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The complexity of protein haze formation in wines

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ABSTRACT

The mechanism responsible for protein haze formation in wines remains essentially to be elucidated. Current knowledge suggests the absolute requirement of one or more as yet unknown non-proteinaceous wine components (termed the X factor) for protein precipitation in wines. Using the single grape variety Arinto wine, naturally containing 280 mg protein/l, a series of heat stability tests were performed over a range of wine-relevant pH values (from 2.8 to 3.8). The results obtained indicate the existence of at least two different mechanisms responsible for the heat-induced precipitation of the Arinto wine proteins: one occurring only at the higher pH values, that appears to result from isoelectric precipitation of the proteins; another prevailing at the lower pH values, but possibly operating also at other pH values, that depends on the presence of the X factor. Therefore, conclusive evidence is provided for the existence of the X factor, here defined as one or more low molecular mass wine components that sensitise proteins for heat-induced denaturation at low wine pH values and whose presence is a pre-requisite for the precipitation of proteins in wines under these circumstances. The chemical nature of protein aggregation was further analysed as a function of pH. Neither of the two proposed mechanisms responsible for the heat-induced precipitation of the wine proteins is electrostatic in nature, lectin-mediated or divalent cation-dependent. Both mechanisms show minimum turbidity at pH 7, but increased turbidity towards lower and higher pH values.

1. Introduction

Wines contain varying amounts of different nitrogenous substances, amongst which are proteins. These polymers do not contribute significantly to the nutritive value of wines since their concentration varies typically from 15 to 300 mg/l (Ferreira, Piçarra-Pereira, Monteiro, Loureiro, & Teixeira, 2002; Waters et al., 2005). However, the presence of a residual amount of unstable protein in wines is of great concern for winemakers. Slow denaturation of wine proteins, possibly resulting from unfavourable storage conditions, is thought to originate protein aggregation and flocculation into a hazy suspension, leading to the appearance of a haze or deposit in the bottled wine. This unattractive haze does not affect the olfactory and gustatory characteristics of the wine. However, translucency is of vital importance to wine quality because this property makes the first impression on the consumer, who will reject wines containing cloudy precipitates regardless of how the wine tastes (Ferreira, Monteiro, Piçarra-Pereira, Loureiro, & Teixeira, 2004). For these reasons, protein precipitation in wines reduces their commercial value and indicates that they are unstable and therefore unacceptable for sale (Bayly & Berg, 1967; Hsu & Heatherbell, 1987a; Waters, Wallace, & Williams, 1991, 1992). Despite significant advances in wine protein research, the precise molecular mechanism of protein haze formation and the factors involved remain largely to be elucidated.

The presence of protein in wine is certainly a pre-requisite for haze formation and it seems generally accepted that the higher the wine total protein content, the higher its tendency to become unstable (Mesquita et al., 2001). For these reasons, for a number of years the study of protein haze formation in wines was essentially focused on the proteins themselves.

Although proteins from *Saccharomyces cerevisiae* (Dambrouck et al., 2003; Dupin et al., 2000) and *Botrytis cinerea* (Marchal, Warchol, Cilindre, & Jeandet, 2006; Marchal et al., 1998) have been detected in wines, the majority of the wine proteins appear to derive from the grape pulp (Ferreira et al., 2000). Wines typically contain a very large number (many tens and possibly many more) of distinct polypeptides, exhibiting similar molecular masses but subtle differences in electric charge (Monteiro et al., 2001). Most of these polypeptides exhibit a very high degree of homology and have been identified as pathogenesis-related (PR) proteins, regardless of the grape variety, region, year or winemaking conditions (Ferreira et al., 2000; Monteiro et al., 2001; Waters, Shirley, & Williams,

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1996). Most notably, these proteins include chitinases, thaumatin-like proteins and osmotins (Monteiro et al., 2001; Waters, Hayasaka, Tattersall, Adams, & Williams, 1998), which are particularly stable under winemaking conditions (low pH, proteolysis), passing selectively into the wine. In this sense, the actual pattern of polypeptides that accumulate in mature grapes and wines is determined by the environmental and pathological conditions that prevail during vegetative growth (Ferreira et al., 2004). Infection with common grapevine pathogens or skin contact, such as occurs during transport of mechanically harvested fruit, results in enhanced concentrations of PR proteins in wine (Cilindre, Castro, Clément, Jeandet, & Marchal, 2007; Waters et al., 2005).

It was initially proposed that wine instability is solely related to its protein content. If this is correct, wine instability can be evaluated by determining its total soluble protein content (Anelli, 1977; Somers & Ziemelis, 1973). Supporting this hypothesis is the study of Koch and Sajak (1959), who used paper electrophoresis to show that wine contains two major protein fractions, both of which decrease upon heat treatment. However, other studies have shown that protein instability does not correlate well with the wine total protein content, and, therefore, the potential of wine to form haze is not predictable from its protein concentration (Bayly & Berg, 1967; Moretti & Berg, 1965). If this is the case, two alternative hypotheses may be advanced to explain the insolubilization of proteins in wines: (a) individual proteins behave differently in their sensitivity to heat denaturation, contributing differentially to haze formation, in which case, only part of the protein mixture is responsible for instability rather than the entire protein content; (b) although protein-dependent, the development of turbidity in wines is controlled by one or more factors of non-protein origin.

