UNIVERSITÀ DEGLI STUDI DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Agronomia Animali Alimenti Risorse Naturali e Ambiente

DOTTORATO DI RICERCA IN:

VITICOLTURA, ENOLOGIA E MARKETING DELLE IMPRESE VITIVINICOLE

CICLO XXIII

NEW APPROACHES FOR PROTEIN STABILIZATION OF WHITE WINES

APPROCCI INNOVATIVI PER LA STABILIZZAZIONE PROTEICA DEI VINI BIANCHI

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Data Consegna Tesi

31 Gennaio 2012

Acknowledgements

This PhD thesis was supported by provincia di Treviso and the international collaboration at “Australian Wine Research Institute” (Australia) in part by Fondazione Aldo Gini (Padova).

A particular acknowledgement to my supervisor Professor Andrea Curioni for giving me the opportunity to work with him during these years, for the discussion about the experiments and for all time spent in the revision of this manuscript. I also thank Professor Francesco Favaron for his support and for giving me the occasion to collaborate with his staff. I want to thank all staff of the “Australian Wine Research Institute” and in particular Elizabeth Waters, Matteo Marangon, Ken Pocock and Steve Van Sluyter for their scientific support and helping.

I would like to thank the laboratory of oenology in Conegliano (Deborah Franceschi, Simone Vincenzi e Diana Gazzola), the laboratory of plant pathology in Padova (Silvana Odorizzi, Luca Sella and Carla Castiglioni), and Giuseppe Laudicina and Francesco Di Giovanni for their collaboration.

Also, I thank the important people who are constantly present in my life: all my family and special friends.

Special thanks to my wife Graziela. Many thanks for your help and your continuous support.

Thesis summary

Protein instability results in the formation of haze and precipitates in bottled white wines with diminution of their commercial value. This alteration is due to the so-called pathogenesis related proteins (PR proteins) of the grape berry, which survive the fermentation process and pass into the wine where they are responsible for protein instability. Nowadays the main technique used to avoid protein stability in wines is their treatment with bentonite, which, however, causes wine losses and a reduction of the organoleptic quality.

The aims of this thesis were to find alternative techniques able to substitute the bentonite treatment in wine stabilization.

The first approach was to exploit the activity of different proteases secreted from phytopathogenic fungi, that were evaluated for their ability to degrade the grape proteins before the fermentation process. Partially purified proteases were obtained from culture media of *Botrytis cinerea* and *Sclerotinia minor*. The protease from *B. cinerea* was not able to degrade the grape proteins and the wine obtained showed increased protein instability. *B. cinerea* however was demonstrated to be able to remove the proteins from the grape juice by an oxidative mechanism involving the enzyme laccase. An aspartyl protease from *S. minor* was then partially purified from a culture medium which was able to increase the protease production. That protease degraded the grape proteins and reduced protein instability both in *in vitro* and microvinification trials. However, protease production by the fungus as well as its effectiveness were inconstant and seemed related to other unknown mechanisms.

The second approach was based on the application of pellets of metal oxides as adsorbents for grape proteins in white wine and juice during fermentation. Different juices and wines were completely stabilized after treatments with

dosages from 15 to 25 g/L of metal oxides. An easy and practicable procedure to regenerate the metal oxides was developed, allowing to use them many times. Moreover, sensory analysis did not show significant differences among wines stabilized with bentonite and metal oxides.

Finally, the third approach was the treatment of the wine with polysaccharides, which are known to affect protein stability in wines. Carrageenan and pectin were added separately or in combination to a Chardonnay juice prior to fermentation. Both adsorbents removed proteins (up to 75%) thus increasing wine protein stability. Carrageenan was more effective than pectin in increasing wine protein stability.

Riassunto

L'instabilità proteica causa la formazione di torbidità e precipitati in vini bianchi imbottigliati con conseguente diminuzione del loro valore commerciale. Questa alterazione è dovuta alle proteine legate alla patogenesi (PR proteins) presenti nell'uva, le quali resistono al processo di fermentazione e si ritrovano nel vino dove sono responsabili dell'instabilità proteica. Attualmente la principale tecnica utilizzata per evitare l'instabilità proteica è il trattamento con bentonite, il quale, purtroppo comporta perdite di vino e riduzione della sua qualità organolettica.

Gli obiettivi di questa tesi sono stati quelli di trovare tecniche alternative capaci di sostituire il trattamento con bentonite nella stabilizzazione dei vini bianchi.

Il primo approccio è stato la valutazione dell'attività di differenti proteasi secrete da funghi fitopatogeni che sono state valutate per la loro abilità nel degradare le proteine dell'uva prima del processo di fermentazione.

Proteasi parzialmente purificate sono state ottenute da culture di *Botrytis cinerea* e *Sclerotinia minor*. La proteasi di *B. cinerea* non si è dimostrata capace di degradare le proteine dell'uva anche se il fungo era comunque capace di rimuovere le proteine del mosto d'uva mediante un meccanismo ossidativo che coinvolge l'enzima laccasi.

Una aspartil proteasi da *S. minor* è stata parzialmente purificata da un mezzo culturale che favoriva la produzione di proteasi. Questa proteasi ha degradato le proteine dell'uva e ha ridotto l'instabilità proteica sia *in vitro* che in prove di microvinificazione. Comunque, la produzione di proteasi da parte del fungo e la sua efficacia erano incostanti e sembrano legate ad altri meccanismi non chiari.

Il secondo approccio ha riguardato l'applicazione di pellets di ossidi di metallo

come adsorbenti delle proteine dell'uva, nel vino e nel mosto durante la fermentazione. Diversi mosti e vini sono stati completamente stabilizzati dopo trattamento con dosi comprese fra 15 e 25 g/L di ossidi di metallo. Si è quindi sviluppata una facile e pratica procedura per rigenerare gli ossidi di metallo, permettendo numerosi trattamenti con la stessa partita di materiale. Inoltre, l'analisi sensoriale non ha evidenziato differenze significative tra vini stabilizzati con bentonite ed ossidi di metallo.

In fine, il terzo approccio è stato il trattamento del vino con polisaccaridi, i quali sono noti per il loro effetto di interferenza sui meccanismi di formazione di torbidità proteica nei vini. Carragenano e pectina sono stati aggiunti separatamente o in combinazione a mosto di Chardonnay prima della fermentazione. Entrambi i polisaccaridi hanno rimosso le proteine (fino al 75%) aumentando la stabilità proteica. Il carragenano è risultato maggiormente efficace nell'incrementare la stabilità proteica rispetto alla pectina.

List of abbreviations

BF = Beginning of Fermentation

BSA = Bovine Serum Albumin

EF = End of Fermentation

GMD = glass-microfibre discs

KHT = Potassium hydrogentartrate

NTU = Nephelometric Turbidity Unit

MO = Metal Oxide

MW = Molecular weight

pI = Isoelectric Point

PR = Pathogenesis Related

RT = Retention Time

TLproteins = Thaumatin like proteins

Tmax = Temperature maximum

Tmin = Temperature minimum

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1. Introduction

The grape proteins have low nutrition value, although they have biological functions, and affect technological and economic aspects of winemaking.

Most of these proteins are only partially degraded during fermentation of the grape juice and remain in solution in the fermented wine. The grape proteins are involved in wine protein instability, a common problem in white wines that causes formation of haze and amorphous sediments on the bottom of the bottle. This defect normally is not accepted by the consumers and causes strong reduction of the commercial value of the wine.

To reach protein stability in white and rosé wines, the main technique is to treat the wine with bentonite. However, the bentonite treatment has some drawbacks, including significant volume loss (3-10%) due to poor settling (Waters *et al.*, 2005) and reduction of the sensorial quality of the wines. Actually, bentonite is not specific for protein adsorption and can remove important aroma and polyphenolic compounds (Lubbers *et al.,* 1993; Ribéreau-Gayon *et al.,* 2000). For these reasons, alternative procedures for protein removal from white wines have been extensively investigated, including use of other adsorbents (Pachova *et al.,* 2002; Sarmento *et al.,* 2000a; Vincenzi *et al.,* 2005a), immobilized tannic acid (Weetal *et al.,* 1984) or proanthocynidins (Power *et al.,* 1988), ultrafiltration (Hsu *et al.*, 1987a; Peri *et al.*, 1988; Flores *et al.*, 1990), polysaccharide addition (Marchal *et al.*, 2002; Cabello-Pasini *et al.*, 2005), use of haze protective factors (Waters *et al*., 1994; Moine-Ledoux and Dubourdie, 1999; Dupin *et al*., 2000) and proteases (Feuillat *et al.*, 1980; Bakalinsky and Boulton, 1985; Lagace and Bisson, 1990; Waters *et al.*, 1992). However, none of these methods has been proven to be a real alternative to bentonite, due to a low effectiveness in protein stabilisation and/or to excessive costs of the treatment.

1.1 Wine proteins

The wine proteins come mainly from grapes, but some of them can derive from micro-organisms, particularly yeasts (Marchal *et. al.*, 1996; Lugera *et al*., 1998; Goncalves *et al.,* 2002) and bacteria (Monteiro *et al.*, 2001). Bayly and Berg (1967) demonstrated that in fermented model juice the yeast contribution to the final protein level was not significant. Instead, several authors (Lee, 1985; Hsu and Heatherbell, 1987b; Ruiz-Larrea *et al.,* 1998; Ferreira *et al.,* 2000; Dambrouck *et al*., 2003) suggested that the main protein source for wines is the grape berry, the final wine protein level being especially affected by the grape variety, the ripening stage of the grapes and the climate.

The protein content in wines is very variable because it is influenced by the protein content of the berries and by the vinification system. According to literature data protein content in wines ranges from less of 1 mg/L up to more than 1 g/L, although the analytical methods used for protein quantification strongly affect the results (Vincenzi *et al.,* 2005b). Realistically, proteins level in wines varies between 10 and 250 mg/L.

These proteins show molecular mass from 9 to 63 kDa and isoelectric points from 3 to 9 (Hsu and Heatherbell, 1987b; Lamikanra and Inyang, 1988; Brissonet and Maujean, 1993). However the majority of the wine proteins have relatively low molecular masses (20-30 kDa) and low isoelectric points (4.1-5.8), thus possessing positive charge at the pH values encountered in wines (Brissonet and Maujean, 1993; Hsu and Heatherbell, 1987b; Ferreira *et al.*, 2000). Some authors suggested also a remarkable presence of proteins of yeast origin (Yokotsuka *et al*., 1991; Monteiro *et al.,* 2001; Kwon, 2004). Waters and colleagues (1994) isolated two mannoproteins from white and red wines fermented with *Saccharomyces cerevisiae* strains. These compounds were released from yeasts during both the exponential phase of growth and wine fining on lees. Also Yokotsuka and colleagues (1997) achieved similar results,

showing that some glycoproteins recovered from red wines were from yeasts and that they appeared during both alcoholic and malolactic fermentations. Lugera *et al.* (1998) by using a chromatographic approach demonstrated that alcoholic fermentation and the successive stabilization process led to a decrease on the protein content of a Chardonnay wine. This study showed that no proteins release occurred from yeasts through the fermentation but only after 18 months of fining on lees. Therefore yeasts can influence the wine protein composition through protein transfer into the wine during the autolysis process and/or through the emission of extracellular proteolytic enzymes that contribute to the must protein hydrolysis (Feuillat *et al.,* 1980).

1.2 Grape proteins

Grape proteins are synthesized during berry ripening, most of these proteins belonging to as the type of the Pathogenesis Related (PR)-proteins (Robinsons and Davies, 2000). PR-proteins are very diffused in the plant kingdom and they involved in the mechanisms of defence of the plants against pathogens (Antoniw *et al*., 1980; Collinge and Slusarenko, 1987; Dong, 2004) and also in the resistance to water stress conditions (Zhou *et al.,* 2004). However, regarding water stress conditions Pocock *et al.* (2000) demonstrated that drought stress in *Vitis vinifera* does not affect the final levels of PR-proteins per berry at maturity. Although in most of the cases the PR-proteins are expressed in the presence of biotic or abiotic stresses, constitutive levels are present in many plants also in the absence of pathogen infection (Bowles, 1990).

The PR-proteins are divided in 17 families and some of them can comprise different groups (basic and acid groups, intracellular and extracellular groups) (Kitajima and Sato, 1999). It is noteworthy that among PR-protein families many proteins homologues to common food allergens can be found (Van Loon and Van Strien, 1999; Hoffmann-Sommergruber, 2002; Pastorello *et. al.,* 2002).

In grapevine, there are evidences of strong constitutive expression of some PR-proteins that are simply regulated by the developmental stage of the berry (Dercker *et al*., 1996; Robinson *et al*., 1997). These proteins demonstrate antifungal activity *in vitro* against common fungal pathogens of the grapevine (Giananakis *et al.*, 1998; Salzman *et al*., 1998; Tattersall *et al*., 2001; Jayasankar *et al*., 2003; Monteiro *et al*., 2003).

The PR-proteins in grapes are consituted by the families 2, 3 and 5, which include Glucanases, Chitinases and Thaumatin like proteins (TL-proteins) respectively. Among these PR-proteins, Chitinases and TL-proteins have found to be the major soluble proteins of grapes (Peng *et al*., 1997; Tattersall *et al*., 1997; Pocock *et al*., 1998, 2000).

Chitinases (PR 3) normally represent around 50% of the total soluble proteins in healthy grapes (Ferreira *et al.,* 2002; Waters *et al.,* 1998). Grape Chitinases are synthetized during the berry ripening, being able to hydrolyse chitin (β-1,4 acetylglucosamine), the main component of the cellular wall in fungi.

The PR 5 proteins are defined Thaumatin Like (TL) proteins for their homology with the thaumatin of the fruit of the African plant *Thaumatococcus danielii*. They have a molecular mass of 24 kDa, with the molecule stabilized by 8 disulphide bonds, this feature making the protein very resistant to degradation by proteases (Roberts and Selitrennikoff, 1990). The mechanism of action of the TL proteins against fungi is not well explained. These proteins seem to modify the permeability of the cell membrane of the fungi but only in the presence of the cellular wall. Indeed, they are inactive if the fungus is deprived of the wall (Roberts and Selitrennikoff, 1990).

Glucanases (PR 2) degrade the β-1,3-glucan of the fungal cell wall and their activity has been demonstrated against different fungi as *Rhizoctonia solani, Candida albicans* and *Aspergillus fumigatus* (Payne *et al.*, 1990). Glucanases and chitinases are often expressed simultaneously in plant tissues and, *in vitro,* they have been shown to operate in synergy inhibiting the growing of

phytopathogenic fungi as *Botrytis cinerea* (Jach *et al.*, 1995) and *Uncinula necator* (Ferreira *et al.*, 2002).

Recently, the defence role of some PR-proteins has been demonstrated in transgenic plants of grapevines where the genes for chitinases and *β*-1,3-glucanases were overexpressed. These plants resulted more resistant against the fungal infection (Jach *et al.*, 1995).

Recently, by using proteomic approach, it was possible to construct a map of the different proteins of grape pulp (cv. Gamay, Sarry *et al*., 2004) and skin (cv. Cabernet Sauvignon, Deytieux *et al*., 2007) of the grapes, showing the presence of around 300 different protein spots (figure 1).

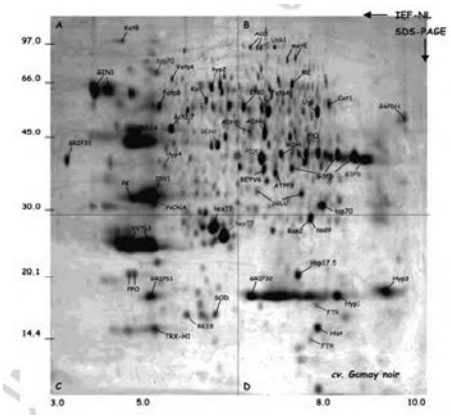

Figure 1. Two-dimensional electrophoretic analysis of the mesocarp proteins from berries of *Vitis vinifera cv.* Gamay (Sarry *et al*., 2004).

82 of these proteins were analysed by Matrix assisted Laser Desorption Ionization- Time of Flight-Mass Spectrometry (MALDI-TOF-MS) recognizing 66 different components, the majority involved in the energetic metabolism (34%), in the response to biotic and abiotic stresses (19%) and in primary metabolism (13%) (Sarry *et al.,* 2004).

1.3 Biochemical and structural characteristics

The grapes proteins pass into wine where they could determine the instability the proteins involved in this phenomenon are, paradoxically, those most stable during winemaking. Actually, only a little part of the several hundreds of protein species contained in the grape juice is capable to survive the vinification effects. The pH reduction, the presence of alcohol and the proteolytic activities are all factors that, in general, provoke protein denaturation and degradation. This causes a "selection" and only the most resistant proteins remain in the wine after fermentation. The proteins able to survive the vinification are the PR-proteins that for their nature have an intrinsic resistance against proteolysis and denaturation (Waters *et al.,* 1992). Indeed comparing the electrophoretic results obtained for the protein in grapes and in wine it is possible to note a strong simplification of the pattern with few main components remaining after fermentation. These components, which have molecular weights between 20 and 30 kDa and p.I. of 4-6 (figure 2), have been identified as chitinases and TL proteins of the grapes (Waters *et al.,* 1996).

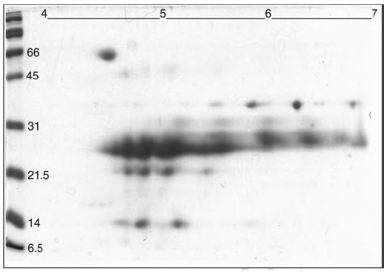

Figure 2. 2D-PAGE of wine proteins (cv. Manzoni Bianco) (from Polesani, 2004).

