). Moreover, the low percentage of protein removal observed in wine B was due to the presence of polysaccharides and mannoproteins released by yeasts during aging on yeast lees. Yeast cell wall polysaccharides and glycosylated cell wall-derived yeast proteins increase during prolonged aging of the wine on yeast lees (Waters et al. 1993), a procedure required by a few specific wine styles and used in wine B. The occurrence of glycosylated proteins in wine is not common (Hsu and Heatherbell 1987, Waters 1991, Waters et al. 1993) and cell wall-derived yeast proteins found in wines are glycosylated (Marchal et al. 1996). Glycosylation confers stability to many proteins by carrying negative charges in the wine pH range. Such proteins are less susceptible to being removed by bentonite. Moreover, polysaccharides released during aging onto lees may establish electrostatic and ionic interactions with other wine components, resulting in the formation of either soluble or insoluble complexes in a process that is strongly dependent on their net electric charge and on the structure of their functional groups (Ferreira et al. 2002). The resulting increase in the colloidal matrix can negatively interfere with the protein's approach to bentonite sheets. Our results showed that the adsorption capacity of the bentonites for wine proteins varied with adsorbent sample and dose, as recently reported (de Bruijn et al. 2009). In Chardonnay A, 20 g/hL stabilized the wine protein as previously observed in Macabeu wine (Salazar et al. 2006), but this bentonite dose can lead to a higher percentage of protein adsorption than observed in our study (Puig-Deu et al. 1999). In wine B, percentage reductions in protein were very low, and the large effect of bentonite on polysaccharides-proteins reported elsewhere (de Bruijn et al. 2009) was not observed.

The different wine pH values modified the efficacy of bentonite clarification. The pH is the most important factor affecting adsorption, as it affects the end surface charge of the bentonite and the degree of ionization and speciation of the protein (Xifang et al. 2007). Thus, pH influences both the cationic charge of the protein and the relative exchange of hydrogen, protein, and sodium in the bentonite. More protein was removed from wine B at pH 3.60 than from wine A at pH 3.30. Since protein is less cationic at a higher pH, this could be due to less competition between hydrogen ions and the protein in a higher pH wine. Bentonites show a strong preference for hydrogen over sodium at similar concentrations and a strong preference for very large cations (Blade and Boulton 1988).

The different ethanol concentration also modified the efficacy of bentonite toward proteins. In water, bentonite swells and its layers separate, enabling molecules to enter the structure. It has been suggested that ethanol molecules, being larger than water molecules, separate layers even more (Blade and Boulton 1988). The mg/L of removed proteins was higher in wine B (14.20% v/v ethanol) than in wine A (11.00% v/v). The increase in bentonite swelling caused by ethanol allowed the protein molecules to enter the bentonite structure more easily, broadening the channel

to the interlayer of bentonite and increasing protein adsorption (Xifang et al. 2007, Achaerandio 2001).

There were significant differences among bentonites in external + internal SSAs determined by methylene blue titration. By this measure, Top Gran should be the bentonite with the strongest subtractive action. In fact, it had the highest efficacy in wine A despite its lowest Na^+/Ca^{2+} ratio, perhaps because of the very high external + internal SSA (402.57 ± 5.02 m^2/g) that provided a high charged surface area per unit weight. This could promote the adsorption of a greater protein fraction that was more cationic at the lower pH of wine A. These results seem to confirm that structural properties such as SSA prevail over Na^+/Ca^{2+} ratio. Superbenton, with the highest Na^+/Ca^{2+} ratio but four times lower SSA than Top Gran, did not adsorb as much protein. At higher pH and alcohol and when yeast-derived material was present (wine B), experimental clay was most effective at removing proteins. In this wine, protein adsorption was enhanced by fining with a bentonite with higher montmorillonite content (90–95%) and which probably contains magnesium smectite. This finding underscores the dependence of protein adsorption on wine style and on structural particularities of bentonite.

Effect of bentonite fining on Chardonnay odor-active compounds. Bentonite fining produced losses in some odor-active compounds. In wine A, richer in grape proteins, a pronounced interaction of ethyl butyrate with the proteic hydrophilic colloid through hydrogen bonds could be hypothesized. In wine B, the major interaction of ethyl butyrate may have been with the proteins released by yeasts, as previously reported (Lubbers et al. 1993). As a consequence, like proteins, ethyl butyrate in wine B would be less susceptible to removal by bentonite. Furthermore, at high protein concentrations, protein-protein interactions could diminish binding of aromatic molecules (Blade and Boulton 1988).