According to the first hypothesis, the molecular properties of each protein influence its natural tendency to precipitate. However, the nature of the proteins responsible for wine turbidity remains unclear. There is also conflicting evidence in the literature as to which proteins are responsible for haze and deposit formation. Thus, some reports suggest that the lower molecular mass, lower p*I* proteins are the major and most important fractions contributing to protein instability in wines (Hsu & Heatherbell, 1987a, 1987b; Hsu, Heatherbell, Flores, & Watson, 1987; Mesrob, Gorinova, & Tzakov, 1983). Other studies indicate that the lower molecular mass and higher p*I* (Heatherbell et al., 1984; Lee, 1986; Ngaba & Heatherbell, 1981) or the higher molecular mass proteins contribute most to heat instability. Yet other investigations revealed that all the major wine protein fractions are present in wine hazes and all have been shown to be heat unstable (Waters, 1991; Waters & Høj, 1999; Waters, Wallace, & Williams,1990, 1991, 1992). Besides isoelectric point and size, glycosylation was another protein property addressed when analysing protein haze formation in wines. It has been generally reported that glycosylation confers stability to many proteins. Indeed, comparison of native glycoproteins to non-glycosylated versions of the same shows that the presence of glycans increases stability, solubility, and resistance to proteases (Helenius & Aebi, 2004). However, there are conflicting reports in the literature about the glycosylation status of wine proteins. Thus, whereas some researchers claimed, but have not proved, that all wine proteins are glycoproteins (Paetzold, Dulau, & Dubourdieu, 1990; Yokotsuka, Ebihara, & Sato, 1991; Yokotsuka, Nozaki, & Takayanagi, 1994), the majority of studies suggest that the occurrence of glycosylated proteins in wines is not common (Hsu & Heatherbell, 1987a; Waters, 1991; Waters, Wallace, Tate, & Williams, 1993). Marchal, Bouquelet, and Maujean (1996) isolated three proteins from a Champenois Chardonnay still wine by concanavalin A affinity chromatography, suggesting the presence of glycosydic side chains. The three proteins were not susceptible to *O*-glycosidases but one of them undergoes a 3.1 kDa variation after treatment with peptide-*N*-glycanase F, indicating that it is a true *N*-glycosyl protein. In addition, some glycosylated, cell wall derived yeast proteins were found in wines.

Only recently was the attention of wine researchers moved towards compounds of non-proteinaceous nature. Indeed, the observations that wines are essentially composed of identical sets of polypeptides that have been identified as PR proteins and that the haze forming wine proteins are PR proteins apparently similar in wines vinified from different grape varieties (Dawes, Boyes, Keene, & Heatherbell, 1994; Ferreira et al., 2000; Hsu & Heatherbell, 1987b; Monteiro et al., 2001; Pueyo, Dizy, & Polo, 1993; Waters et al., 1992, 1996) support the view that protein insolubilization is not determined by the protein molecules themselves, depending on some other non-protein factors. In other words, protein haze formation in wines is controlled by factors of non-protein origin (Ferreira et al., 2002). Current knowledge indicates that there appears to be an absolute requirement for one or more as yet unknown non-proteinaceous wine component(s) (termed the X factor) if a visible protein haze is to be formed (Mesquita et al., 2001; Waters et al., 2005). In this respect, using artificial model wine solutions, Waters and collaborators recently proposed sulfate anion as a candidate for the missing essential X factor (Pocock, Alexander, Hayazaka, Jones, & Waters, 2007). Nevertheless, these authors showed that PVPP fining of commercial wines results in a reduction in protein haze, an observation that suggests that phenolic compounds play a modulating role in wine haze formation.

In this work, we have used the single grape variety Arinto wine to study the pattern of protein haze formation over a wide range of typical wine pH values. The wine was subsequently fractionated in an attempt to explain the molecular mechanisms involved in protein instability.

2. Materials and methods

2.1. Preparation of wine

The varietal white wine used in this work was prepared from Arinto grapes (2002). Ripened grapes (25 kg) were harvested and processed into wine by a conventional microvinification procedure, according to the classical white wine technology. Fermentation was carried out at about 16 °C for eight days. Bentonite was not added during the fermentation. The wine was found to contain 280 mg protein/l. The wine was divided in 100 ml aliquots and stored at −20 °C until used. To avoid repeated thawing and freezing, a new aliquot was used for each experiment.

2.2. Fractionation of the wine into <3 kDa and >3 kDa components

The wine was fractionated into <3 kDa and >3 kDa components using 3 kDa cut-off ultrafilters (Centriplus YM-3 3,000 MWCO membranes, Millipore), according to the manufacturer's instructions. Separation was achieved by centrifugation at 3000*g* for 8 h. Each ultrafilter retentate was washed twice with water (pH adjusted to 2.8). All fractions were reconstituted with water (pH adjusted to 2.8) to their original wine concentrations, centrifuged at 10000*g* for 5 min and assayed for protein.

2.3. Purification and concentration of the wine soluble proteins

Wine aliquots (100 ml) were thawed and centrifuged at 10000*g* for 5 min to remove insoluble particles. The resulting supernatant was desalted at 4 °C on PD-10 prepacked Sephadex G-25M columns (9.1 ml bed volume; GE Healthcare), previously equilibrated with water (Milli-Q plus, Millipore). The desalted wines (140 ml) were subsequently lyophilised (Edwards Micro Modulyo freeze

drier) and the dried residues were resuspended and solubilised in 4 ml of 20 mM citrate–NaOH buffer, pH 2.5.