Also the invertase enzyme (MW 62-64 kDa) is considered one of the most abundant proteins of the wine, representing from 9 to 14 % of the total protein content (Dambrouck *et al.*, 2005). However, for each MW, it is possible to

observe many protein spots (figure 2) suggesting a certain micro-heterogeneity, which probably derive from limited chemical modification or proteolysis of the grape proteins occurring during fermentation (Monteiro *et al.*, 2001). However, recently this idea was not confirmed because it has been shown that many forms of the same protein exist also in the grape berry (Monteiro *et al.*, 2007). In addition to the molecular weight and isoelectric point, proteins differ also for their hydrophobicity, an important functional characteristic influencing the interactions with other wine components such as the tannins (Marangon *et al.,* 2010).

Indeed, many different fractions can be obtained when the wine proteins are separated according to this characteristic (figure 3), several of these fractions containing mainly grape chitinases and TL proteins, as assessed by MS identification.

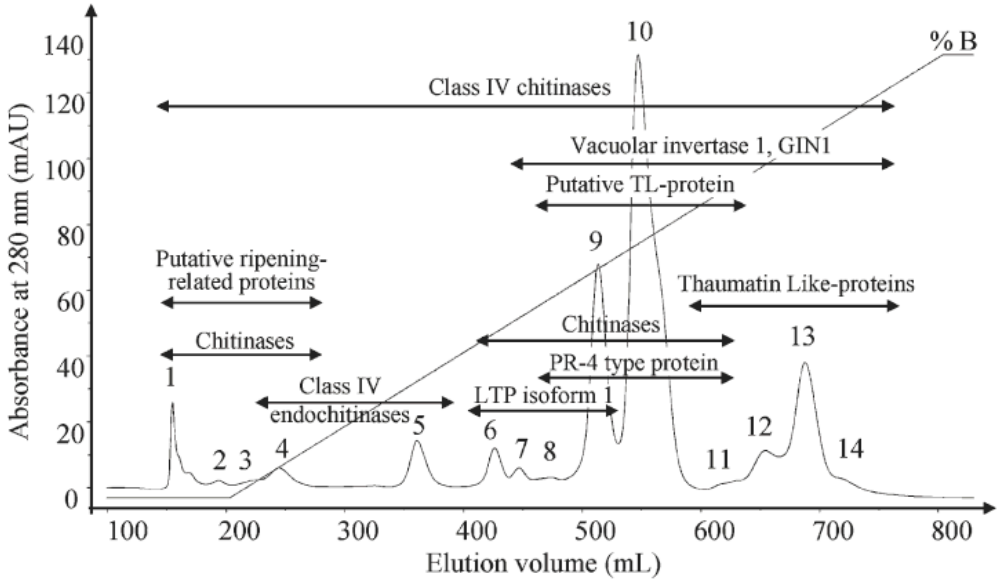

Figure 3. Protein separation by hydrophobic interaction chromatography and identification of protein peaks (from Marangon *et al.,* 2009).

The wine proteins are characterized for having globular and compact structures stabilized by numerous intramolecular disulphide bonds (between 6 and 8)(Roberts and Selitrennikoff, 1990). The importance of such bonds to keep the molecular compactness can be demonstrated by electrophoresis in reducing and not reducing conditions. Indeed, the reducing condition results in an electrophoretic mobility lower than that of the unreduced protein indicating a loss of compactness of the protein molecule upon reduction (figure 4).

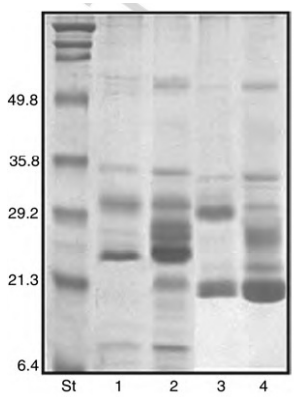

Figure 4. SDS-PAGE analysis of grape (1 and 3) and wine proteins (2 and 4) in reducing (1 and 2) and non reducing conditions (3 and 4). The molecular weights (MW in kDa are indicated on the left side (from Vincenzi and Curioni, 2005).

The loosening of the protein structure by thermal denaturation, (unfolding), a step considered necessary for proteins aggregation and the haze formation in wines was studied by differential scanning calorimetric (DSC) and circular dichroism (Falconer *et al*., 2010). These techniques are able to follow the changes of protein structures occurring during the temperature increase. From the results obtained using these techniques it was demonstrated that chitinases have a lower temperature of denaturation (Tm) than the TL proteins. Besides, TL proteins denaturation, but not that of chitinase, is a phenomenon partially reversible (Table 1).

Protein	T_m (°C)	*Denaturation reversibility*
Chitinase (class IV)	55	No
TLP (VVTL1)	62	Yes (partial)

Tabella 1. Denaturation Temperature (Tm) of purified chitinase and TL protein (Falconer *et al.*, 2010).

Considering these data it was possible to calculate that chitinase has a half life of 9 years at 15 °C, 1.3 months at 25 °C and only 17 minutes at 45 °C.

1. 4 Technical problems in white and rosé wines

Protein instability in white and rosè wines is the most common non microbiological defect in commercial wines (Bayly and Berg, 1967; Hsu *et al.*, 1987a; Waters *et al.*, 1992).

This is due to the fact that the wine proteins can precipitate thus forming amorphous sediments or aggregates that give turbidity in the bottled wine. Waters *et al.* (1996) were the first to identifie the PR-proteins as the responsible of the protein instability. The mechanism of protein instability in wines is classically described by the general scheme of flocculation of a hydrophilic colloid when deprived of the two factors allowing its stability, charge and hydration (Ribéreau- Gayon, 1976). In particular, in the presence of tannins mixed tannin-protein aggregates are formed giving rise to a negatively charged hydrophobic colloid that flocculate after interacting with the cations of the medium. However, the mechanism involved in protein flocculation seems to be

more complex than that hypothesised by Ribéreau- Gayon, as pointed out in recent papers.

The protein haze formation in white wines is almost certainly due to a mechanism involving protein denaturation as the first step followed by interactions with other components leading to aggregation and flocculation of visible precipitates (Falconer *et al.,* 2010).

Protein denaturation seems to be the limiting step for aggregation, and when the proteins are denatured the aggregation and the formation of particles easily occur.

Beyond the temperature, other factors seem to be involved in phenomenon of protein precipitation in wines. These factors can influence the denaturation step and/or the consequent aggregation of the denatured proteins, some of these factors (called X factor) being necessary to develop the turbidity. Indeed, the purified wine proteins are not able to produce turbidity if they are heated alone in model wine (aqueous solution with 12% of ethanol and containing tartaric acid at pH 3.2), but they can form turbidity when they are added in an ultrafiltered wine (deprived of its macromolecules). Other low molecular wine components (the X factor(s)) seem then to be necessary for protein aggregation. Indeed, it was demonstrated that ions, polyphenols and polysaccharides can influence the haze formation in white wines. The phenomenon is very complex and consequently not completely clear.

Salts can influence protein stability by modifying the ionic strength of the solutions and interacting with the protein charges. These aspects facilitate the denaturation of the protein molecule and promote interactions between proteins. The ionic strength of the wines is between 10 to 100 mM and consequently it is possible to suppose that differences in this parameter could strongly influence the electrostatic interactions. Indeed, this was demonstrated in model system containing purified chitinases of the wine (Marangon *et al.,* 2011). Besides, the mechanisms involved could be influenced also by the type of ion. For example,

the sulphate ion was indicated as the X factor necessary to cause haze formation in wines (Pocock *et al.,* 2007). A further study examined more in detail the effect of sulphate on the wine proteins confirming major susceptibility of chitinases to form turbidity in presence of this ion and a model describing the different behaviour of the two classes of proteins (chitinases and TL proteins) upon heating was reported. According to this model, Chitinases undergo total irreversible denaturation and aggregation during the heating step, while TL proteins partially aggregate only during cooling of the heated solution. Also another study confirmed that chitinases are the protein components most involved in protein haze formation, especially in the presence of sulphate (Marangon *et al.*, 2011). Actually, the proteins mainly present in the precipitates obtained in wine incubated at 30 °C for 22 hours (then in realistic conditions) are chitinases, while the TL proteins are capable to remain in solution in these conditions.

Also the presence of tannins in wine must be considered a factor with possible strong effects in protein precipitation, as reported in literature from several years (Siebert *et al.*, 1996; Dawes *et al.*, 1994; Waters *et al.*, 1995; Siebert, 2006).

In model solution it was observed that the turbidity formed depends on the ratio between polyphenols, with maximum aggregation when the proteins/polyphenols ratio reaches certain values and the quantity of proline present in the proteins has been correlated with their reactivity with tannins (Siebert *et al.*, 1996). Indeed, polyphenols establish hydrophobic bonds with residues of proline in polypeptide chains and voluminous aggregates are formed when the concentration of protein and polyphenols are similar. When polyphenols are in excess respect the proteins, as occurs in white wines, the aggregates are small but enough to cause turbidity (Siebert *et al.,* 2006).

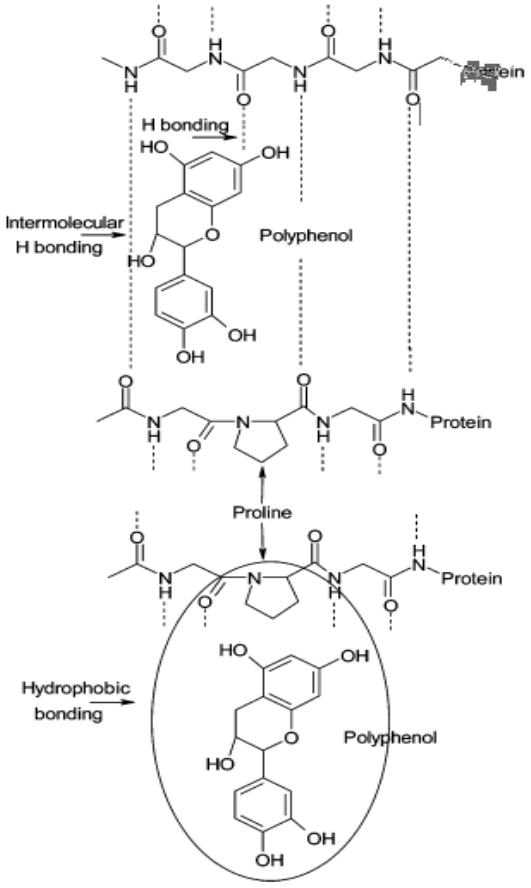

Figure 6. Model of protein – polyphenol interaction leading to haze formation (Asano *et al.,* 1982)

Other studies confirmed that haze formation in wines is due to hydrophobic interactions occurring among proteins and tannins. These interactions should occur on hydrophobic tannin-binding sites, whose exposition on the proteins can depend on both protein heating and reduction (Marangon *et al.,* 2010). The separation of wine proteins according to their hydrophobicity allowed producing different fractions that reacted with tannins (purified of the same wine) at an extent, which increased with the degree of hydrophobicity of the fraction (Marangon *et al.*, 2010). The highest turbidity developed when the

reaction with tannins was done at temperatures higher than the temperature of protein denaturation, while at room temperature the turbidity developed was much lower.

Based on the experimental data, the following interaction model was proposed to explain haze formation in white wines (Curioni *et al.* unpublished): the wine proteins have a compact structure at room temperature, with the hydrophobic portions buried inside the molecule. However, few tannin molecules (which can be present also in white wines) are linked on external surface of the molecules, but not in such an extent to allow reticulation among protein molecules, because in this situation all the few tannin binding sites are occupied. When the mixture is heated proteins unfold (thermal denaturation) causing the exposition of additional hydrophobic portions, which initially were unexposed. These protein regions could now operate as new sites for tannin binding which interact with the tannins initially present on the external structure of the proteins, thus provoking the reticulation and the precipitation of the complex (haze formation) (figure 7).

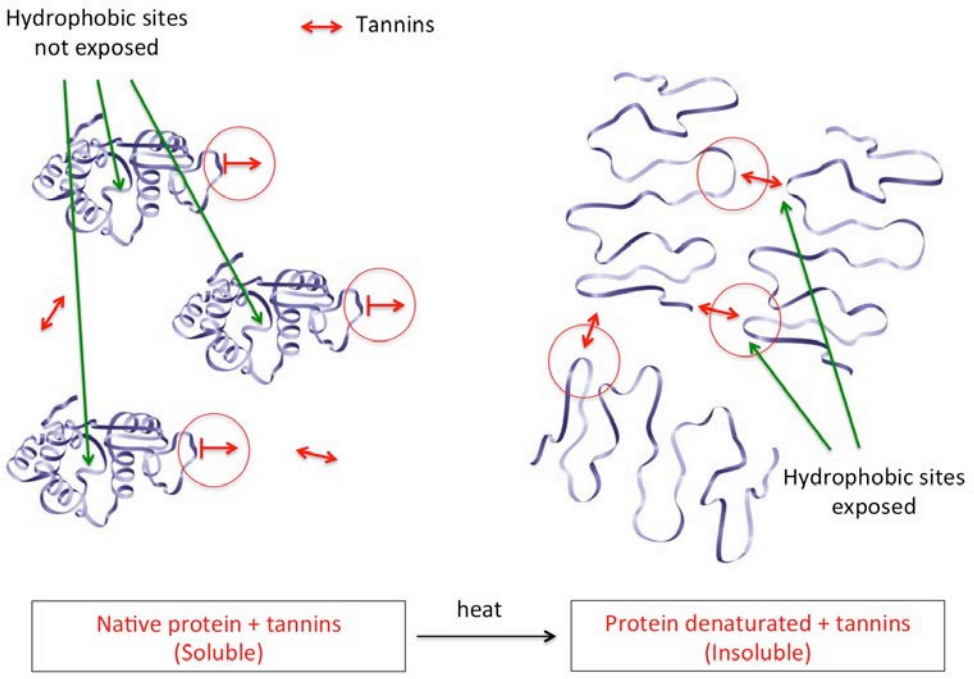

Figure 7. Interaction model between protein and tannins in white wines (from Curioni *et al.,* unpublished).

1. 5 Test for the evaluation of protein instability in wine

Several tests have been developed to check the risk of protein haze formation in wines. Actually the possibility to estimate protein instability of white and rosé wines by simple and quickly tests is of great importance to decide which is the dose of bentonite to use in wine stabilisation before bottling.

Common protein stability tests are based on the (1) measurement of the protein content (total protein assays ad Biuret, Coomasie blue; (2) addition of tannins, (3) chemical denaturation of the proteins (trichloroacetic acid, phosphomolybdic acid) and measurement of the turbidity formed; (4) heat denaturation and measurement of the turbidity formed (het test); (5) decrease of

solubility, which can be achieved by increasing the ethanol content (Boulton, 1980) or the tannin content (Mesrob *et al*., 1983).

The trichloroacetic acid test precipitates all the proteins and can also lead to overfining (Berg and Akiyoshi, 1961; Rankine and Pocock, 1971; Dubourdieu *et al.*, 1988, Toland *et al.,* 1996), while the effect of tannin addition depends on the wine composition and is considered unreliable (Sarmento *et al.*, 2000b).

Several temperatures and times for heat tests are reported in literature (Pocock and Waters, 2006). These can be divided into long or short incubation at different temperatures. The long incubation tests could be divided into the following:

- 35 °C for 10 days (Dubourdieu *et al.*, 1988);
- 49 °C for 2 days (Moretti and Berg, 1965);
- 60 °C for 1 day (Toland *et al.,* 1996);
- 60 °C for 4 days (Sarmento *et al.*, 2000b).

The short incubation methods proposed could be divided into the following:

- 70 °C for 15, 30 and 60 minutes and for 6 and 24 hours (Pocock and Rankine, 1973);
- 80 °C for 5 minutes (Dubourdieu *et al.,* 1988);
- 80 °C for 30 minutes (Ribéreau-Gayon and Peynaud, 1961; Pocock and Rankine, 1973; Dubourdieu *et al.,* 1988; Ledoux *et al.,* 1992);
- 80 °C for 2 hours (Pashova *et al.*, 2004a);
- 80 °C for 6 hours (Pocock and Rankine, 1973; Hsu and Heatherbell, 1987a);
- 90 °C for 1 hour (Sarmento *et al.,* 2000b).

One of the most used heat test analysis is the test proposed by Pocock and Rankine (1973), in which the wine is heated in bain-marie at 80 ºC for 6 hours and then cooled down. The wine is considered to be stable when the turbidity formed in these conditions is lower than 2 NTU.

Other tests exploited the addition of tannins from oak galls (0.5 g/L) associated with heating at 80 ºC for 30 minutes, which gave values more elevated compared to the heat test without tannins (Ribèrau-Gayon *et al.*, 2003) or the addition of a reactive based on Phosphomolybdic acid (concentration 10%) or Bentotest (Jakob, 1962). In the ethanol test (Boulton *et al.,* 1996 one volume of absolute ethanol is added to one volume of wine and turbidity formed is measured. However, in general all these methods have the tendency to overestimate protein instability of the wine.

1.6 Techniques for preventing wine protein instability

The presence of proteins in white wines and the related problem of haze development are studied since the beginning of the nineteenth century. Several methods have been proposed in order to remove the grape proteins and to reach the protein stability. Laborde *et al.* (1904) suggested heating the wine at 70-80ºC for 15 minutes to eliminate proteins and consequently to reach the stability. The use of cation exchanger was firstly proposed in 1932 by using caolin. However, the caolin required high dosages in order to eliminate proteins. Finally, in 1934 Saywell proposed bentonite for protein removal because of its net negative charge at wine pH that allowed reaction with positively charged molecules.