Our results showed greater depletion of ethyl hexanoate than observed elsewhere (Voilley et al. 1990): ~5% loss during bentonite stabilization. Moreover, the depletion was generally higher in wine A than in wine B, as with percentages of protein removed. We hypothesize the same removal mechanism proposed for ethyl butyrate: a linkage in wine A with the proteic hydrophilic colloid through hydrogen bonds and an interaction in wine B with macromolecules released by yeasts. These assumptions agree with reported observations (Langourieux and Crouzet 1997). Moreover, since each adsorbent material produced a different reduction of ethyl hexanoate, a direct interaction of this molecule with bentonite is proposed.

Ethyl octanoate removal was not affected by bentonite type or wine style (Table 6), but did differ by bentonite dose (Figure 3). For this molecule, binding with proteins should be similar for the two wines and is probably due to hydrophobic interactions (Lubbers et al. 1993, Landy et al. 1995). Bentonite fining was reported to produce 65% losses of ethyl octanoate (Voilley et al. 1990), a depletion significantly greater than the less than 40% shown by this study.

Removal of isoamyl acetate during bentonite fining, as reported previously (Voilley et al. 1990), was confirmed by our results. It could be hypothesized that in wine A, aroma removal involved both free molecules and those bound to proteins. In wine B, the reduced removal of isoamyl acetate during fining led to the assumption that this molecule is predominantly linked to macromolecules less susceptible to removal by bentonite. The linkage between macromolecules and isoamyl acetate was not broken during fining (Voilley et al. 1990).

In wine A, the percentage removal of phenylethyl acetate is similar to that of proteins (Table 3), implying a possible interaction between the aromatic ring of the molecule and hydrophobic protein sides as already reported for benzaldehyde (Fares et al. 1998). In wine B, 100 g/hL bentonite removed 90% of the phenylethyl acetate, implying direct adsorption of this compound onto bentonite sheets. A similar dose-dependent behavior was observed for β-phenylethanol, implying that this compound also interacts with proteins through the same mechanism.

Percentage removal of 1-hexanol was higher, as its initial concentration was lower, and generally greater than reported (Voilley et al. 1990). This implies a direct interaction of 1-hexanol with bentonite. In wine A, Top Gran demonstrated a strong specificity for this aroma compound, which was extensively removed at each concentration.

Among fatty acids, the removal of hexanoic acid in wine A varied with protein removal, implying that it was bound to proteins. However, in wine B, the percentage reduction was higher than for proteins, indicating a direct adsorption of the molecule onto bentonite. Even if it was not possible to find a simple explanation for the behavior of aromatic compounds from different chemical classes, our results show that octanoic acid behaved similar to isoamyl acetate, but varied more strongly. Because octanoic acid is more hydrophobic than isoamyl acetate (Log*P* of 3.05 and 2.26, respectively), it could be bound by proteins to a greater extent and thus be removed more by bentonite during fining. The hydrophobicity of aroma compounds as a key factor that promotes linkage with proteins and their removal during fining treatment has been widely studied (Damodaran and Kinsella 1980, Fares et al. 1998, Landy et al. 1995, Langourieux and Crouzet 1997, Lubbers et al. 1993, Voilley et al. 1990).

Conclusions

The effect of bentonite treatments on aroma substances in white wine depended on the chemical nature and initial concentration of the volatile compounds and on the abundance and nature of proteins in the wine. Only a few odor-active compounds were directly adsorbed by the bentonite; most were removed as an indirect effect of deproteinization. Wine proteins are normally classified as macromolecular colloids with a positive charge and hydrophilic character that confer stability. Some hydrophilic odor-active compounds undergo weak hydrogen binding onto protein surfaces, while more hydrophobic aromatic molecules

can link to interior protein sites with a stronger affinity for hydrophobic substances. When yeast-derived material represents an important fraction of wine macromolecules, colloids with the same electric charge as bentonite are held in suspension. Consequently, they are held apart from bentonite by electrostatic forces and do not precipitate. In this situation, increased opportunities for direct adsorption of odor-active substances onto bentonite sheets were hypothesized. The low adsorbent concentrations (20 g/hL) generally used to stabilize wine did not significantly affect the concentration of most aromatic substances. This result has important practical applications in selecting which bentonite dose and type are best for fining a particular wine style. Nevertheless, further studies on the mechanism of interaction among wine proteins, odor-active compounds, and bentonite should be encouraged.

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