2.4. Isolation of the wine total soluble protein

A sample (2 ml) containing the wine total protein was purified by FPLC cation exchange chromatography on the Mono S HR5/5 column (GE Healthcare) previously equilibrated in 20 mM citrate–NaOH buffer, pH 2.5. The flow rate was 1.5 ml/min and the bound proteins were eluted with a step gradient (0–1 M) of NaCl. The fraction containing the wine total soluble protein was desalted into water in PD-10 columns and lyophilised.

2.5. Two-dimensional electrophoresis

Isoelectric focusing (first dimension) was performed using the IPGphor System (GE Healthcare). Immobiline Drystrip gel strips (IPG strips) (13 cm, pH 3–10) were obtained from GE Healthcare. IPG strips were rehydrated with 250 μl of a solution containing 0.5% (v/v) IPG-buffer pH 3–10, 7 M urea, 2 M thiourea, 2% (v/v) NP-40, 1% (v/v) dithiothreitol and protein samples in the IPGphor strip holders. The program utilised for isoelectric focusing included the following steps: rehydration – 30 V, 12 h; step 1 – 200 V, 1 h; step 2 – 500 V, 2 h; step 3 – 1000 V, 2 h; step 4 – 8000 V, 3.5 h. After focusing, the gel strips were immediately frozen at −80 °C.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; second dimension) was performed by a modification (Christy, Latart, & Osterhoudt, 1989) of the methods described in Weber and Osborn (1969) and Laemmli (1979), except that the gel contained only the separating gel. The gel strips were thawed and equilibrated for 15 min, with agitation, in 50 mM Tris–HCl buffer pH 8.8, containing 6 M urea, 26% (v/v) glycerol, 2% (w/v) SDS and 1% (w/v) dithiothreitol. The strips were subsequently equilibrated for another 15 min, with agitation, in a similar solution that contained 2.5% (w/v) iodoacetamide (instead of the dithiothreitol), placed on top of the SDS–PAGE gel, sealed with 0.5% (w/v) agarose and electrophoresed (220 V, 15 mA, for 15 min followed by 220 V, 30 mA). Protein spots were visualised by staining the gels with Coomassie Brilliant Blue (CBB) R250. The molecular mass polypeptide standards used ranged from the 45 kDa ovalbumin to the 14.2 kDa bovine milk α-lactalbumin.

2.6. Protein determination

Protein in wine and samples was measured by a modification of the Lowry method (Bensadoun & Weinstein, 1976), using bovine serum albumin as the standard.

2.7. Particle size analysis

Particle size distribution patterns were estimated with laser diffraction particle size analyser (model LS 130; Coulter Instruments) using the Fraunhofer optical model. This instrument presents results for a sample over the particle diameter range of 0.1–900 μm as either the percentage of the total number of particles (number%) or the percentage of the total particle volume (volume%) in each of 100 channels of logarithmically increasing diameter.

2.8. Preparation of samples for turbidity measurements

Wine samples were thawed, centrifuged at 10000*g* for 5 min to remove insoluble particles and the pH adjusted at the appropriate values. The dried residue containing the total wine protein, previously isolated by FPLC cation exchange chromatography as described above, was dissolved in enough water (pH adjusted to 2.8) to give a final protein concentration of 280 mg/l. The resulting solution was centrifuged at 10000*g* for 5 min and the supernatant used for protein quantification and pH adjustment at selected values. The >3 kDa (containing 280 mg protein/l) and the <3 kDa (containing no protein) wine fractions were adjusted to the desired pH values.

Where appropriate, a model wine solution (Waters et al., 2007) was used for turbidity measurements. This solution was prepared as follows: tartaric acid (4 g/l) was first dissolved in water. Then, ethanol (12% v/v) was added, the pH adjusted with NaOH to 2.8 and the protein (280 mg/l) dissolved. Finally, aliquots of the model wine were adjusted to the desired pH values and centrifuged at 10000*g* for 5 min to remove any insoluble material.

2.9. Heat stability tests

The heat stability of wine samples was determined by the procedure recommended by Pocock and Rankine (1973). All measurements were made in triplicate and appropriate controls were performed. Five milliliter samples were saturated with nitrogen and sealed in test tubes with screw caps. The tubes were heated at 80 °C in a water bath for 6 h, held at 4 °C for 16 h, and allowed to warm to room temperature. The increase in turbidity was detected spectrophotometrically (Shimadzu UV-2100 spectrophotometer) at 540 nm and 25 °C in 1 ml plastic cuvettes. Occasionally, colour development in the heated samples was observed independent of the increase in temperature. Whenever colour developed the experiment was repeated, so that the data presented is entirely based on experiments in which colour development was not detected.

3. Results and discussion

A number of non-protein factors, recently reviewed by Waters et al. (2005), have been proposed as absolutely required for the formation of visible protein haze in wines subjected to the industry standard heat test (80 °C, 6 h). Among these are the wine pH, ethanol, polysaccharides, metal ions and phenolic compounds. None of them has been analysed in sufficient depth to understand its precise role in wine protein haze formation. Sulfate anion has recently been proposed as a candidate for the non-protein factor required for wine haze formation (Pocock et al., 2007). In addition, interactions between these factors are likely to influence protein behaviour in wines.

Few and incomplete studies have been performed on the role exerted by the wine pH on the solubility comportment of wine proteins and much of the published work has not directly involved wine (Waters et al., 2005). This is particularly important in the case of amphoteric substances, such as proteins, whose net electrical charge is determined by the surrounding pH. Therefore, the isoelectric point (p*I*) of a protein is the pH value at which the protein bears a zero net charge.