1.7 Bentonite

The most used and practicable method for the protein stabilization of white and rosé wines is bentonite treatment (Ferreira *et al.,* 2002). Bentonite clay is an aluminium phyllosilicate consisting mostly of montmorillonite ($Al_2O_3\ 4\ SiO_2\ nH_2O$), which is used as suspension in water or in wine where it forms a colloidal suspension whose negatively charged particles have the propriety to

fix the positively charged proteins of the wine.

Bentonite adsorbs principally proteins with isoelectric points > 6, the elimination of proteins with lower isoelectric point requiring larger amounts of bentonite (Hsu *et al.*, 1987b; Paetzold *et al.,* 1990).

Bentonite treatment is applied in winemaking through discontinue systems and there are different studies about the capability to stabilize white and rosé wines by using of different types of bentonite (Dawes *et al.*, 1994; Blade and Boulton, 1998).

The level of bentonite addition required for stabilization is determined by stability tests. These levels have increased during the last 20 years. Actually, in wine making process are often employed bentonite doses of 100-200 g/hL (Dubourdieu and Canal-Lìaubère, 1989).

Bentonite, especially if used with high dosages, can negatively influence the sensorial characteristics of the white wines and sometime causes colour alteration (Miller *et al.*, 1985; Bayonove *et al*., 1995; Puigdeu *et al.*, 1996; Cabaroglu *et al.,* 2002). For example, it has been demostrated that the strong aromatic intensity of Sauvignon wines is often impaired by bentonite treatments, due to the elimination of part of 4-mercapto-4-methylpentan-2-one (Ribéreau-Gayon, 2003).

Moreover the bentonite treatment cause a loss of volume of wine that it is difficult to recover (Hoj *et al.*, 2001) and the disposal of spent bentonites constitutes a non negligible source of waste. Finally, bentonite handling is also of concern for occupational health and safety issues. For these reasons, currently the attention is focused on developing alternative practices for protein stabilization. Moreover, those practices need to maintain the quality, reduce costs and be sustainable (Waters *et al.*, 2005).

1.8 Other techniques for wine protein stabilisation

1.8.1 Alternative adsorbents

Ion exchange resins have been proposed to remove proteins from wine (Sarmento *et al.*, 2000a; Gump e Huang, 1999). However, these resins caused the decreasing of several compounds other than unstable proteins with loss of wine colour and aroma.

Vincenzi *et al.* (2005a) proposed to remove proteins from wine in a continuous flow application using a column filled with chitin. Chitin, the natural substrate of chitinases, was able to bind these proteins, which are one of the main haze forming components (Waters *et al.,* 1998). However, the wines obtained were not completely stabilized because the presence of other heat unstable components.

Cabello-Pasini *et al.* (2005) suggested the addition of polysaccharides of seaweed origin. They evaluated the binding capability of negatively charged polysaccharides such as agar, carrageenan and alginic acid. However the protein removal was not effective to reach wine protein stability.

Finally, De Bruijn and co-workers (2009) evaluated the use of trisacryl resins and the possibility to use this material for continuous wine stabilization. Trisacryl resins were able to stabilize a Chardonnay wine through batch adsorption and the members of a sensorial panel were not able to detect statistically significant differences between wines treated by bentonite and trisacryl resins. The negative aspect of this method was the high dosage required.

1.8.2 Flash pastorization

The flash pasteurization could have negative effects on wine quality (Ferenczy, 1966), but several researchers stated that heating the wine for a short time at 90ºC did not have negative effects on the organoleptic quality (Francis *et al*., 1994; Pocock *et al.,* 2003).

Pocock et al. (2003) proposed the treatment of wines with proteolytic enzymes and heating at 90 ºC for 1 minute in a tubular heat exchanger, followed by immediate cooling to 19 ºC. This combined heat and enzyme treatment reduced the protein level in all wines from 40% to 80% of the original levels. Heat treatment alone reduced the protein levels from 50% to 90%. Sensory evaluation of the wines showed that the treatments had negligible effect on aroma and no effects on taste. However, the wines treated were not completely stable. Moreover, these treatments are expensive in large scale applications in terms of energy and apparatus.

1.8.3 Haze protective factors

Another method of protein stabilization is based on the addition of polysaccharides, rather than on the removal of the proteins. Among polysaccharidic compounds, yeast mannoproteins have a strong stabilizing action (Waters *et al.*, 1993; Dupin *et al.*, 2000). The concentration of mannoproteins in the wine can be improved by extending the contact time of the wine with the yeast lees (Moine-Ledoux, 1996). These proteoglycans, termed *haze protective factors*, reduce the visible haziness by decreasing the size of the haze particles (Waters *et al*., 1993). The improvement of the concentration of mannoproteins in wine can be reached also by adding yeast invertase (McKinnon, 1996; Moine- Ledoux and Dubourdieu, 1999). Moreover, the addition other polysaccharides with stabilizing effect such as Arabic gum

and arabinogalactan peptides from grapes (Waters *et al.,* 1994) and apple (Pellerin *et al.,* 1994) could decrease the wine protein instability.

1.8.4 Immobilized phenolic compounds

Weetal *et al.* (1984) studied the use of immobilized tannins to remove proteins from wine. These researchers observed that proteins could be eliminated without modification of the sensorial quality of the treated wines, but, this method results quite expensive. Later, Powers *et al.* (1988) immobilized proanthocyanidins in an agarose matrix and prepared a column for continuous wine stabilisation. However, the regeneration of the column matrix showed reduction in protein-binding capacity after a small regeneration cycles.

1.8.5 Metal Oxides

Other studies demonstrated the capability to remove proteins from the juice by adsorption on particles of metals oxide, also in continuous systems (Fukuzaki *et al.*, 1996; Pachova *et al.*, 2002; Pashova *et al.*, 2004a and b; Salazar *et al.,* 2007; Marangon *et al.*, 2011). These studies showed that a material such as zirconia (zirconium oxide) could be a promising technology because the capability to remove the proteins and to be easily regenerated (Marangon *et al.,* 2011). Moreover, the organoleptic quality of the wine was slightly influenced and no differences were found in comparison with the wine stabilised by a bentonite treatment (Pachova *et al*., 2002; Pashova *et al.,* 2004a; Pashova *et al.*, 2004b; Marangon *et al.*, 2011).

1.8.6 Proteolytic enzymes

Proteolytic enzymes are largely studied for the possibility to degrade the heat

unstable proteins without compromising the organoleptic quality of the wine, leading to elimination or reduction of the need of bentonite (Lagance and Bisson, 1990; Waters *et al.,* 1992; Dizy and Bisson, 2000).

Several researchers have studied the addition of proteases from *Saccharomyces cerevisiae* (Feuillat *et al*., 1980; Lurton *et al*., 1988), *Aspergillus niger* (Posada *et al.,* 1971; Bakalinski and Boulton, 1985; Edens *et al.*, 2005) and *Botrytis cinerea* (Marchal *et al*., 1998; Girbau *et al.,* 2004; Marchal *et al.,* 2006; Cilindre *et al.,* 2007). *Botrytis cinerea* was studied because the juice obtained from infected berries showed a dramatic reduction of the PR proteins content, which was confirmed *in vitro* tests. This action was ascribed to the activity of proteolytic enzymes secreted by the fungus (Marchal *et al.,* 1998). However, in *Botrytis cinerea* the mechanism of PR-proteins removal seems to be due to a different mechanisms, based on the insolubilisation of the proteins bound by polyphenols after oxidation by laccase (Favaron *et al.,* 2009).

The use of proteases could be considered a promising technology in order to stabilize wine without compromising its organoleptic quality. However, proteases able to degrade effectively the grape proteins have not be found yet.

1.8.7 Ultrafiltration

Ultrafiltration (Hsu *et al.,* 1987a; Dumon and Barmier, 1992) could be utilized to eliminate the proteins from wine, but unfortunately also this method causes elimination of other important wine components, including aroma compounds.

1.8.8 Genetic methods

The possibility to modify the genes responsible for expression of the grape PR proteins, the main responsible for haze formation in wines, has been also proposed in order to produce grapes giving stable wines. However, this

alteration could produce vines highly susceptible to fungal attacks or to stresses in general (Ferreira *et al.,* 2002; Waters *et al.*, 2005).

Schmidt *et al.* (2009) studied the effect of Hpf2 yeast mannoprotein in wine haze formation and produced this molecule in *Pichia pastoris.* However, the transgenic molecule was able to increase protein stability in wine only at very high concentrations.

1.9 Aims of the thesis

This thesis aims to find and develop new methods and tools to be applied in order to reach protein stability in white and rosé wines. The main target was to study methods able to preserve the wine quality better than what can be obtained by bentonite treatments. This was done with different approaches.

A method to stabilize wine would be the degradation of the instable grape proteins, in such a way to make them unable participate in the mechanisms of haze formation. The most logical approach to reach this aim is obviously to use proteolytic enzymes. Unfortunately the grape proteins remaining in the wine, which are pathogenesis related (PR) proteins are characterized for being highly resistant to proteolysis and all the attempts made to date by using commercial proteases have been unsuccessful (Feuillat *et al*., 1980; Lurton *et al*., 1988 Posada *et al.,* 1971; Bakalinski and Boulton, 1985; Edens *et al.*, 2005; Marchal *et al*., 1998; Girbau *et al.,* 2004; Marchal *et al.,* 2006; Cilindre *et al.,* 2007). Starting from the idea that the natural organisms that can attack the grape berries or other fruits must have a set of biochemical tools, including proteolytic enzymes, able to degrade the fruit components in order to provide nutrients for growth, the first approach used in this thesis was based on the idea that some phytopathogenic fungi must be able to degrade the plant PR-protein and therefore also the grape proteins causing haze formation in wines. The aim was then to assess the possibility to exploit the proteases of selected phytopathogenic fungi growth in a medium containing the grape proteins as the

sole nitrogen source in order to enhance the production of proteases able to degrade these particular proteins. The fungi selected for this goal were *Botrytis cinerea*, a natural pathogen of the grape berries, and *Sclerotinia minor*, a naturally grape unrelated fungus. In particular the aim was purify and the proteases produced by the fungi and to use them in microvinification trials in order to evaluate the possibility to degrade the grape proteins and to stabilize white wines.

The second approach to obtain stable wines was to exploit the strong protein adsorption capacity of metal oxides. These type of materials, and in particular zirconium oxide (zirconia), used in pellets during winemaking have been already proposed for white wine stabilization (Fukuzaki *et al.*, 1996; Pachova *et al.*, 2002; Pashova *et al.*, 2004a and 2004b; Salazar *et al.,* 2007; Marangon *et al.*, 2011). However, the operating condition for the use of zirconia have to be improved to render its use suitable for practical application in winemaking. Moreover, an other metal oxide, titania, which potentially also have optimal characteristics for being used in winemaking to remove the unstable proteins was chosen. Therefore the aim was to develop and optimize the use of metal oxides as adsorbent materials for unstable protein removal and to stabilize white wines, without affecting wine quality in an inexpensive and simple way. This was done with the aim to find a practical alternative to bentonite treatments, which, as well known, although widely used, have some drawbacks, including negative effects on wine quality, losses of wine, waste problems and so on.

Finally, as the third approach to prevent protein haze formation in white wines, the possibility to use polysaccharides, which in general are known to interfere with protein haze formation in wine (Waters *et al.*, 1993), was studied. To this aim the effects of carrageenan and pectin addition to wine were tested either in model wine and in wine making trials.

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2. Protein stabilization of white wines: *Botrytis cinerea* as remover of grape proteins

This work evaluated the ability of *Botrytis cinerea* to produce enzymes able to degrade the grape proteins and to decrease the protein instability in white wines. A proteolytic enzyme purified from *B. cinerea* cultures was not able to degrade the grape proteins in the standard winemaking conditions. The mechanism used by fungus to remove the grape proteins was demonstrated, involving polyphenols oxidation by the fungal laccase activity. Purified laccase may be then a potential tool to prevent protein haze formation in wines.

2.1 Introduction

As described in the fist chapter, the proteins from grapes can provoke one of the most common non-microbial defects of commercial white wines: the proteins instability leading to haze formation during wine storage in the bottle (Waters *et al.,* 1992). This phenomenon greatly reduces the commercial value of the wine, making it unacceptable for sale. The proteins causing this problem are pathogenesis-related (PR) proteins of the grape (Robinson and Davies, 2000), which are involved in the mechanisms of plant defence against pathogens (Monteiro *et al.,* 2003).

Botrytis cinerea causes grey mould disease on grapevine, resulting in loss of grape production and wine quality (Pearson and Goheen, 1994). This fungal pathogen attacks the host plant tissue to acquire the nutrients necessary for growth and reproduction. This process is assisted by the secretion of cell-wall-degrading enzymes and phytotoxic compounds (Williamson *et al.,* 2007). However, to successfully colonize the plant tissue, *B. cinerea* must also

neutralize several plant anti-fungal compounds; in the case of grape berries, these compounds are primarily the stilbenic phytoalexins (trans-resveratrol), but also the pathogenesis-related (PR) proteins. Proteomic and transcriptomic analyses have shown that ripening grape berries constitutively contain large amounts of chitinase, β-1,3-glucanase, osmotin, and thaumatin-like (TL) proteins, which all are PR proteins (Pocock *et al.*, 2000; Sarry *et al.*, 2004; da Silva *et al.*, 2005; Monteiro *et al*., 2007). These proteins may contribute to protect the berries from fungal infections and are considered resistant to fungal digestion (Ferreira *et al.,* 2002). However, grape berries infected with *B. cinerea* show strong reduction of the protein content in comparison to healthy ones (Marchal *et al.,* 1998) and this finding was ascribed to a putative proteolytic activity secreted by the fungus during colonization. Moreover, the enzyme laccase seems to be involved in the pathogenic process of *B. cinerea* (Viterbo *et al.*, 1994). The enzyme laccase is an oxidase extensively spread in nature (Mayer *et al.,* 2002). It is a multi-copper ezyme because the catalytic site is characterized by the presence of copper ions. Recently two laccase genes (Bclcc1 and Bclcc2) have been characterized in *B. cinerea* (Schouten *et al.,* 2002). The fungus seems to detoxify the phenolic compounds present in leaves and berries of the grapevine with a oxidative mechanism which affect phytoalexins as resveratrol and other stilbene compounds (Breuil *et al.,* 1999).

Based on this knowledge, the precise mechanism responsible for protein removal from juices of *B. cinerea* infected berries, previously reported to be the result of a proteolytic phenomenon (Marchal *et al*., 1998; Cilindre *et al.*, 2007) was studied.

2.2 Materials and Method

2.2.1 *Botrytis cinerea* cultures

B. cinerea strain PM-10, isolated from grape berries, was kindly provided by Prof. Giuseppe Firrao, University of Udine (Italy). The fungus was grown on Petri dishes on potato dextrose agar (PDA, Difco) at 24°C. For spore production, completely colonized plates were incubated under near UV light for 16 h as reported by Schouten *et al.* (2002). After 15 days, conidia were collected into 5 mL of sterile water by gently scraping the plates with a glass rod. Conidia were filtered through a sterilized gauze and counted using a haemocytometer. Liquid cultures were produced in a modified Czapek-Dox medium (2 g/L $NaNO_3$, 0.5 g/L KCl , 0.5 g/L $MgSO_4$ x H_2O, 1 g/L K_2HPO_4, 0.01 g/L $FeSO_4$, 1 mL of a solution of 1% $ZnSO_4$ plus 0.5% $CuSO_4$, and glucose 20 g/L), adjusted to pH 3.5 with tartaric acid (about 10 mM), which was sterilized by autoclaving. The medium was prepared at a double concentration, and 2 mL aliquots were plated onto Petri dishes (3 cm diameter) and diluted 1:2 with filter-sterilized stock solutions of purified grape proteins, grape polyphenols, *trans*-resveratrol (Sigma-Aldrich, purity >99%), and/or water, and with the concentrated spore suspension to obtain a final concentration of 10^4 conidia mL.

Proteins, polyphenols, and *trans*-resveratrol were supplied at 100 mg/mL, 200 mg/mL, and 200 mg/mL, respectively. The *trans*-resveratrol stock solution (25 mg/mL) was in 95% ethanol, and the same amount of ethanol (0.8%, v/v) was added to the cultures not treated with *trans*-resveratrol. Crystals formed when *trans*-resveratrol was added at the beginning of the experiment, but the compound dissolved during the course of the culturing. A higher ethanol amount (4%, v/v) has been recommended to increase the *trans*-resveratrol solubility (Adrian *et al.*, 1998), but this was not used because we observed a

reduced growth of the fungus at this ethanol concentration. Two levels (absence and presence) of each factor (proteins, polyphenols, and *trans*-resveratrol) were compared in an experimental factorial design. Each treatment was replicated three times.

The cultures were maintained in the dark at 24°C for 4 days. Aliquots of 100 mL were harvested daily from each culture to assess laccase activity and the electrophoretic protein pattern. At the end of the experiment, the content of each culture medium was transferred into 5 mL pre-weighed tubes and centrifuged at 12000 ×g for 30 min. In addition, each plate was rinsed with 3 mL of water, which was then added to each tube, and the mycelium mat was briefly vortexed and centrifuged again. The supernatant was discarded, and the tubes were oven dried at 80°C for 3 days and then weighed.

2.2.2 Grape berry source and inoculation

Ripe white grapes (*Vitis vinifera*, cv. IM 6.0.13) were harvested in the first week of September 2008 from a typical vineyard area near the city of Conegliano (TV, Italy) and stored for 3–6 days at 4°C before protein and polyphenol extraction and *B. cinerea* inoculation.

For inoculation, the berries were detached from the bunch and their surface was sterilized with ethanol (99%) for 1 min, then rinsed with sterile water. A piece (3 × 2 mm) of PDA-colonized agar, cut from the marginal zone of an actively growing fungal colony, was placed on small wound created on the berry surface with a razor blade. The berries were placed above a moist filter paper and closed in a plastic bag at about 22°C. After 7 days, the inoculated berries appeared extensively brownish, and they were harvested and stored at -20°C until extracted. Berries inoculated with non-colonized PDA were incubated in a similar way and used as the control.

Moreover, healthy and naturally infected berries with grey mould symptoms were harvested from the vineyard at the beginning of November 2008.

2.2.3 Grape protein extraction and analysis

Proteins were extracted from about 1 L of grape juice obtained by hand crushing about 1.6 kg of berries at 4°C in a beaker. The grape juice was immediately filtered through a nylon gauze, and cysteine was added to give a final concentration of 4 mM to prevent polyphenol oxidation. All successive operations were also performed at 4°C. The juice was centrifuged at 30000 ×g for 30 min, and the supernatant was filtered in succession through glass-microfibre discs (GMD) and cellulose acetate filters (0.8 µm) (Sartorius) and dialyzed overnight (10 kDa cut off) against 10 mM potassium tartrate buffer, pH 3.5. The dialyzed material was adjusted to 20% saturation with $(NH_4)_2SO_4$ and stirred for 1 h. After centrifugation at 30000 ×g for 30 min, the supernatant was filtered in succession through 0.8, 0.45, and 0.2 µm membranes, concentrated 8 times with a VivaFlow 5000 apparatus (Sartorius), and loaded on a Sephadex G-25 column (PD-10, GE Healthcare). Proteins were eluted with water and further concentrated with a VivaFlow 5000 apparatus and then assayed for protein (Bradford, 1976) and phenol (Folin-Ciocalteau assay) contents using BSA and gallic acid as the standards, respectively. The protein recovered, about 20 µg/g of berry fresh weight, was approximately one-third that measured initially in the grape juice. The estimated weight ratio between proteins and phenols in the final protein preparation was about 5:1. This protein was used for *B. cinerea* cultures and *in vitro* assays.

The protein profile was analysed after precipitation of the protein with four volumes of cold ethanol at -20°C for 2 h. The pellet containing the precipitated protein was washed once with cold 70% ethanol. After centrifugation at 12000 ×g for 15 min, the precipitated protein was air dried and analysed by sodium

dodecyl sulphate gel electrophoresis on 16% (w/v) polyacrylamide gels (SDS-PAGE). Gels were stained with the colloidal Coomassie blue G-250 or the silver method (Candiano *et al.*, 2004).

To compare the protein patterns of healthy and infected berries, 10 healthy and 10 infected berries were crushed, and the juice was centrifuged, filtered, and passed through a PD-10 column as described above. A volume of extract of healthy berries containing 5 µg of protein and an identical volume from the infected berries were precipitated and analyzed by SDS-PAGE, as described above.

2.2.4 Extraction of Grape polyphenols

Once crushed and divested of seeds, the solid grape residue was mixed with potassium metabisulphite at a concentration of 0.5 g/kg of fresh grape and stored at -20°C. Grape polyphenols were extracted by the method of Kammerer *et al.* (2004) with some modifications. A total of 50 g of the stored material was stirred with four volumes of methanol / 0.1% HCl (v/v) for 2 h under nitrogen at room temperature. The extract was filtered through filter paper and vacuum dried using a Rotavapor at 30°C. The residue was dissolved in 100 mL of acidified methanol, centrifuged at 8600 ×g for 20 min, and dried again. The residue was dissolved in 40 mL of deionized water brought to pH 3.5 with HCl. This suspension was centrifuged at 8600 ×g for 15 min. The supernatant was filtered through 0.45 µm membranes, and 10 mL aliquots were loaded onto SPE DSC-18/6 mL columns (Supelco) equilibrated with deionized water. After washing with 5% methanol in water, phenols were eluted with absolute methanol and concentrated using the Rotavapor, as described above. The residue was dissolved in 2 mL of deionized water, and the phenol concentration was determined by the Folin-Ciocalteau assay, using gallic acid as the standard.

About 500 µg of phenols per gram of grape fresh weight were obtained, which were stored at -20°C until required.

2.2.5 *B. cinerea* laccase production, purification, and assay

Laccase of *B. cinerea* was extracted as described by Slomczynski *et al.* (1995). After culturing for 7 days, several 250 mL Erlenmeyer flasks, each containing 50 mL of culture, were pooled, and the content was filtered through GMD and then through a cellulose acetate membrane (0.8 and 0.45 µm) (Sartorius). The filtrates were dialysed against deionized water, concentrated to 40 mL using a VivaFlow 5000 apparatus, adjusted to pH 6.0 with 10 mM potassium tartrate, and loaded onto a Q-sepharose column (16 × 120 mm, Pharmacia). Bound protein was eluted with a 60 min linear gradient of 0–0.5 M NaCl dissolved in the 10 mM K tartrate buffer. Laccase activity was assayed in the eluted fractions (2 mL), and the fraction with the highest activity was used in the following assays. This fraction showed a 96 kDa band when analyzed by SDS-PAGE, similar in size to the protein purified by Slomczynski *et al.* (1995).

Laccase activity was determined spectrophotometrically as described by Wolfenden and Willson (1982) in a total volume of 0.8 mL containing 0.7 mL of 0.1 M acetate buffer, 0.1 mL of 2 mM 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) substrate, and variable volumes of samples (1–30 µl). The enzyme assay was performed at 30°C by monitoring the A_{420}. One laccase unit (U) was defined as the amount of enzyme that oxidizes 1 µmol ABTS per min.

2.2.6 Polyphenols, protein and *trans*-resveratrol interactions

Grape proteins, grape polyphenols, and *trans*-resveratrol were mixed in the same weight ratio of 1:2:2 as used for the *B. cinerea* cultures. Grape proteins

(50 µg/mL), *trans*-resveratrol (100 µg/mL), and polyphenols (100 µg/mL) were dissolved in 0.1 M potassium-tartrate buffer pH 3.5 in the presence or absence of the purified laccase (0.02 U mL). After 24 h of incubation at 24°C, 100 µL aliquots of the mixtures were centrifuged at 16000 ×g for 20 min, and the supernatant was separated from the pellet. The pellet containing the precipitated protein was washed once with cold 70% ethanol. The protein in the supernatant (soluble protein) was precipitated with 4 volumes of cold ethanol at -20°C for 2 h and then washed once with cold 70% ethanol. Both soluble and insoluble proteins were recovered by centrifugation and air dried. Proteins were run on SDS-PAGE gels and stained as described above.

2.2.7 Juice treatment by purified proteolytic enzyme from *B. cinerea*

A purified proteolytic enzyme was obtained from liquid medium after growing of *B. cinerea.* Twenty agar plugs (Ø 5 mm) containing active growing fungal mycelium were used to inoculate flasks containing 400 mL of Czapeck medium (0.5 g/L KCl, 0.5 g/L $MgSO_4*7H_2O$, 1g/L K_2HPO_4, 0.01 g/L FeSO4, 1mL/L solution 1% $ZnSO_4$ - 0.5 % $CuSO_4$), glucose (20 g/L), hydrolysate of casein (5 g/L) as nitrogen source and acidified with tartaric acid until pH 3.5. The flasks were stirred at 100 rpm for 6 days at 25 °C in an orbital shaker. The medium was 0.45 µm filtered and dialysed against deionized water, concentrated to 40 mL using a VivaFlow 5000 apparatus and loaded onto a Q-sepharose column (16 × 120 mm, Pharmacia). Bound protein was eluted with a 60 min linear gradient of 0–0.5 M NaCl dissolved in 10 mM K tartrate buffer. Protease activity was assayed in the eluted fractions (2 ml), and the fraction with the highest activity was used for treatment of the juice. The protease was added in the juice at 180 µg/L in a flasks containing 100 mL of juice. After 24 hours the DV10 strain was inoculated and the fermentation was monitored by

refractometric analysis. At the end of fermentation the samples were put at 4°C for 4 days and then 0.45 μm filtered and added of SO_2 (30 mg/L) to protect the wine from oxidation. Samples were analysed by the heat test (Waters *et al.,* 1992) and SDS- PAGE.

2.2.8 Heat Test

Wines were heated at 80°C for 2 h and cooled in ice for 2 h. After equilibration at ambient temperature the haze was measured by calculating the difference in adsorbance at 540 nm (Waters *et al*., 1992) or in NTU (Pocock *et al.*, 1973) between the heated and unheated samples.

2.2.9 SDS–PAGE

SDS–PAGE analysis was performed according to Laemmli (1970) using 0.75 mm thick gels with the mini-Protean® II apparatus (Bio-Rad). The stacking gel contained T = 4% and C = 2.6% and the separating gel T = 16% and C = 2.6%.
The proteins, precipitated with 3 volumes of ethanol, were suspended in two-fold concentrated 0.5 m Tris-HCl, pH 6.8, containing 10% SDS, 0.1% blue of bromophenol, 10% glycerol and 10 mg/mL DTT and boiled for 5 minutes before being loaded on the gel. Standard proteins (Biorad) with molecular weights in the range 14.4 - 97.4 kDa were used.
Electrophoresis was run at constant 200 millivolts for 60 minutes.
Protein bands were stained with colloidal Coomassie® R-250, following the method developed by Candiano *et al.* (2004).

2.2.10 Statistical analysis

Data for laccase activity and mycelium dry matter were subjected to analysis of variance according to a multifactorial design with three replicates.

2.3 Results and Discussion

In the juice obtained from grape berries infected with *B. cinerea* the protein content was dramatically reduced in comparison to the juice from healthy berries, giving different SDS-PAGE profiles (figure 1). In infected berries many bands disappeared and only a few bands were barely detectable, confirming previous results (Marchal *et al.,* 1998).

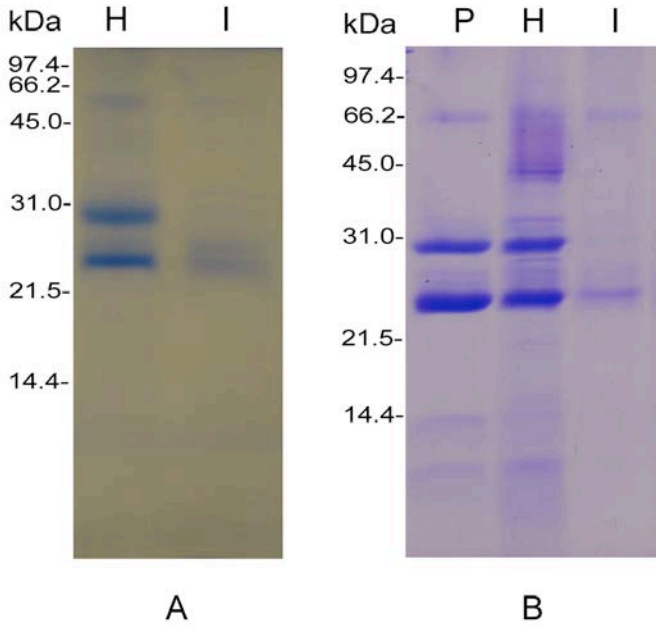

Figure 1. SDS-PAGE of the proteins extracted from healthy or *B. cinerea* infected grape berries. A: Protein extracted from healthy (lane H) and artificially infected berries (lane I) at 7 days from inoculation. B; Protein extracted from healthy (lane H) and infected berries (lane I) harvested from a vineyard. A sample (approximately 5 mg) of the partially purified grape proteins is also shown (lane P). MW in kDa are indicated on the left of each panel.

B. cinerea cultures were prepared in a medium supplied with partially purified grape proteins as sole nitrogen source. The residual protein content in 8 days-old *B. cinerea* cultures indicated only a limited protein degradation even in the presence of a measurable proteolytic activity (figures 2 and 3). In detail the band ascribable to chitinases disappeared, while new bands at 34 kDa and 42 kDa were detected.

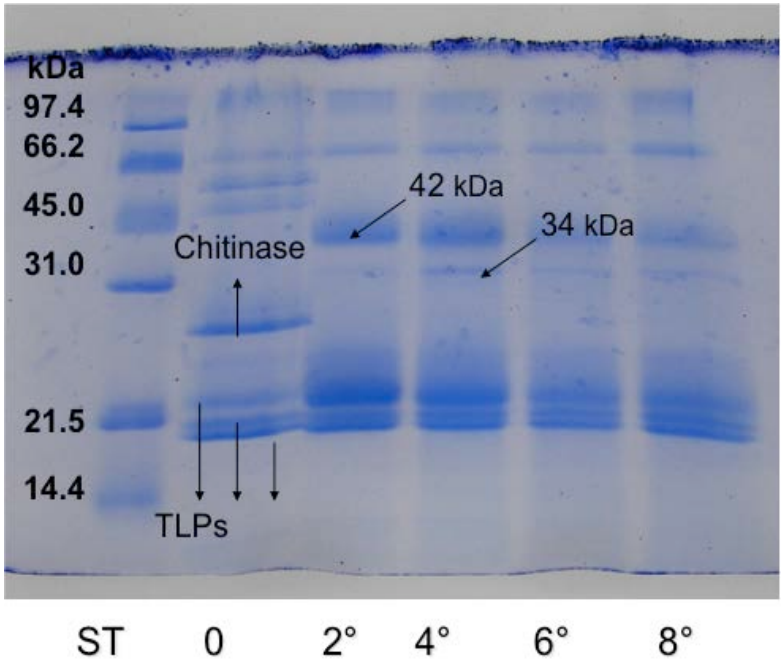

Figure 2. SDS-PAGE of the residual grape proteins in liquid medium (with only purified grape proteins as the sole nitrogen source) during the development of *B. cinerea*. The fungus changed the pattern but did not remove the proteins.

This results are in contrast with the previous data (Marchal *et al.,* 1998; Cilindre *et al*., 2007) and with the possibility to use the proteases of *B. cinerea* to remove the grape proteins and reduce protein instability in white wines. Furthermore, we observed that grape proteins induce strong mycelium

fragmentation in *B. cinerea* (figure 3). This event can be prevented by the addition of grape polyphenols (Favaron *et al.*, 2009).

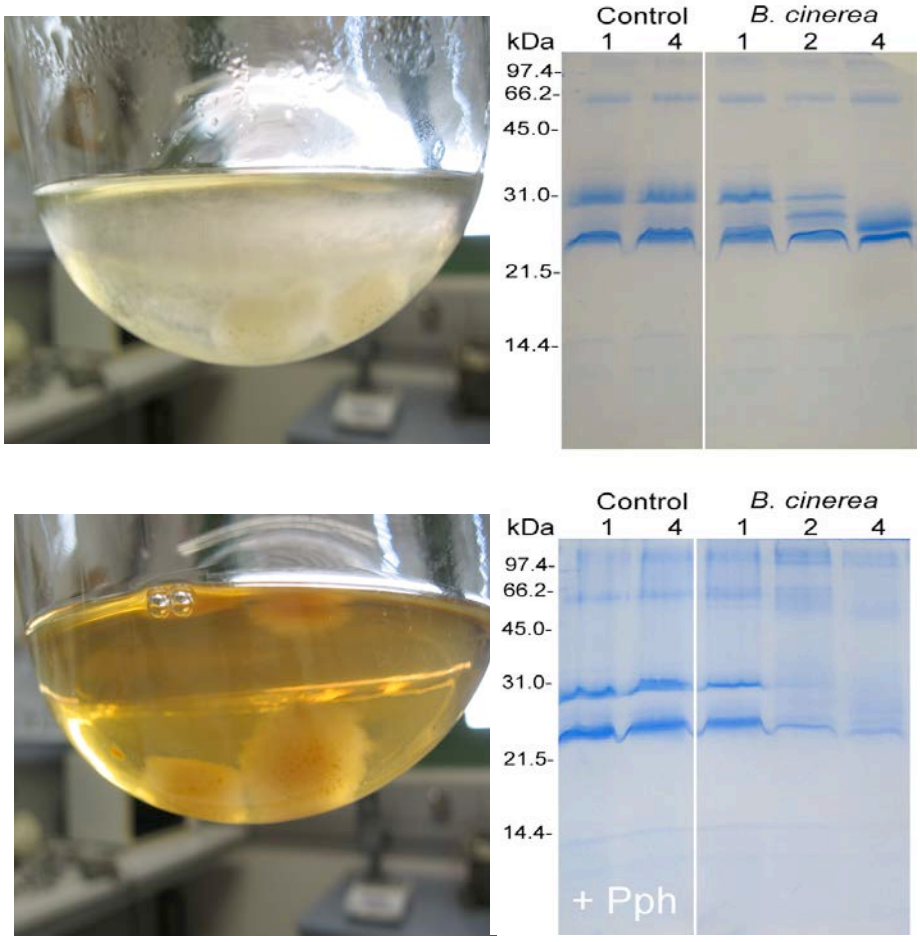

Figure 3**.** SDS-PAGE patterns of proteins collected from *B. cinerea* cultures. The cultures were incubated in 4 ml Czapek-Doxmedium at pH 3.5 supplied with grape proteins (upper panel) and grape proteins plus grape polyphenols (+ Pph, bottom panel). In the culture treated with protein only (upper panel), the band at 31 kDa disappears and a new 28 kDa band can be detected. In the culture supplied with grape polyphenols (bottom panel), the 31 kDa band gradually disappeared and the 25 kDa band faded, becoming poorly visible by the end of the culture period. The days of incubation are indicated on top of the gels.

In vitro experiments (figure 4) showed laccase as the factor responsible for the insolubilisation of grape proteins when polyphenols and/or resveratrol are present in the solution. From these results it can be deduced that *B. cinerea* uses the oxidative laccase activity as the mechanism to remove the grape proteins and the polyphenols, which are toxic for the fungus.