Using artificial model solutions that mimicked beer, wine and fruit juice, and gelatin and catechin as the protein and phenolic compounds, respectively, Siebert and collaborators (Siebert, Carrasco, & Lynn, 1996; Siebert & Lynn, 2003) studied the effect of pH on the formation of protein–polyphenol complexes and concluded that maximum haze occurred at pH 4.0–4.5 when ethanol was 12% (v/v), with less haze at lower and higher pH values. In a different study, Mesquita et al. (2001) used actual wine samples and observed that the wine became increasingly heat stable as the pH rose from 2.5 to 5.5 and to 7.5, indicating that pH does play an important role in protein haze formation and that a higher pH reduces the potential to form protein haze in response to heat. However, it is not apparent from this study the magnitude of importance that pH variation exerts on protein haze formation

within the range encountered in wine (typically between 2.8 and 3.8).

The single grape variety Arinto wine, naturally containing 280 mg protein/l, was subjected to the heat stability tests performed at different pH values, ranging from 2.8 to 3.8. The results obtained, presented in Fig. 1 (◇), show that the pattern of protein haze formation, as measure by the absorbance at 540 nm, does not change significantly over the range of pH values tested. In a subsequent experiment, the total soluble protein from the Arinto wine was isolated by fast desalting, to remove the <5 kDa compounds fraction, followed by FPLC cation exchange chromatography to remove polysaccharides and polyphenols (Monteiro et al., 1999). The isolated protein was desalted into water, lyophilised, dissolved in water (pH adjusted to 2.8) and centrifuged to give an approximate protein concentration of 280 mg/l. Aliquots of this solution were adjusted to the desired pH values (2.8, 3.0, 3.2, 3.4, 3.6 and 3.8) and submitted to the heat stability test. When compared to the data presented for the Arinto wine, the results obtained, also shown in Fig. 1 (o), produce a pattern of protein haze formation that is virtually identical at high pH values, but strikingly different at low pH values. To disclaim the possibility that the protein haze differences in wine versus water seen in Fig. 1 derive from the low ionic strength, pH adjusted-water, a similar experiment was performed in a model wine solution containing isolated protein (280 mg/l), ethanol (12% v/v) and tartaric acid (4 g/l), providing an ionic strength similar to that of wine (Waters et al., 2007). The results obtained, presented in Fig. 1 (Δ), clearly indicate that the protein haze difference observed in wine versus water can not be attributed to the low ionic strength of the pH adjusted-water alone. In addition, the model wine solution appears to exert a stabilizing effect upon the wine proteins, an effect attributed to the presence of tartaric acid in the model wine solution and that is currently under investigation (results not shown). As a whole, the data presented in Fig. 1 suggests the existence of at least two different mechanisms responsible for the heat-induced precipitation of the Arinto wine proteins: one operating at the lower and possibly other pH values, requiring the presence of a substance of non-proteinaceous nature (that may be a <5 kDa compound or a large molecular mass polysaccharide or polyphenol); another occurring only at the higher pH values, that appears to result from an inherent property of the proteins themselves. To get an insight into the two mechanisms, the following set of experiments were undertaken.

As described above, the pattern of turbidity of the Arinto wine does not change significantly with pH within the range 2.8–3.8 (see Fig. 1). However, the turbidity formed at pH 2.8 looks quite different to the naked eye than that formed at pH 3.8 (Fig. 2). In other words, the heat-induced haze is smooth and homogeneous in the pH 2.8 Arinto wine, but coarse and flocculated in the pH 3.8 Arinto wine. These observations were confirmed by particle size analysis, as shown Table 1 and in Fig. 3A for the turbidity formed at pH 2.8 and in Fig. 3B for the turbidity formed at pH 3.8. There is clearly an increment in the average particle size when the pH increases from 2.8 to 3.8.

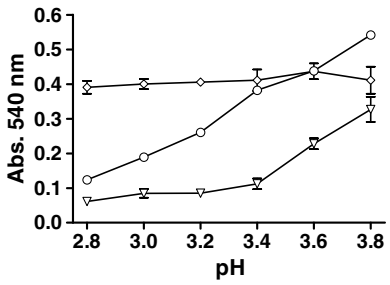

Fig. 1. Heat stability tests of the Arinto wine (naturally containing 280 mg/l; ◇) or of an aqueous solution containing 280 mg/l of the isolated Arinto wine protein (○) or of a model wine solution composed of isolated protein (280 mg/l), ethanol (12% v/v) and tartaric acid (4 g/l) (Waters et al., 2007; ▽) were performed at different pH values (2.8, 3.0, 3.2, 3.4, 3.6 and 3.8), as described in Section 2. All experiments were performed in triplicate. Vertical bars represent plus or minus the standard deviation, shown when bar is bigger than symbol.

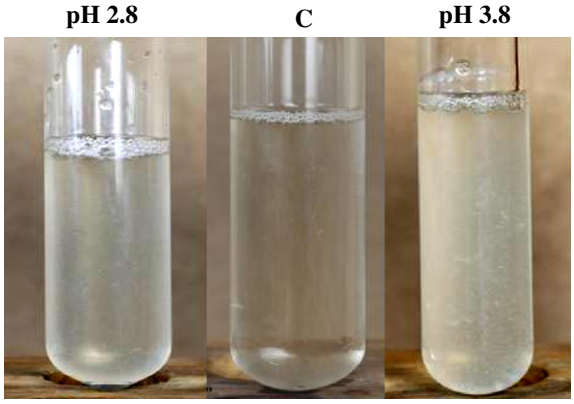

Fig. 2. Samples of Arinto wine were adjusted to pH 2.8 or to pH 3.8 and subjected to the heat stability test. Control (C): Arinto wine that was not submitted to the heat treatment.