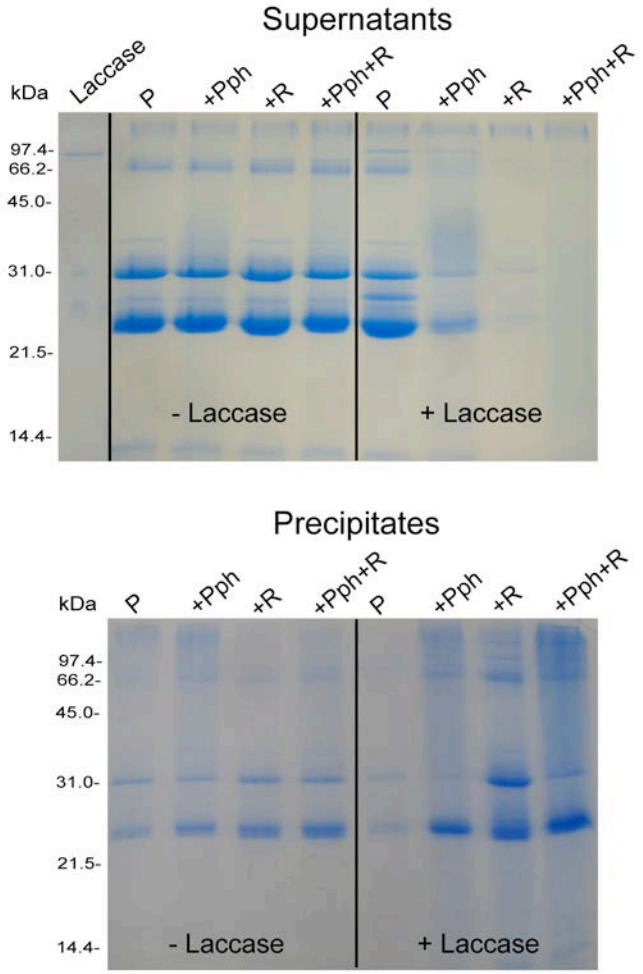

Figure 4. SDS-PAGE results of an in vitro experiment performed by mixing grape proteins (P) with *B. cinerea* laccase (+Laccase) and polyphenols (+Pph), and/or resveratrol (+R). After 24 h of incubation, the presence of laccase caused insolubilisation of most grape proteins when polyphenols, resveratrol or both were present. Mixtures without laccase (-Laccase) have been used as controls.

To confirm these results, partially purified proteolytic enzyme from *B. cinerea* was used for the treatment of a Manzoni bianco juice before the alcoholic fermentation. SDS-PAGE analysis of the culture medium used to induce the proteases production in *B. cinerea* is shown in figure 5. The medium used was able to produce an adequate quantity of protease. Indeed, in the SDS-PAGE analysis is visualized a band at 34 kDa ascribable to a protease isoforms (Favaron, unpublished).

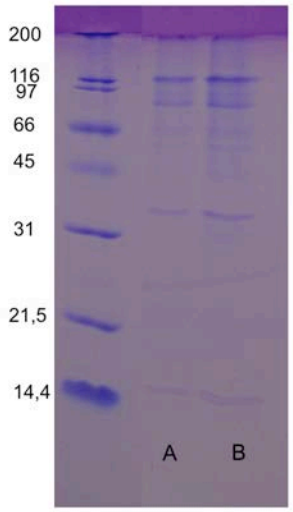

Figure 5. SDS-PAGE of culture medium of *B. cinera* for the production of proteases. The medium was filtered and concentrated 6 times. In A and B 50 μL and 80 μL of concentrated culture medium were loaded respectively.

This enzyme was not able to reduce haze formation in the finished wine (figure 6), but caused increased protein instability.

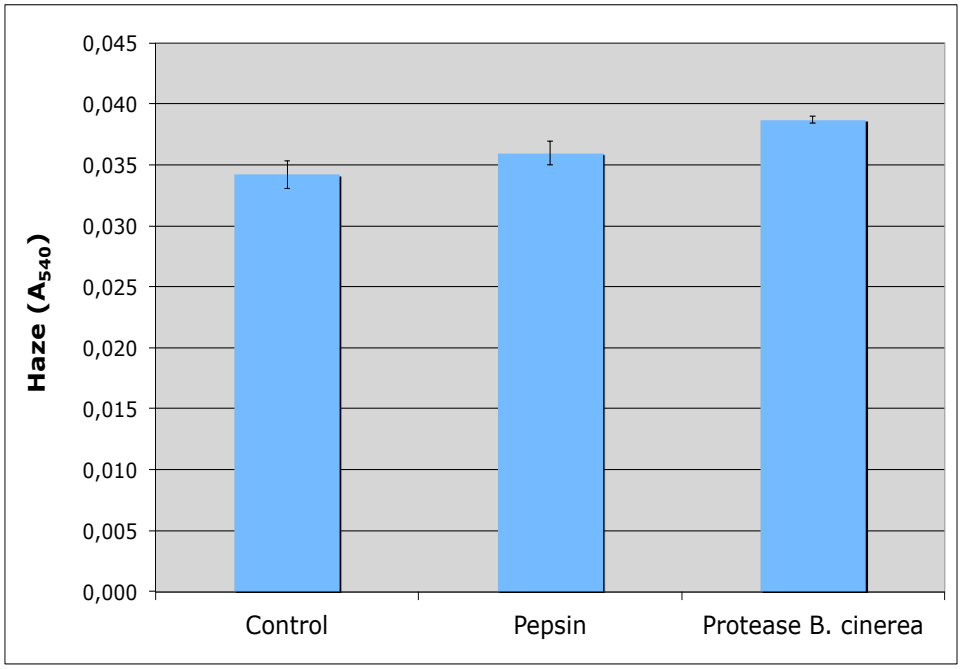

Figure 6. Heat Test results for the wines obtained from the fermentation of Manzoni bianco juice treated with the protease from *Botrytis cinerea*, and compared with a sample treated with pepsin and with the control (no protease added). The protease from *B. cinerea* increased wine instability.

2.4 Conclusions

In the presence of grape polyphenols, *B. cinerea* laccase, probably through the production of oxidized phenol intermediates, modifies the solubility of grape PR proteins. This mechanism seems to be responsible for grape protein removal in infected berries. These studies showed that the proteolytic activity produced *in vitro* by *B. cinerea* is not effective in degrading the grape proteins. Also a partially purified proteolytic enzyme from *B. cinerea* was not able to reduce the wine protein instability. Therefore, in contrast with what was thought before, proteolysis is not the reason for protein removal in grapes, which is actually due to an oxidative process deriving from laccase activity in the presence of polyphenols. The mechanism is likely to be related to the production of

oxidised polyphenols derivatives with high reactivity towards proteins leading to formation of insoluble complexes which in this way are eliminated by precipitation. Laccase may be then a candidate tool able to reduce the protein instability in white wine. However, other studies are needed to verify this possibility mainly in relation to wine quality.

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3. Protein stabilization of white wines: *Sclerotinia minor* as remover of grape proteins

The fungus *Sclerotinia minor* has the capacity to remove wine proteins when these are added to the culture of the fungus. This capacity is due to the presence in the culture filtrate of fungal proteinase activity. It was observed that fungus growing in liquid culture with casein as the nitrogen source produced high levels of proteolytic activity due to the presence of an aspartyl protease enzyme. Microvinification experiments were performed by adding variable amounts of this concentrated and purified protease preparation to two different musts from white grapes, containing 79 and 36 mg/L of protein. In comparison to an untreated control wine, the addition of a minimal amount of proteinase preparation, as low as 60 μg/L of fungal protein, removed approximately one half of the wine protein without affecting the biological activity of the yeast *Saccharomyces cerevisiae*. The protein stability of the wine was increased consistently. Unfortunately *S. minor* protease preparations obtained in other cultures were unable to degrade the grape protein and to increase the protein stability in wine, indicating inconsistent results. It was observed that the most effective fungal protease preparation contained a moderate residual laccase activity which may be involved in the mechanism of wine protein removal.

3.1 Introduction

The use of proteolytic enzymes could be an alternative to bentonite treatment for wine protein stabilisation. The proteolysis could degrade the grape proteins without compromising the organoleptic quality of the wine. Unfortunately the studies performed so far have shown that the proteases isolated from yeasts and

others microorganisms are poorly effective in grape protein removal from musts or wines (Ferreira et al., 2002). Also the plant protease papain, used to soften meat, was not effective for wine treatment (Posada *et al.,* 1971). A recent patented procedure for eliminating grape proteins utilises proteases from *Aspergillus niger*. That procedure is questionable because the amount of enzymatic protein to be added to wine must be even larger than that of the proteins to be degraded (Edens *et al.*, 2005).

Some researchers have hypothesized the possibility to exploit the proteolytic enzymes of phytopathogenic fungi to degrade the grape proteins responsible for protein instability in wines. In particular *Botrytis cinerea*, the necrotrophic fungus that attacks the berry at maturity, could be able to produce proteases capable to degrade the grape proteins. In experiments performed in liquid culture, *B. cinerea* decreased by only 30% the grape juice proteins, but it is not clear if this reduction is due by proteolytic action of the fungus or others mechanism (Gibrau *et al.,* 2004), as also described in the previous chapter of this thesis.

The preliminary results here presented focused on other phytopathogenic fungus, *Sclerotinia minor*, which is not a natural pathogen for grapes, as a possible source of proteases capable to degrade the grape proteins and to allow wine protein stabilisation.

3.2 Materials and methods

3.2.1 Fungal culture

The fungus *Sclerotinia minor* was grown on Potato Dextrose Agar (PDA, Difco laboratories) plates (85% potato extract, 8.5% dextrose, 6.5% agar) at 24 ºC.

The medium was prepared dissolving 39 g of PDA in 1 litre of distilled water and sterilized in autoclave at 121 ºC for 15 minutes.

3.2.2 Purification of grape proteins

Proteins were extracted from about 1 L of juice obtained by hand crushing about 1.6 kg of grape berries (cv. Manzoni bianco) at 4°C in a beaker. The grape juice was immediately filtered trough nylon gauze, and cysteine was added to final concentration of 4 mM to prevent polyphenol oxidation. All successive operations were also performed at 4°C. The juice was centrifuged at 30000g for 30 min, and the supernatant was filtered in succession with GMD and cellulose acetate membranes (0.8 µm) (Sartorius) and dialyzed overnight (membrane cut off 10 kDa) against potassium tartrate buffer 10 mM at pH 3.5. The dialyzed material was adjusted to 20% of saturation of $(NH_4)_2SO_4$ and stirred for 1 h. After centrifugation at 30000g for 30 min, the supernatant was filtered in succession with 0.8-, 0.4-, and 0.2-µm membranes, concentrated 8 times with a VivaFlow 5000 apparatus (Sartorius), and loaded on a Sephadex G-25 column (PD-10, GE Healthcare). The protein was eluted with water and further concentrated with a VivaFlow 5000 apparatus and then assayed for protein (Bradford, 1976) and phenol (Folin-Ciocalteau assay) amounts using BSA and gallic acid as the standards, respectively.

The protein profile was analysed after protein precipitation with four volumes of cold ethanol at -20°C for 2 h and the pellet containing the precipitated protein was washed once with 70% cold ethanol. After centrifugation at 12000g for 15 min, the precipitated protein was air dried and separated by sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) on 16% (w/v) total polyacrylamide gels (SDS-PAGE). Gels were stained with the colloidal Coomassie G-250 blue silver method (Candiano *et al.*, 2004).

In the protein product was analysed the protein content by Amido Black method (Weiss *et al.,* 2001) and the polyphenols content by Folin-Ciocalteau (Singleton *et al.,* 1965).

3.2.3 Production and purification of proteases from *S. minor*

Twenty agar plugs (Ø 5 mm) containing active growing fungal mycelium were used to inoculate flasks containing 400 mL of Czapeck medium (0.5 g/L KCl, 0.5 g/L $MgSO_4$ x $7H_2O$, 1g/L K_2HPO_4, 0.01 g/L $FeSO_4$, 1mL/L of a 1% $ZnSO_4$ - 0.5 % $CuSO_4$ solution), glucose (20 g/L), casein hydrolysate (5 g/L) as the nitrogen source and to pH 3.5acidified with tartaric acid.

The flasks were stirred at 100 rpm for 6 days at 25 °C in an orbital shaker. At harvest, the culture medium was filtered with 0.2 μm and loaded on a PD10 desalting column (Amersham Biosciences AB, USA). Proteins were eluted with 20 mM of sodium succinate pH 4,concentrated bya VivaFlow 5000 apparatus and assayed for protein content determination (Bradford, 1976).

3.2.4 Proteolytic activity assay

The proteolytic activity produced in fungal cultures was checked with the following protocol: 100 μL medium or pepsin (Sigma) (0.5 g/L in glycine buffer – HCl 0.2 M pH 3) were mixed with 450 μL of 1% haemoglobin pH 3. The samples were incubated at 37 °C for 8 hours and the reaction was stopped with 450 μL of 20% trichloroacetic acid (TCA). After centrifuging at 14000 rpm for 10 minutes and addition of an equal volume of 0.5 M NaOH, the samples were read spectrophotometrically at 280 nm. The absorbance of blank samples (obtained with the same procedure but adding the TCA 20% before the substrate) was subtracted to the sample values. The protease activity was expressed in AU/mL.

3.2.5 Degradation of BSA by *S. minor* protease

Ten µL filtered and 8 fold concentrated culture medium of *S. minor* were added to 1 mL of 0.2 M glycine HCl buffer pH 3.22 containing 1mg Bovine Serum Albumin (BSA). The samples were incubated at 20 °C. The proteolytic activity was stopped at different times by adding 10 µLof 0.5 M Tris-HCl buffer at pH 6.8 containing 15 % (v/v) glycerol (Sigma) and 1.5% (w/v) SDS (Bio-Rad), and heating at 100 °C for 5 minutes. These samples were loaded the loaded in SDS-PAGE.

3.2.6 Laccase activity assay

Laccase activity was determined spectrophotometrically as described by Wolfenden and Willson (1982) in a total volume of 0.8 mL substrate [0.7 mL of 0.1 M acetate buffer, 0.1 mL of 2 mM2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS)], and variable volumes of sample to be tested (1–30 μL). The enzyme assay was performed at 30°C by monitoring the A_{420}. One laccase unit (U) was defined as the amount of enzyme that oxidizes 1 μmol ABTS per min.

3.2.7 *In vitro* Test

200 μL (30 mM sodium succinate buffer at pH 3.2) containing 50 μg/mL of purified grape proteins were incubated with partially purified protease of *S. minor* (0.001 U/mL). Purified proteins (50 μg/mL) and partially purified protease (0.001 U/mL) in 30 mM sodium succinate buffer at pH 3.2 were singularly prepared as controls.

The samples were incubated at 24 °C for 72 hours. Then, 4 volumes of ethanol were additioned to the samples. Successively, they were centrifuged and the supernatant was eliminated. Then, 40μL of 0.5 M Tris-HCl buffer pH 6.8

containing 15 % (v/v) glycerol (Sigma) and 1.5% (w/v) SDS (Bio-Rad) was added to the pellets. The samples were heated at 100 °C for 5 minutes and loaded in SDS-PAGE gels.

3.2.8 Fermentation trials

The experiments were conducted on juices of Manzoni bianco and Glera grape varieties by pressing the berries manually and adding 40 mg/L of sulphur dioxide. The grapes were collected at physiological maturity (stage) in 2009 in the vineyard of Lucchetta Marcello farm (Conegliano – Italy).
Fermentations were carried out at 18 ˚C in 150 mL flasks containing 100 mL of juice. The protease was added 24 hours before yeast inoculation. The yeast DV10 preparation (Lallemand) was hydrated and inoculated according to the manufacturer instructions.
Fermentations were monitored through the measure of soluble solids in solution using the refractometer PR-101 (ATAGO CO. LTD). In the middle of fermentation 300 mg/L of di-ammonium phosphate (Sigma) were added as nutrient for the yeast. At the end of fermentation the samples were stored at 4 °C for 7 days, then filtered by 0.45 μm membranes and, after addition of sulphur dioxide (30 mg/L), were subjected to heat test and assayed for protein content.

3.2.9 Heat Test

Sample aliquots (18 mL) were incubated at 80 °C for 2 hours in 20 mL glass tubes, cooled in ice for 2 hours, maintained at room temperature for 30 minutes and transferred into quartz cuvette (1 mL capacity). Haze was measured spectrophotometrically (Shimadzu UV 6010) at 540 nm (Waters *et al.,* 1992) and nephelometrically (Pocock&Rankine, 1973) using a turbidimeter (2100P,

HACH). Values from untreated samples were subtracted to those of each treated sample.

3.2.10 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoretic analyses were performed according to Laemmli (1970). Samples to be analysed were dissolved in a 0.5 M Tris-HCl pH 6.8 buffer containing 15% (v/v) glycerol (Sigma) and 1.5% (w/v) SDS (Bio-Rad) (loading buffer) and heated at 100 °C for 5 minutes before loading. For SDS-PAGE in reducing conditions, 3% (v/v) of 2-mercaptoethanol (Sigma) was also added to the loading buffer. Electrophoresis was performed in Mini-Protean III apparatus (Bio-Rad) with T = 14% (acrylamide-N, N' metylen-bisacrylamide 29:1; Fluka) gels. The molecular weight standard proteins were Myosin (200,000 Da), β-galactosidase (116,250 Da), Phosphorylase b (97,400 Da), Bovine Serum Albumin (66,200 Da), Ovalbumin (45,000 Da), Carbonic anhydrase (31,000 Da), Trypsin inhibitor (21,500 Da), Lysozyme (14,400 Da) and Aprotinin (6,500 Da) (Broad Range Molecular Weight Markers, Biorad). After electrophoresis, gels were stained for 18 h with Coomassie brilliant blue R-250 (Sigma) and then destained with 7% acetic acid for 24 h (Koenig et al. 1970).

3.2.11 Protein Determination by Reverse Phase (RP)-HPLC

The protein composition of wine fractions was determined by HPLC, according to the method proposed by Peng *et al.* (1997).

One hundred μL of sample was loaded at 1 mL/min onto a Vydac 218 MS 54 4.6 x 250 mm (Grace Davison Discovery Science^{TM}) column equilibrated with a mixture of 83% (v/v) solvent A [8% Acetonitrile, 0.1% trifluoroacetic acid (TFA)] and 17% solvent B [80% Acetonitrile, 0.1% (v/v) TFA] and held at

35ºC. Proteins were eluted by a gradient of solvent B from 17% to 49% in the first 7 minutes, 49% to 57% from 7 to 15 minutes, 57% to 65% from 15 to 16 minutes, 65% to 81% from 16 to 30 minutes and than held at 81% for 5 minutes before re-equilibrating the column in the starting conditions for 6 more minutes. Peaks were detected at 220 nm. Thaumatin (Sigma) was used as standard.

3.3 Results and Discussion

S. minor growing in the liquid culture with hydrolysed casein produced a main protein band with a molecular weight of about 34 kDa which was further purified by differential ammonium sulphate precipitation (Figure 1 A). The protein was identified by the Plant Pathology laboratory of the University of Padova as an aspartyl protease. The purified protease activity was assayed on BSA and the resulting mixture was separated by SDS-PAGE. Results at increasing the times of incubation showed that the purified protease was able to progressively degrade BSA (Figure 1 B).

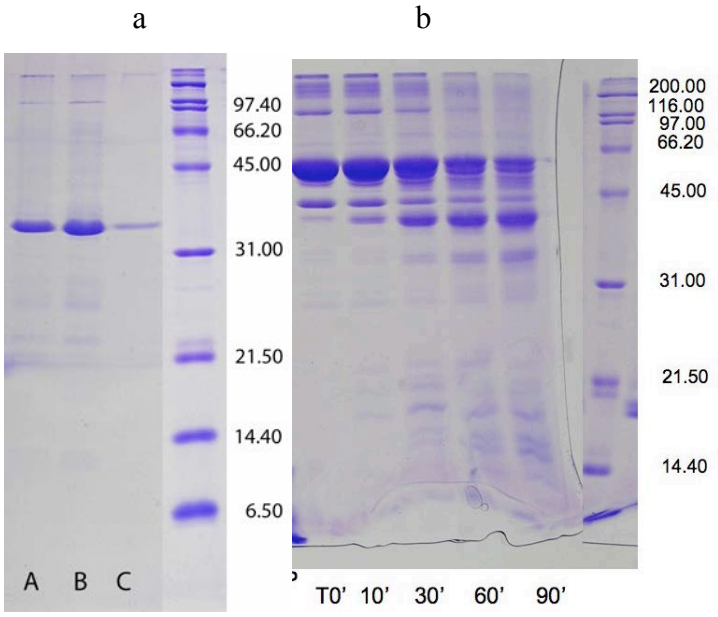

Figure 1. **(a)** SDS-PAGE analysis of the protease produced by *S. minor* in liquid culture before (lanes A and B) and after purification (lane C). The loading volumes of precipitated (with 4 volumes of cold ethanol) samples were 250 μL (lane A), 500 μL (lane B) and 100 μL (lane C). **(b)**SDS-PAGE analysis of BSA solutions (10 μL) after incubation from 10 to 90 minutes with the *S. minor* protease.

A similar test performed using the purified grape proteins as the substrate showed that, after 72 hours of incubation, 1 μg of protease decreased dramatically the intensity of the major bands of 5 μg of grape proteins (Figure 2). The main bands around 21.50 and 31 kDa are ascribable to grape TL proteins and Chitinases, respectively (Figure 2).

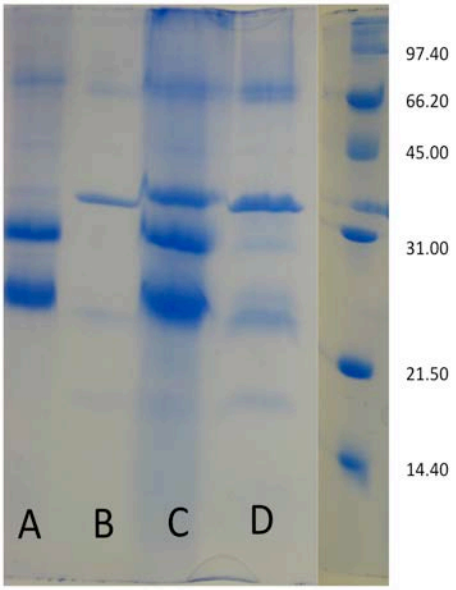

Figure 2. SDS-PAGE of purified grape proteins incubated for 72 hours with the protease from *S. minor*. Lane A: 5 μg of purified grape proteins (control); lane B: 1 μg protease of *S. minor* (0.001 U); lane C: 5 μg of purified grape proteins plus 1 μg protease of *S. minor* (0.001 U) mixed and precipitated immediately with addition of 4 volumes of cold ethanol; lane D: 5 μg of purified grape proteins plus 1 μg protease of *S. minor* (0.001 U) after 72 hours of incubation.

Different amounts of the partially purified protease were added to grape juice before fermentation. Successively the wines obtained were checked for the presence of grape proteins and protein instability. Pepsin was used for comparison.

Both the protease from *S. minor* and pepsin did not affect the kinetic of fermentation and all samples completed the fermentation (data not shown).

In comparison to treatments with pepsin and with the untreated control, the juices treated with 0.001 U (corresponding to 960 μg/L) of *S. minor* protease preparation became more intensely coloured (figure 3).

Figure 3. Flasks containing the grape juices after 24 hours from the treatment with protease of *S. minor* (left), pepsin (middle) and untreated control (right).

The addition of protease to the juice before the fermentation altered the protein instability of both Manzoni bianco and Glera wines. Figure 3 shows heat test results after fermentation of the Manzoni bianco juice. Both pepsin and the *S. minor* protease preparation at 0.001 U increased wine protein instability probably because this amount of enzyme increased the total amount of protein in wine. Instead, lower amounts of *S. minor* protease (0.0005 U corresponding to 480 μg/L and 0.00025 U corresponding to 240 μg/L) decreased the protein instability and the wine was almost stable to the heat test (figure 4). The SDS-PAGE analysis of the wine samples showed decreased protein bands intensity after the *S. minor* protease treatments independently from the dose used, while, as expected, the intensity of the protease band was proportional to the dose used (Figure 5). Instead, in wine samples obtained after the pepsin treatment, the intensity of the bands did not decrease and was comparable to that observed in untreated controls (Figure 5).

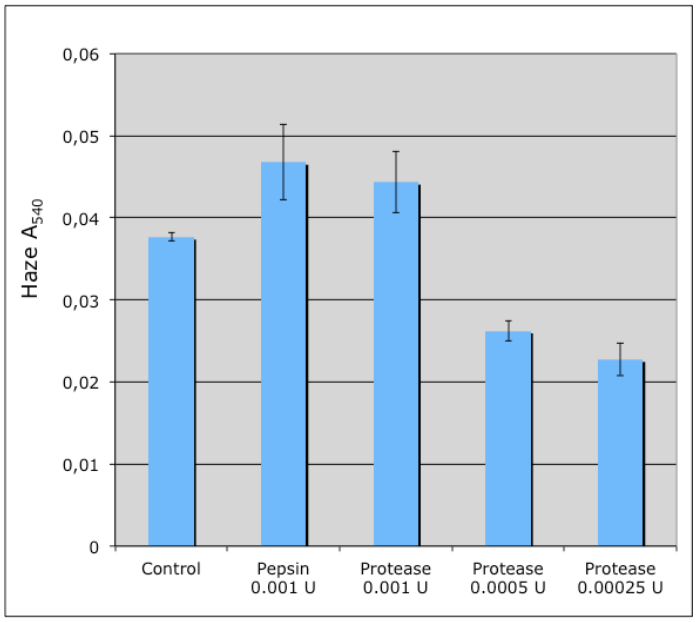

Figure 4. Heat Test results of the Manzoni bianco wine obtained after different enzymatic treatments. The haze was determined spectrophotometrically at 540 nm after heating and values measured before heating were subtracted. The wine is considered stable when the net absorbance is below 0.02. The tests were repeated 3 times.

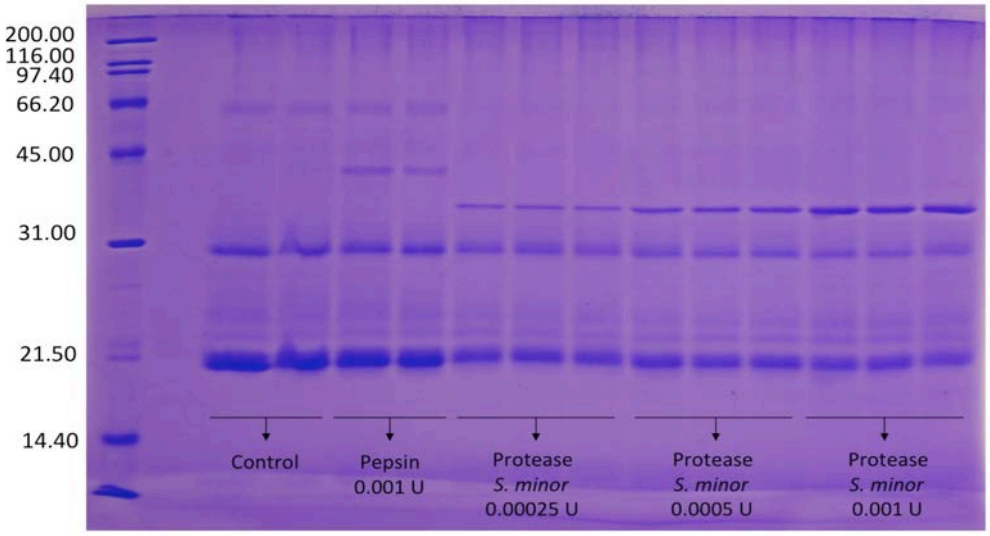

Figure 5. SDS-PAGE of wine proteins of Manzoni bianco after enzymatic treatment with *S. minor* protease or pepsin. The main bands are Thaumatin like proteins (bands around 21.50 kDa) and chitinases (bands around 31.00 kDa). The bands of pepsin (41 kDa) and of *S. minor* protease of (34 kDa)are visible. Controls were prepared without protease addition.

The heat test results after fermentation of Glera juice are shown in figure 6. In this experiment the *S. minor* protease doses were lowered in comparison to experiment performed with the Manzoni bianco juice. Although the initial protein amount of the Glera juice was much lower than that of Manzoni bianco and mostly represented by TL proteins (Figure 7), also in this case the *S. minor* protease treatments decreased the wine haze, allowing to reach complete protein stability at the end of the experiment (Figure 6). The SDS-PAGE analysis confirmed that grape proteins were partially removed only by the *S. minor* protease treatment.

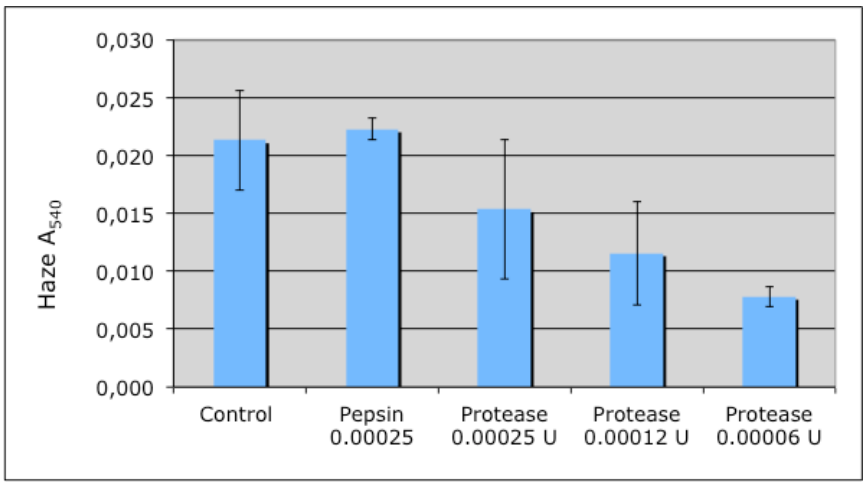

Figure 6. Heat Test for the Glera wine obtained after different enzymatic treatments. The haze was determined spectrophotometrically at 540 nm after heating and values determined before heating were subtracted. The wine is considered stable when the net absorbance is below 0.02. The tests were repeated 3 times.

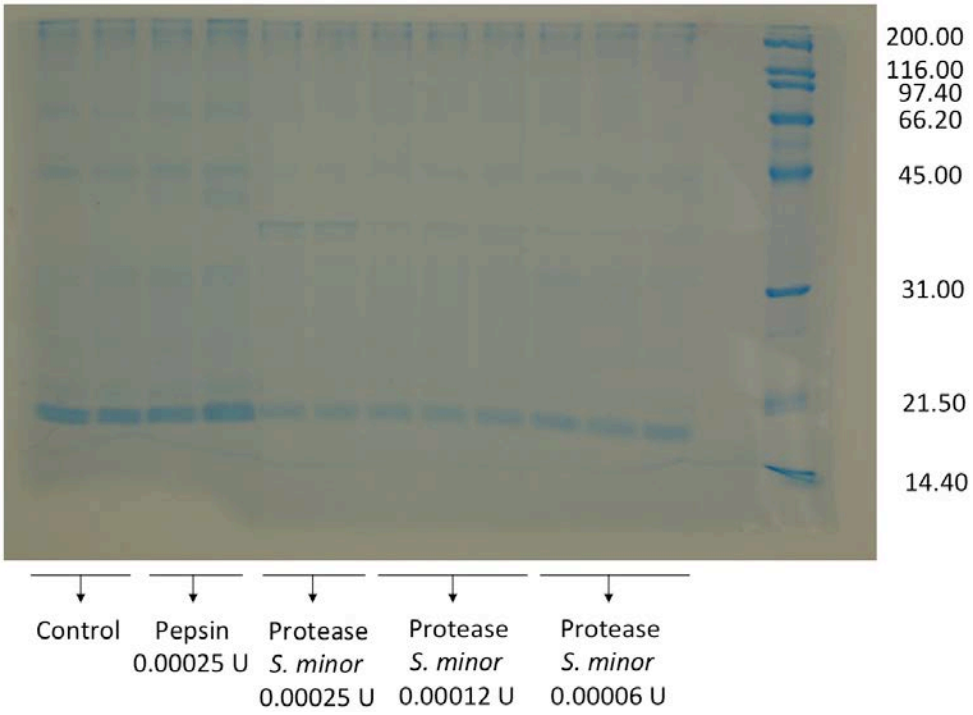

Figure 7. SDS-PAGE of wine proteins of Glera juice after enzymatic treatment with *S. minor* protease or pepsin. The main bands are Thaumatin like proteins (bands around 21.50 kDa). The band of pepsin is barely visible at 41 kDa and that of *S. minor* protease is visible at about 34k Da. Controls were prepared without addition of protease.

The protein separation performed by RP-HPLC (Figure 8 and 9) confirmed the results obtained by heat test and SDS-PAGE analysis. The protease of *S. minor* treatment was able to decrease of about 50% the protein content compared with control, acting on all the main protein components.

The darkening of samples fermented with the higher dose of *S. minor* protease preparation (Figure 3) suggested the presence of possible secondary reactions likely due to the presence of other enzymatic activities. Moreover protease preparations produced with two further *S. minor* cultures showed different capacity to degrade the grape proteins and to decrease the protein instability (Table 2) although these preparations maintained the capacity to degrade BSA (data not shown).

Laccase activity was measured in the different *S. minor* protease preparations. The preparation capable to increase the wine protein stability contained the highest residual laccase activity, from 3 to 10 times more than the other two preparations (Table 2).