Table 1
Observed size distribution of 0.1–900 μm diameter particles in samples of Arinto wine adjusted to pH 2.8 or 3.8 and subjected to the heat stability test

% of particles bigger than	Particle diameter (μm)	
	pH 2.8	pH 3.8
10	82.08	116.4
25	49.02	72.65
50	15.79	49.04
75	8.15	29.59
90	2.31	20.05
Mean	31.7	55.72
Median	15.79	49.04

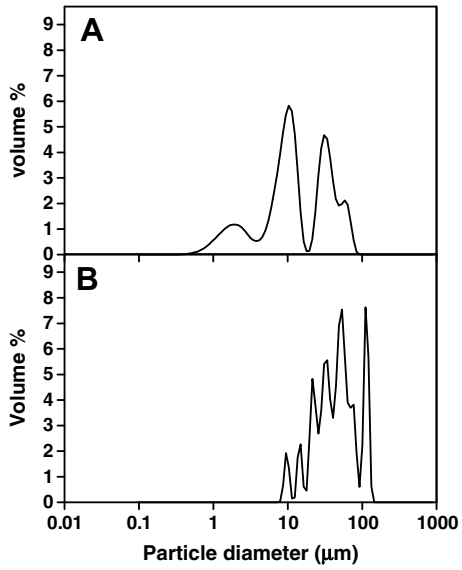

Fig. 3. Observed size distribution of 0.1 to 900 μm diameter particles in samples of Arinto wine adjusted to pH 2.8 (A) or 3.8 (B) and subjected to the heat stability test. Measurements were made as described in Section 2.

Thus, the previous results indicate that within the range pH 3.4–3.8, the Arinto wine proteins precipitate by themselves upon heat treatment, apparently with no need for the presence of other wine components. Moreover, the flocculate-type insolubilization suggests that isoelectric precipitation may be involved. To test this hypothesis, the experiment illustrated in Fig. 1 was extended into pH values that are not technological relevant in what wines are concerned, i.e. within the range 4.0–7.0. The results obtained, displayed in Fig. 4, show that the proteins in the Arinto wine are heat unstable between pH 2.8 and 6, but become rapidly resistant to heat precipitation above pH 6, confirming our previous results (Mesquita et al., 2001). In contrast, the isolated Arinto wine proteins dissolved in water show a broad peak of instability centered around pH 4.0 when subjected to the heat stability test, becoming gradually more stable towards lower and higher pH values and reaching heat stability at pH 2.8 and pH 6.0. Not surprisingly, this peak of instability coincides with the isoelectric point of most Arinto wine proteins, as determined by two-dimensional electrophoresis (Fig. 5). The large effect of wine pH on the solubility of the wine proteins and on their relative stability in wines as determined by the protein p*I*s was proposed by Bayly and Berg, as early as 1967. Proteins in wines are cationic if the wine pH < protein p*I*, anionic if the wine pH > protein p*I* or neutral if wine pH ~ protein p*I*. The smaller the difference between the wine pH and the protein p*I*, the lesser the net charge carried by that protein and the lower its stability. Therefore, the isoelectric properties of proteins greatly influence their tendency to precipitate according to the wine pH. All these observations strongly support the view that the mechanism responsible for the heat-induced precipitation of the Arinto wine proteins at high pH values (i.e. 3.4–3.8) is due to isoelectric precipitation. Fig. 4 further suggests that one or more non-proteinaceous wine components are required for the Arinto wine protein instability detected between pH 5 and pH 6.5. However, due to the lack of technological significance, this observation was not further investigated.

The two-dimensional gel illustrated in Fig. 5 further suggests the interaction of the wine proteins with non-protein wine components during the heat treatment performed at pH 3.2 – compare the polypeptide patterns of Arinto wine total protein (Fig. 5A) with that of the precipitated protein obtained after the heat stability test (Fig. 5B). Interestingly, all Arinto wine proteins appear to have been precipitated by the heat treatment, as shown by the absence of proteins in the supernatant obtained after the heat stability test (Fig. 5C).

The analysis of the mechanism underlying the heat-induced denaturation of the Arinto wine proteins at low pH values revealed to be more complex. In a first approach, the Arinto wine was subjected to ultrafiltration using a 3 kDa cut-off ultrafilter membrane.

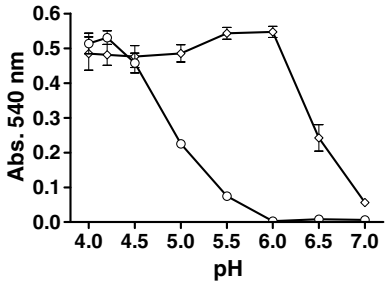

Fig. 4. Heat stability tests of the Arinto wine (naturally containing 280 mg protein/l; ◇) or of an aqueous solution containing 280 mg/l of the isolated Arinto wine protein (○) were performed at different pH values (4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0), as described in Section 2. All experiments were performed in triplicate. Vertical bars represent plus or minus the standard deviation, shown when the bar is bigger than the symbol.

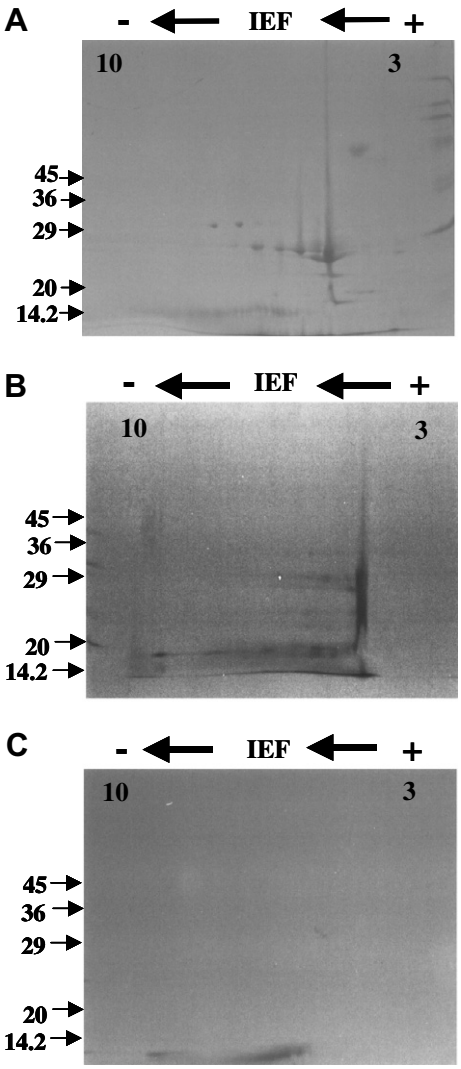

Fig. 5. Two-dimensional electrophoresis of the Arinto wine proteins. (A): Arinto wine under control conditions. (B) and (C): pellet and supernatant, respectively, obtained after subjecting the Arinto wine to the heat stability test performed at pH 3.2 (the natural Arinto wine pH) followed by centrifugation. Total proteins were stained with Coomassie Brilliant Blue. IEF: isoelectric focusing performed between pH 3 and 10, as indicated. The molecular masses of standards are given in kDa.

The fraction containing the >3 kDa components was thoroughly washed with water (pH adjusted to pH 2.8) to ensure the complete absence of <3 kDa compounds. The volume of each of the fractions (<3 kDa and >3 kDa) was always adjusted with water (pH adjusted to 2.8) to their original wine concentrations. When the fraction containing the Arinto wine <3 kDa components was subjected to the heat stability test over the range of pH values 2.8–3.8, the result presented in Fig. 6 was obtained. No turbidity was detected for any of the pH values analysed, an expected result if we consider that this fraction contains no protein at all. When the fraction containing the Arinto wine >3 kDa components was analysed in a similar experiment, a gradual increase in turbidity was observed from the lower (pH 2.8) to the higher (pH 3.8) pH values studied (Fig. 6). However, this increase is not as sharp as the one observed for the isolated Arinto wine protein dissolved in water (280 mg protein/l; Fig. 1). This discrepancy may be tentatively explained if we consider that the >3 kDa fraction contains proteins (280 mg/l) and polysaccharides whereas the isolated Arinto wine protein dissolved in water is free from these polymers (Monteiro et al., 1999). Waters and collaborators (Dupin et al., 2000; Waters,

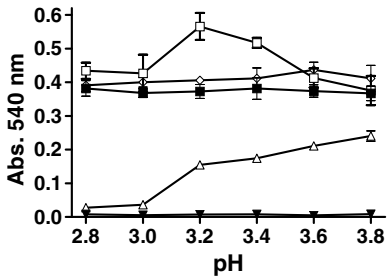

Fig. 6. Heat stability tests of the Arinto wine individual or combined fractions. Arinto wine (control; naturally containing 280 mg protein/l; ◇), the Arinto wine <3 kDa components fraction (containing no protein; ▼), the Arinto wine >3 kDa components fraction (containing 280 mg protein/l; Δ), the combined Arinto <3 kDa components fractions and >3 kDa components fractions (containing 280 mg protein/l; ■), and the Arinto <3 kDa components fraction back-added with 280 mg/l of previously isolated Arinto wine protein (□) were subjected to the heat stability test performed in a range of pH values as described in the Section 2. All experiments were performed in triplicate. Vertical bars represent plus or minus the standard deviation, shown when the bar is bigger than the symbol.

Pellerin, & Brillouet, 1994a; Waters, Pellerin, & Brillouet, 1994b; Waters et al., 1993) described mannoproteins termed "haze – protective factors" that protect wines from protein haze.

As expected, the combination of the <3 kDa and >3 kDa fractions restored the pattern of protein haze formation of the Arinto wine over the range of pH values tested (Fig. 6). A similar result was achieved when an identical experiment was performed after adding the <3 kDa Arinto fraction to isolated Arinto wine protein (280 mg/l; Fig. 6). However, in this particular case, an increment in turbidity is observed at pH 3.2 and 3.4 (□ in Fig. 6) when compared to the pattern of turbidity of the Arinto wine (◇ in Fig. 6). Once again, this difference may be tentatively explained by the presence of haze-protective factors in the wine (Waters et al., 1993, 1994a, 1994b). As a whole, the data presented in Fig. 6 indicate that at low pH, and possibly at other pH values, protein haze formation in the Arinto wine exhibits an absolute requirement for a low molecular mass (<3 kDa) Arinto wine component – the so called X factor, that may now be defined as one or more low molecular mass wine components that sensitise proteins for heat-induced denaturation at low wine pH values and whose presence is a pre-requisite for the precipitation of proteins in wines under these circumstances. This conclusion is in good agreement with the recent proposal that sulfate anion is a candidate for the missing essential factor (Pocock et al., 2007).