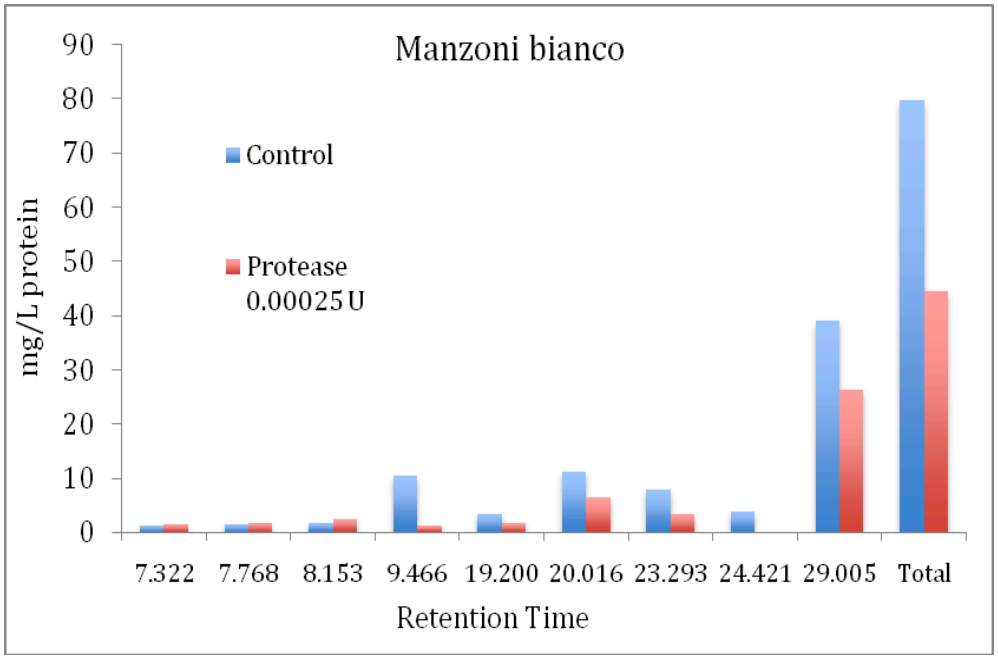

Figure 8. Separation of the Manzoni wine proteins by RP-HPLC after treatment of grape juice with or without *S. minor* protease. The protein concentration (mg/L of Thaumatin) of the main peaks was determined from their chromatographic area using Thaumatin as the standard

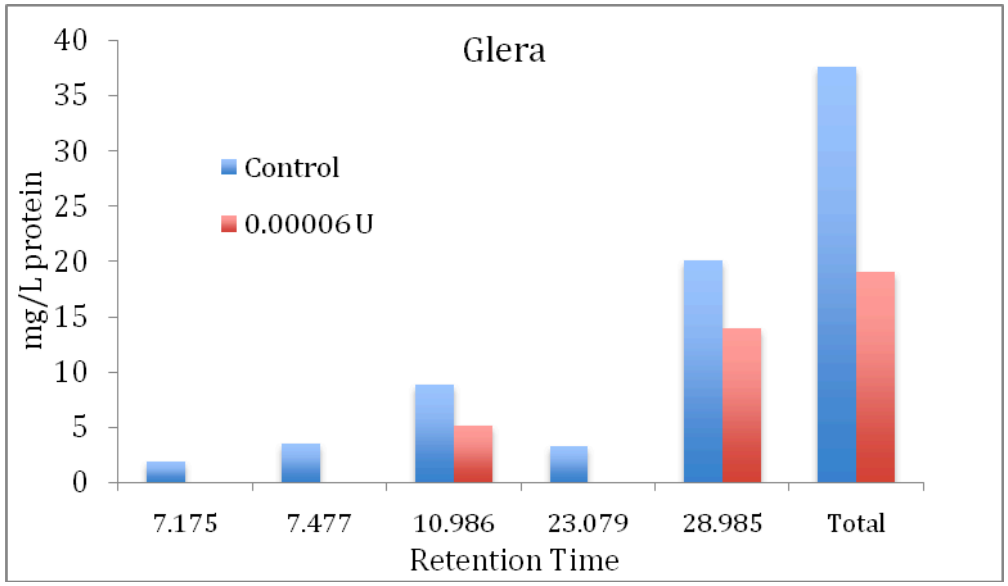

Figure 9. Separation of the Glera wine proteins by RP-HPLC after treatment of grape juice with or without *S. minor* protease. The protein concentration (mg/L of Thaumatin) of the main peaks was determined from their chromatographic area using Thaumatin as the standard

	Protease activity U/mL	**Laccase activity U/mL**	**Effect on grape protein and wine stability**
Culture 1	180	0.26	Degradation of grape proteins and reduction of protein instability
Culture 2	230	0.023	No effect against grape proteins and no reduction of protein instability
Culture 3	223	0.08	No effect against grape proteins and no reduction of protein instability

Table 2. Comparison of protease and laccase activities in different *S. minor* protease preparations and effects on grape protein degradation and wine stability.

3.4 Conclusions

A protease preparation obtained from a culture of *Sclerotinia minor* was able to degrade the grape proteins in *in vitro* tests and in microvinification trials. In particular, the protein instability decreased and in some trials the wine reached complete protein stability. Also the protein profile obtained by SDS-PAGE and the protein content determined by HPLC confirmed the effect of the protease preparation against the grape proteins. However, others culture preparations were not active against the grape proteins although showing proteolytic activity against BSA. One factor that could influence the activity of the protease of *S. minor* is the laccase activity released in the culture medium. In fact, the only protease preparation able to degrade the grape proteins was that containing the highest laccase activity. This mechanism should be better clarified and also the evaluation of organoleptic quality of the wines obtained after protease treatments is required.

In conclusion, the approach here used for developing a method for degradation of wine unstable proteins by exploiting the enzymatic activities of a phytopathogenic fungus should be taken into account as a possible strategy to stabilise white wines. This is because of the potentiality of the phytopathogens to attack fruits by expressing biological tools able to contrast the natural defence mechanisms of the plant, including the grape PR-proteins, which are responsible for protein instability in wines. The results here shown demonstrated that this strategy can be potentially exploited. However the first trials here attempted in that direction showed inconsistent results, indicating that some unknown factors, probably acting during the growth of the fungus, should be identified and controlled to be successful.

3.5 References

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4. The use of zirconium dioxide during fermentation as an alternative method for protein removal

Zirconium dioxide (zirconia) could be a valid alternative to bentonite in protein stabilization of white wines (Pachova *et al.,* 2002; Pashova *et al.,* 2004a; Pashova *et al.,* 2004b; Salazar *et al.,* 2006; Salazar *et al.*, 2007; Marangon *et al.*, 2011), but the application of this materials does not seem to be simple and would need complex equipment.

The aim of this work was to find a system allowing to exploit the capacity of zirconia to stabilize wines to be used also in small wineries in a simple and effective way. To do that, the application of zirconia pellets during the fermentation in order to exploit the natural mixing of the must by the carbon dioxide released by the yeast was evaluated. The treatment was achieved by enclosing zirconia pellets into a simple metallic cage submerged in the fermenting grape juices. Three juices (from grapes of the cvs. Riesling, Sauvignon and Semillon) were treated during fermentation and the wines obtained were found to be fully stabilized after the treatment. Indeed zirconia treatment of the juices was very effective in removing grape unstable proteins. Zirconia did not affect the fermentation and partially removed some organic acids and ions. This work confirmed the effectiveness of zirconia in removing proteins and stabilize wines. The proposed method could be applied in a simple way to the industrial wine making process. Moreover a practical and inexpensive protocol regenerate the pellet materials was developed.

4.1 Introduction

The Grape proteins remaining in finished wines could cause haze formation during wine storage (Bayly and Berg, 1967). This fault is not accepted by the consumers and results in a strong reduction of the commercial value of the wine.

Nowadays, bentonite clay (a cation exchanger with strong protein adsorption capacity) is mostly used in winemaking as the main technique to stabilize wines. Bentonite is still extensively used because of its established efficacy as well as its low cost. However, bentonite fining has some drawbacks such as significant wine volume loss (3-10%) due to poor settling (Waters *et al.,* 2005). Bentonite could also provoke reduction of wine sensory quality, since it is not a specific adsorbent and can remove important aroma, flavor and polyphenolic compounds (Lubbers *et al.,* 1993, Ribéreau-Gayon *et al.,* 2000). For these reasons, alternative procedures for protein removal from white wines have been extensively investigated, including the use other adsorbents (Sarmento *et al.,* 2000, Vincenzi *et al.,* 2005), immobilized tannic acid (Weetall *et al.*, 1984) or proanthocynidins (Powers *et al.*, 1988), ultrafiltration (Hsu *et al.,* 1987), and proteases (Waters *et al.,* 1992), but no alternative has proven sufficiently cost effective compared to bentonite.

One alternative technique is the use of zirconium oxide, a metal oxide commonly known as zirconia. The surface of zirconia is able to adsorb the unstable grape proteins (Pachova *et al.,* 2002; Pashova *et al.,* 2004a; Pashova *et al.,* 2004b; Salazar *et al.,* 2006; Salazar *et al.*, 2007). This material is characterized by low potential corrosion, low thermal conductivity and strong thermal and mechanical resistance (Piconi and Maccauro *et al.*, 1999; Liu *et al.*, 2005; Manicone *et al.*, 2007). Because of its characteristics zirconia has many applications such as catalyst or support material, refractory material, ceramic material and biomechanical support in medical implants (Piconi and Maccauro 1999; Rovira-Bru *et al.,* 2001; Chevalier, 2006).

The first application of zirconia in the oenological sector has been proposed by Pachova *et al.* in 2002. The authors demonstrated the efficacy of zirconia to adsorb the proteins in a model white wine. Later, Pashova *et al.* (2004) proposed a continuous method for the protein stabilization using a column filled with zirconia, and this treatment was compared with that based on bentonite (Salazar *et al.* 2006). However, at the moment, wine stabilization with zirconia has not found practical using in the wine industry.

The discontinuous process was suggested again in a previous work (Marangon *et al.,* 2011), with zirconia enclosed in metallic cages to be submerged in the wine. In this way, the protein stability of the wine was increased dramatically, but the wine needed to be mixed permanently with the zirconia cages for 3-5 days. This procedure is difficult to perform in the wineries, in particular in high volume tanks. To overcome this problem, the zirconia treatment during fermentation could be an alternative, since the natural movement of the fermenting must can be exploited.

The aim of this work was to confirm the ability of zirconium dioxide in protein stabilization of white wines and to test the capacity of zirconia to remove the unstable grape proteins during the fermentation of the must. Moreover a practical protocol for the regeneration of the zirconia pellets after their use was developed.

4.2 Materials and Methods

4.2.1 Materials

Three unfined 2007 grape juices (from cv. Riesling, Semillon and Sauvignon Blanc) sourced from Adelaide Hills (South Australia) were used. The Chardonnay was used to develop the method for zirconia regeneration. Zirconia was used in the form of pellets (Saint-Gobain, Ohio, USA) and was donated by Prof. Francisco Lopez (University Rovira y Virgili, Spain). Zirconia

pellets were disks with a diameter of 3 mm and a thickness of 1 mm, a pore size of 6.2 nm, a surface area of 108.5 m^2/g and with a tetragonal morphology (Salazar, 2007). The yeast strain used was the AWRI 796 (Maurivin).

4.2.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Marangon *et al.* (2011). Briefly: NuPage (4-12% Bis-tris), 1.5 mm thick, 15-well gels (Invitrogen, Mt Waverley, Victoria, Australia) and an XCell SureLock Mini Cell (Invitrogen) were used following manufacturer instructions. Proteins from 100 µL of wine were precipitated adding 2 volumes of cold trichloroacetic acid (10%) in acetone. The pellet obtained after centrifugation (13 000 x *g*, 15 min, 0°C) was washed twice with 80% acetone. After centrifugation (13 000 x *g*, 15 min, 0ºC) pellets were dissolved in 40 µL of loading buffer (Invitrogen NuPage recipe) with 5% (v/v) 2-mercaptoethanol and boiled for 5 min. Precision Plus Protein unstained standards were from Bio-Rad (Bio-Rad Laboratories Pty.Ltd, Regents Park, New South Wales, Australia). Proteins were stained with Pierce Imperial Protein Stain (Quantum Scientific, Sidney, New South Wales Australia) according to the manufacturer's microwave instructions.

4.2.3 Protein content determination

Protein content was determined by EZQ® protein quantitation kit (Invitrogen) following the manufacturer's instructions. The calibration curve was built using serial dilution from 0 to 250 mg/L of Thaumatin from *Thaumatococcus daniellii* (Sigma-Aldrich, Castle Hill, New South Wales, Australia). Fluorescence measurements were taken using excitation/emission settings of

485/590 nm with a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, California, USA).

4.2.4 Protein content determination by HLPC

Protein concentration and composition were determined by reverse-phase (RP) HPLC with a Vydac 2.1 x 250 mm C8 column (208TP52 Grace Davison Discovery Sciences, Australia) on an Agilent 1200 system according to the method of Marangon *et al.*(2009) with modifications as suggested by Van Sluyter *et al.* (2009). Injection volumes were 25 µL. From the 210 nm chromatogram, protein identity was assigned by comparison of the retention times (RT) to those of purified grape PR proteins (Marangon *et al.,* 2009) as follows: peaks with a RT between 9.00 and 12.00 min were assigned to the TL protein classes, whereas peaks eluted from 19.00 and 24.50 min were assumed to be chitinases. Thaumatin from *Thaumatococcus daniellii* (Sigma-Aldrich, Australia) was used as the standard for protein quantification.

4.2.5 Heat test

Wines were heated at 80°C for 2 h and cooled in ice for 2 h. After equilibration at ambient temperature the haze was measured by calculating the difference in the absorbance values at 540 nm (Waters et al. 1992) and in a nephelometric turbidity unit (NTU) by means of a nephelometer (Pocock and Rankine, 1973) between the heated and unheated samples.

4.2.6 Analytical Methods

Alcohol, specific gravity, pH, titratable acidity, glucose/fructose and volatile acidity analysis were performed by the Commercial Service of The Australian

Wine Research Institute using a Foss WineScan FT 120 as described by the manufacturer (Foss, Hillerød, Denmark). Free and total SO_2 were measured by the aspiration method (Rankine and Pocock, 1970). Brix was measured by refractometry and Baumé by densitometry.

4.2.7 Organic acids by HPLC

The concentrations of organic acids (citric, tartaric, malic, succinic and lactic) were determined by HPLC as described by Marangon *et al.* (2011).

4.2.8 Metal analysis

Wine metal contents were determined by Inductively Coupled Plasma - optical emission spectrometry performed by the Waite Analytical Services (Glen Osmond, South Australia, Australia http://www.adelaide.edu.au/was/), School of Agriculture, Food and Wine, University of Adelaide.

4.2.9 Regeneration of Zirconia

Stirred Chardonnay wine was treated with 25 g/L of zirconia for 24 hours. The same zirconia pellets were used for 11 consecutive times. At the end of each treatment the zirconia pellets was regenerated by washing the pellets twice with 3 M NaOH at 50 °C for 2 h and then with 5% citric acid at room temperature for 30 minutes. The pellets were rinsed three times in distilled water before use.

4.2.10 Experimental design

Three heat unstable juices were fermented with or without 25 g/L of zirconia in pellet form. Smallscale fermentation (100 mL volume of juice in a Schott

bottle) were conducted in triplicate for each of the juices. Zirconia pellets were applied to juice samples in a stainless steel, 4 cm diameter tea infuser ball. Control wines were made using an empty tea infuser. The infusers were inserted in the juices at day 3 from the beginning of fermentation. The experiment was conducted at 18 °C. After yeast inoculation, fermentation rate was monitored daily by refractometric analysis. An addition of 300 mg/L of DAP (Diammonium phosphate) was done to all fermentations approximately halfway through the fermentations. In the latter stages of the fermentations, reducing sugars were estimated with Clinitest tablets (Rowe Scientific, Melbourne, Victoria, Australia), and the fermentations were considered finished when the reducing sugars was 0.25 % (w/v). The resulting wines were stored at 4°C for 5 days before being filtered (0.2 μm) and added with sulphur dioxide (30 mg/L) to prevent oxidation. Each of the six fermentation was done in triplicate, and analyses for each replicate were done in triplicate too.

4.2.11 Statistical analysis

Data were subjected to analysis of variance in according to a multifactorial design with three replicates.

4.3 Results and Discussion

4.3.1 Physicochemical parameters of the wines

The general composition of the three wines used in this study is described in table1.

	Riesling		Sauvignon Blanc		Semillon	
Parameter	*Control*	*Zirconia*	*Control*	*Zirconia*	*Control*	*Zirconia*
Alcohol (% v/v)	10.8	10.6	13.5	13.4	10.7	10.5
Specific gravity	0.995	0.994	0.992	0.991	0.995	0.994
pH	3.02	3.12	3.13	3.21	2.94	2.98
Titratable acid to pH 8.2 (g/L)	8.9	7.8	8.4	7.5	8.3	7.9
Glucose + Fructose (g/L)	0.3	0.6	0.5	0.3	0.3	0.6
Volatile acidity as acetic acid (g/L)	0.27	0.24	0.48	0.47	0.23	0.22
Sulfur dioxide free/total (mg/L)	< 4/47	< 4/44	< 4/27	< 4/22	< 4/38	< 4/41

Table 1. Main physicochemical parameters of the three wines produced with (zirconia) or without (control) 25 g/L of zirconia pellets.

Contrarily to what observed previously by adding zirconia to wines (Marangon *et al*., 2011), the addition of the adsorbent pellets during fermentation always resulted in lower alcohol content of the wines, which also showed higher pH and lower acidity values. The drop in titratable acidity is explained mainly by the decrease in tartaric acid (~ 10%), while the other organic acids measured were mostly unaffected (Table 2).

	Zirconia (g/L)	Citric (g/L)	Tartaric (g/L)	Malic (g/L)	Succinic (g/L)	Lactic (g/L)	Total Acidity (g/L)
Riesling	0	0.15	3.50	2.38	4.06	0.36	10.47
	25	0.12	3.10	2.50	3.96	0.34	10.05
Sauvignon Blanc	0	0.20	1.70	1.96	2.44	0.18	6.49
	25	0.16	1.31	1.86	2.34	0.17	5.85
Semillon	0	0.09	3.65	1.76	1.45	0.17	7.05
	25	0.07	3.20	1.69	1.31	0.14	6.43

Table 2. Organic acid concentration determined by HPLC of wines after treatment with zirconia.

In a previous study it has been demonstrated that zirconia application to finished wines resulted the decrease of the content of many metals (Marangon *et al.,* 2011). When zirconia is added during the fermentation (Table 3) the resulting wines contained 5 to 10 % less K, Mg, and S. Moreover there was a reduction in P (-10 to -60%) Ca, Mn and B, while only Na was increased.

Metal (mg/L)	Riesling		Sauvignon Blanc		Semillon	
	Control	*Zirconia*	*Control*	*Zirconia*	*Control*	*Zirconia*
K	773.30	700.00	713.33	650.00	646.66	613.33
Ca	88.57	78.56	72.93	65.78	53.14	53.47
Mg	71.54	67.76	60.27	57.78	65.47	65.23
P	243.33	104.46	253.33	106.70	91.99	84.53
S	88.43	85.13	91.14	86.94	85.98	78.19
Na	64.67	78.06	12.58	27.86	15.30	27.41
Mn	0.28	0.19	1.01	0.68	0.70	0.61
B	4.48	4.03	7.72	7.08	5.95	5.25
Cu	< 0.03	< 0.03	0.04	< 0.03	0.11	< 0.03
Zn	0.03	0.08	0.04	< 0.02	0.22	< 0.02
Al	0.04	< 0.01	0.03	0.04	0.10	0.11
Fe	0.18	< 0.02	0.05	< 0.02	< 0.02	< 0.02

Table 3. Metal concentration of wines after treatment with 25 g/L zirconia.