To further characterise the chemical nature of protein aggregation within the range of pH 2.8–3.8, samples of Arinto wine were subjected to the heat stability test at pH 2.8 and 3.8 and subsequently treated with increasing concentrations of ethylenediaminetetraacetic acid (EDTA; Fig. 7A), ethyleneglycol bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA; Fig. 7B), sodium chloride (Fig. 7C) or sodium hydroxide (Fig. 7D). Indeed, there are many examples of the participation of electrostatic interactions in protein aggregate formation, all of which are typically broken by high ionic strength or a change in pH. In some cases, divalent cations, such as Ca^{2+} or Mg^{2+}, are involved, acting as bridges between adjacent, negatively charged protein molecules. Bridging of calcium ions between negatively charged protein molecules is observed, for example, during cheese making, in the cross-linking between milk submicelles, where the calcium ions form bridges between the negatively charged phosphate groups of α- and β-casein molecules present in adjacent submicelles (Coultate, 1989). A similar mechanism was proposed to explain the typical insolubility of legume globulins in water, which is apparently due to the electrostatic involvement of calcium and magnesium ions in the macromolecular aggregation of legume seed storage proteins (Ferreira, Freitas, & Teixeira, 2003). Calcium may also participate in the self-aggregation of glycosylated, multivalent lectins. A lectin isolated from the bark of *Sophora japonica*, for example, is self-aggregatable due to the binding activities of all its four subunits, which enable them to recognise and bind N-linked oligosaccharide chains on three of the four subunits (Ueno, Ogawa, Matsumoto, & Seno, 1991).

The results illustrated in Fig. 7 clearly show that neither of the two proposed mechanisms responsible for wine protein precipitation at low or high wine pH is electrostatic in nature, lectin-mediated or divalent cation-dependent.

Although not relevant from the technological point of view, surprising results were obtained when samples of Arinto wine, both

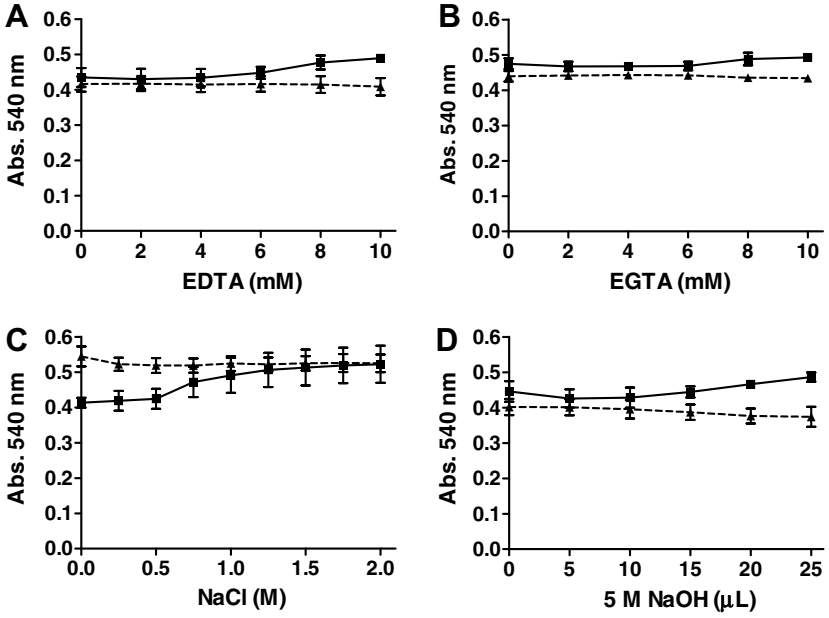

Fig. 7. Samples of Arinto wine were adjusted to pH 2.8 (--■--) or to pH 3.8 (---▲---) and subjected to the heat stability test, as described in the methods section. Subsequently, increasing concentrations of EDTA (A), EGTA (B), NaCl (C) and 5 M NaOH (D) were added and their effect on wine protein turbidity measured. All the experiments were performed in triplicate. Vertical bars represent plus or minus the standard deviation, shown when bar is bigger than symbol.

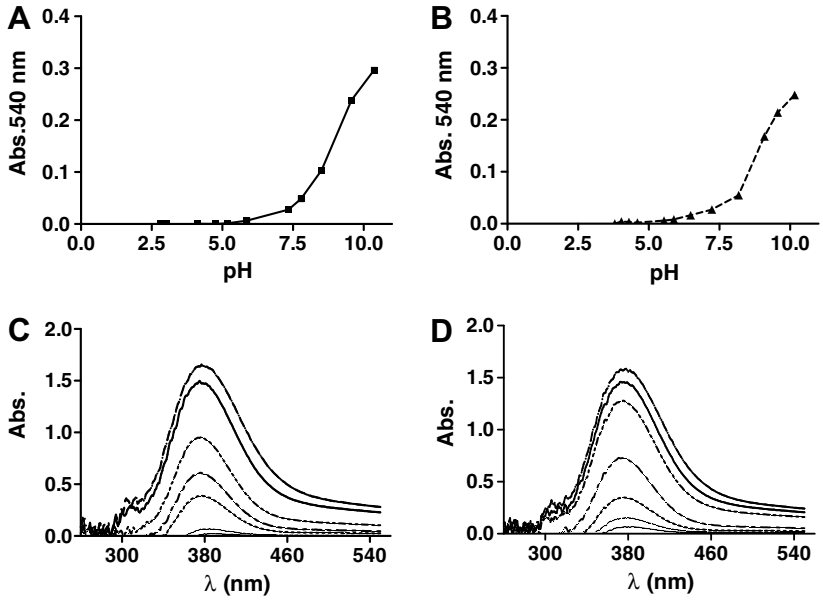

Fig. 8. Effect of pH on the absorption spectrum of Arinto wine. Samples of Arinto wine were adjusted to pH 2.8 (A and C) or 3.8 (B and D). The wine pH was gradually increased by stepwise addition of minute volumes of a concentrated NaOH solution. (C) ––– 5.18; - - - - 5.87; ..... 7.34; ·–·–· 7.78; ··–··– 8.51; 9.57; –10.37. (D) ––– 5.89; - - - - 6.48; ..... 7.23; ·–·–· 8.18; ··–··– 9.09; –9.56; –10.16.

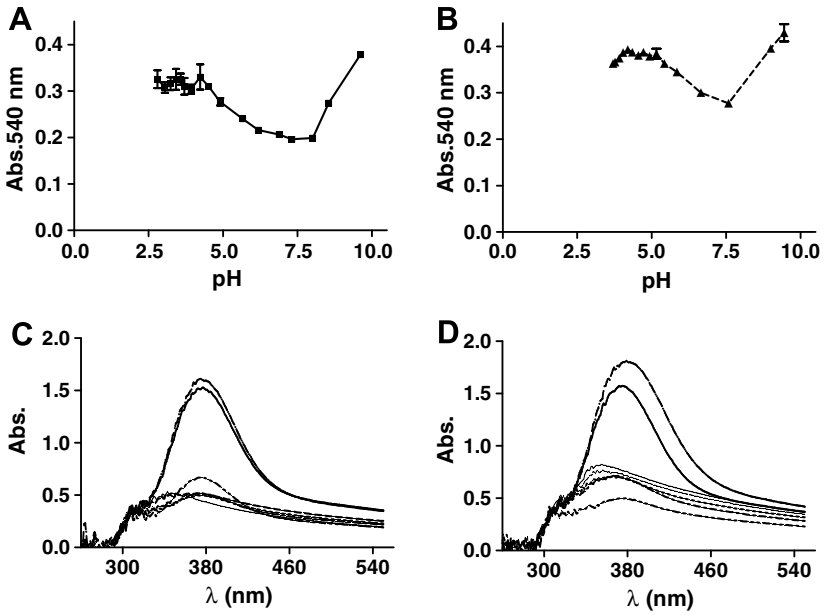

Fig. 9. Effect of pH on the absorption spectrum of Arinto wine previously subjected to the heat stability test. Samples of Arinto wine were adjusted to pH 2.8 (A and C) or 3.8 (B and D) and then subjected to the heat stability test as described in the methods section. The wine pH was subsequently gradually increased by the stepwise addition of minute volumes of a concentrated NaOH solution. (C) ––– 3.45; - - - - 4.79; ..... 5.96; ·–·–· 6.93; ··–··– 8.45; –9.10; –10.13. (D) ––– 4.08; - - - - 4.65; ..... 5.63; ·–·–· 6.28; ··–··– 7.39; –8.85; –9.93.

before or after the heat stability test, were incubated at very high pH values. The data illustrated in Fig. 8 indicates that above pH 7.5 the Arinto wine colour changes to yellow and becomes particularly intense above pH 9.5, concomitantly with the appearance of an absorption peak at approximately 380 nm. At the same time, there is a visual and gradual increase in wine turbidity, as supported by the increment at A_{540}. Nevertheless, all these effects are readily reversible by a subsequent drop in the wine pH.

Fig. 9 depicts a similar experiment but starting with Arinto wine that was previously subjected to the heat stability test performed at pH 2.8 (Fig. 9A and C) or at pH 3.8 (Fig. 9B and D). There is a similar yellowing for the higher pH values tested, as judged for the absorption peak at 380 nm. Two major differences are detected when Figs. 8 and 9 are compared: (i) for the heat-treated wine (both at pH 2.8 and 3.8; Fig. 9A and B, respectively), turbidity reaches a minimum at pH 7; (ii) due to the heat-induced turbidity, the absorption spectra shown in Fig. 9C and D are shifted upwards when compared to those in Fig. 8C and D.

As a whole, these results may be interpreted to mean that pH exerts a multitude of effects on wine components, some of which

are highly relevant form the technological point of view as they exert a direct influence on the protein solubility.

The data reported in this manuscript contradicts the initial proposal that wine instability can be evaluated by determining its total soluble protein content (Anelli, 1977; Somers & Ziemelis, 1973), but supports the currently accepted view that although protein-dependent, the development of turbidity in wines is controlled by one or more factors of non-protein origin (Dawes et al., 1994; Ferreira et al., 2000; Ferreira et al., 2002; Hsu and Heatherbell, 1987b; Mesquita et al., 2001; Monteiro et al., 2001; Pocock et al., 2007; Pueyo et al., 1993; Waters et al.,1992, 1996, 2005). Furthermore, in contrast to a considerable number of reports (Heatherbell et al., 1984; Hsu and Heatherbell, 1987a, 1987b; Hsu et al., 1987; Lee, 1986; Mesrob et al., 1983; Ngaba & Heatherbell, 1981), the data presented in Fig. 5 supports the finding reported by a number of researchers (Waters, 1991; Waters & Høj, 1999; Waters et al., 1990, 1991, 1992) that all the major wine protein fractions are present in wine hazes and all have been shown to be heat unstable.

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