Since zirconia showed the ability of remove metals, some of which important for the yeast fermentation, the fermentation rate were monitored daily (Figure 1).

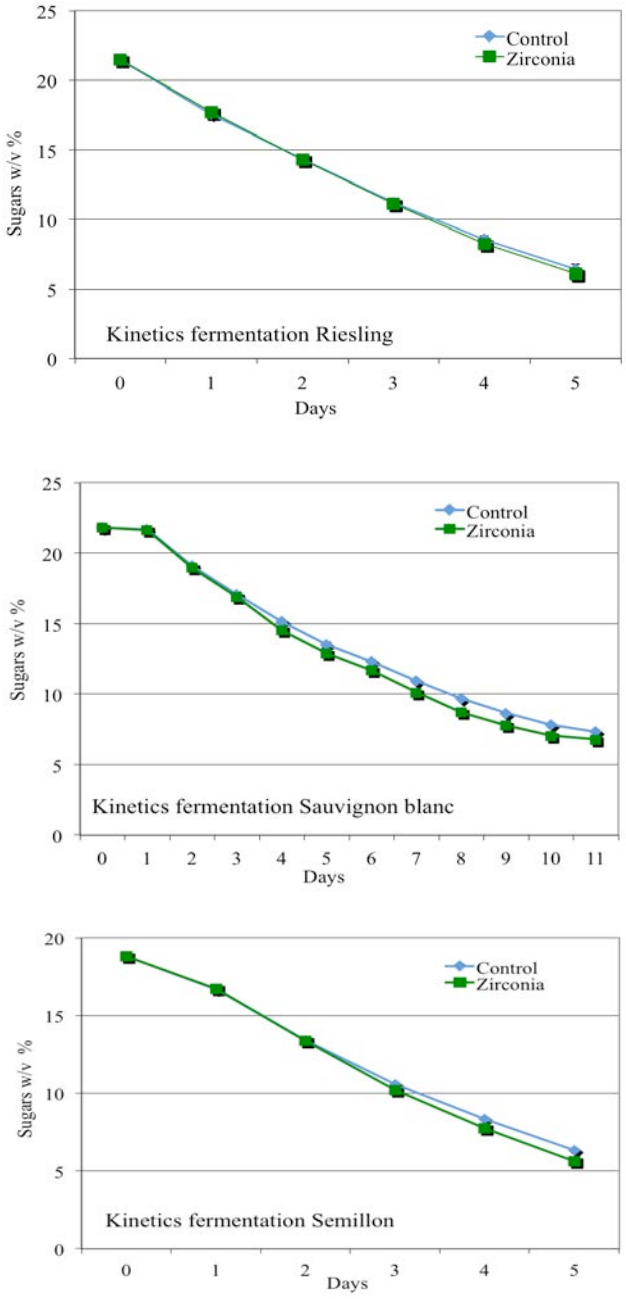

Figure 1. Fermentation rates in Riesling, Sauvignon blanc and Semillon musts measured daily by refractometry. Mean values are shown ($n \geq 3$).

The addition of zirconia, made at day 3 of fermentation, did not have any significant effect on the fermentation rate for all the three musts tested (Figure 1). Conversely, the presence of zirconia always tended to speed up the fermentation process. This result suggests that zirconia can: i) act as support for the yeast, a fact that is considered as positive for the fermentation and ii) remove some compounds, such as ions (iron, copper), with negative effects on yeast activity.

4.3.2 Protein removal and heat stability

The protein content was monitored daily during the fermentation (Figure 2).

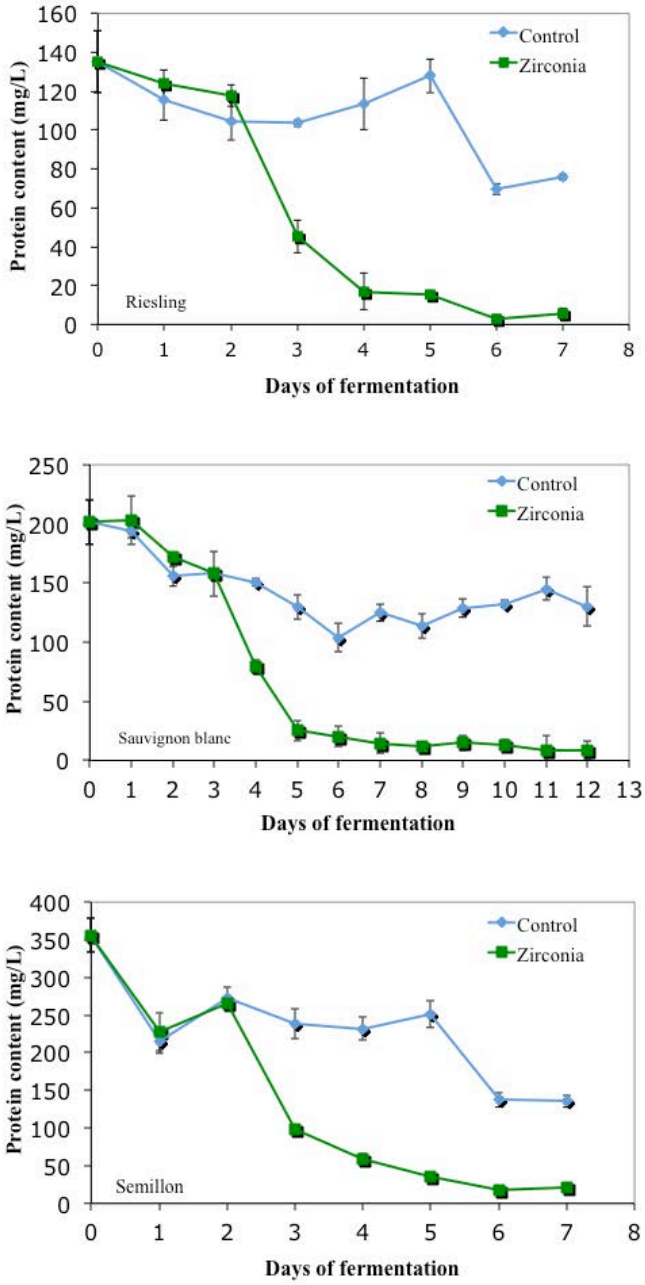

Figure 2. Kinetics of protein removal from the three musts during fermentation. Protein removal was measured by the EZQ kit. Zirconia was added at day 3. Zirconia addition rates: 0 g/L (Control) and 25 g/L (Zirconia). Each point is the average of at least three replicates (n = 3).

The effect of zirconia addition on protein removal is very clear. Indeed, from day 3 there was a sharp decrease in soluble proteins for the three wines that slowed down after 2 days from the application. In general the protein quantity left in the wines was 10-15 mg/L, with a reduction around 95%. From these data it seems that, at this addition rate, 2 days are sufficient to remove most of the proteins. Since in our experiment zirconia was left in contact with the wines for much longer, it is possible that a shorter contact would result in wines less affected by the treatment, particularly with regard to their metals and organic acid contents.

From a qualitative point of view, RP-HPLC (figure 3 and 4) and SDS-PAGE (Figure 5) analyses indicated that protein removal was not specific for particular protein types.

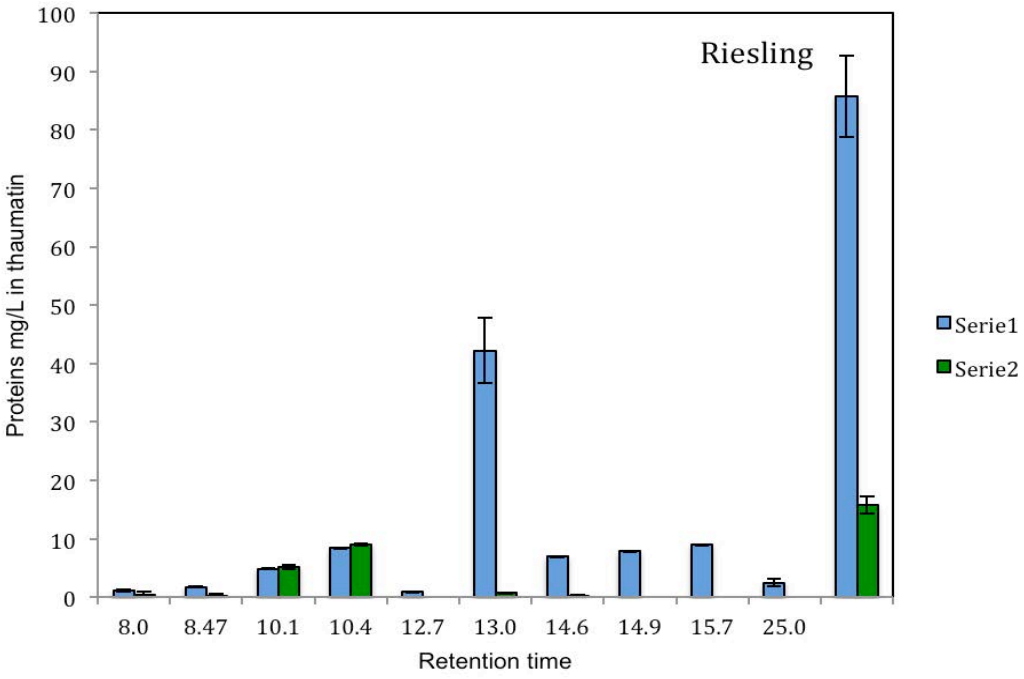

Figure 3. Proteins of Riesling wines separated in the main RP-HPLC peaks. Series 1: no addition of zirconia; series 2 wine from must treated with 25 g/L of zirconia pellets during fermentation. Protein quantity is expressed in mg/L of Thaumatin.

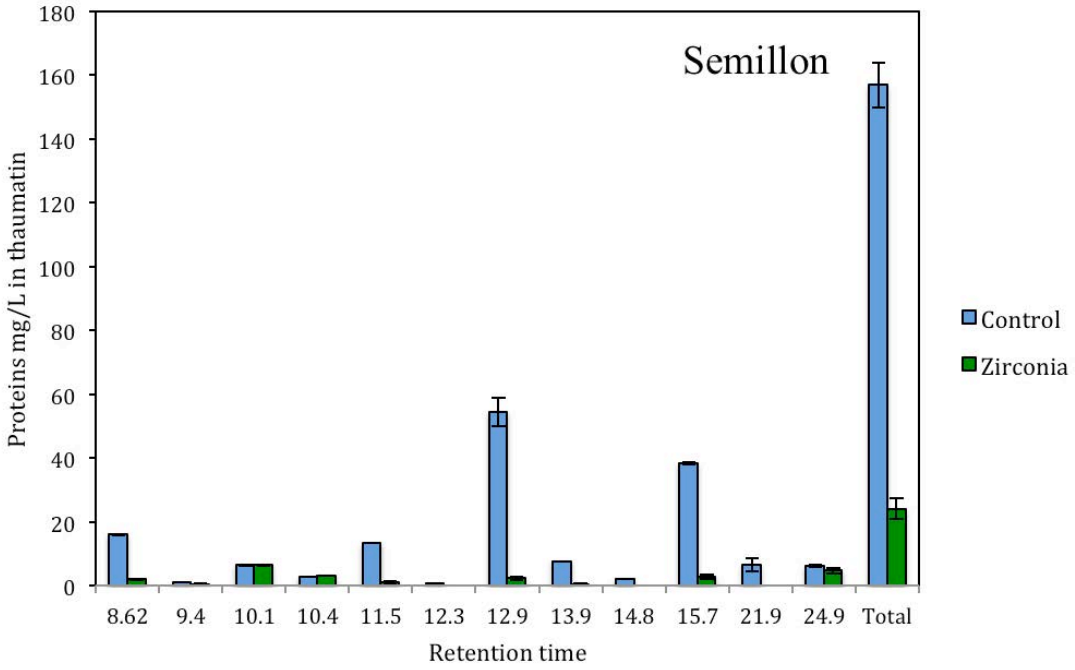

Figure 4. Proteins of Semillon wines separated in the main RP-HPLC peaks. Control no addition of zirconia; Zirconia: wine from must treated with 25 g/L of zirconia pellets during fermentation. Protein quantity is expressed in mg/L of Thaumatin.

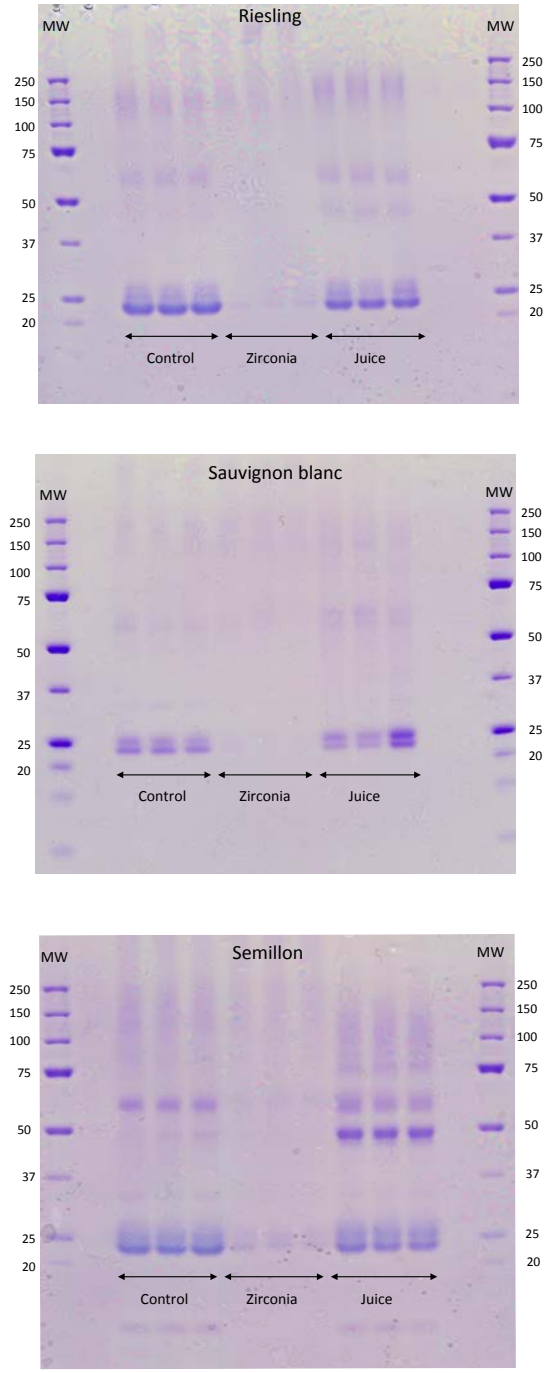

Figure 5. Effect of zirconia treatments on the protein composition of the three wines shown by NuPAGE. Proteins from 100 µL of wine were loaded per lane. The three replicates made for each of the treatments are shown. Starting juice: juice before the beginning of fermentation; Zirconia: wine obtained after treatment with 25 g/L of zirconia; Control: wine obtained without use of zirconia; MW: molecular weight standards (in kDa)

In particular the proteins considered responsible for haze formation, namely Thaumatin-like proteins and chitinases, were almost fully removed by zirconia in all the wines, as assessed by the disappearance of the protein bands at 20-25 kDa.

Despite the observed reduction in protein content (about 95%), an essential step to assess the effectiveness of an adsorbent in relation to winemaking is to challenge the treated wines by a heat test and to measure the haze formed.

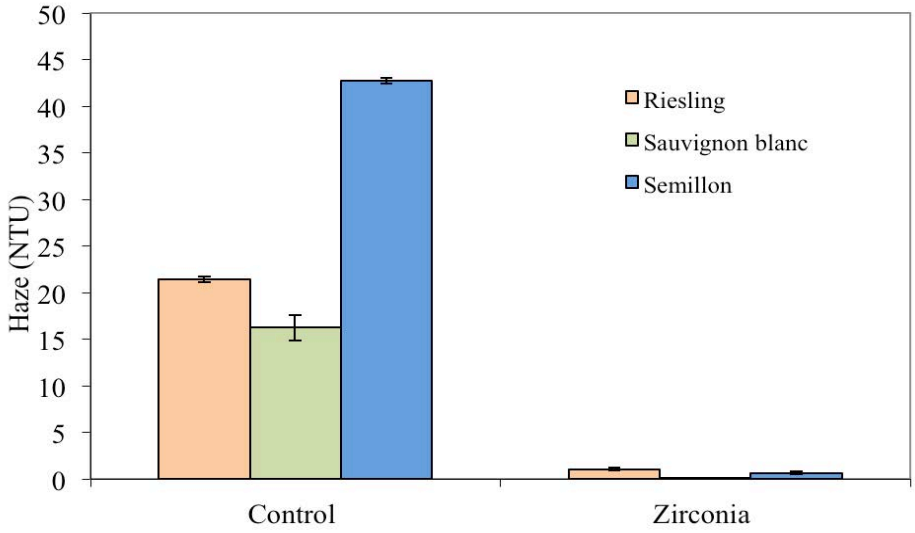

Figure 6. Haze formed by the heat test of the three wines obtained from the fermentation of juices without (Control) and with 25 g/L zirconia (Zirconia). The haze is expressed in Nephelometric Turbidity Units (NTU). Each point is the average of at least three replicates (n = 3).

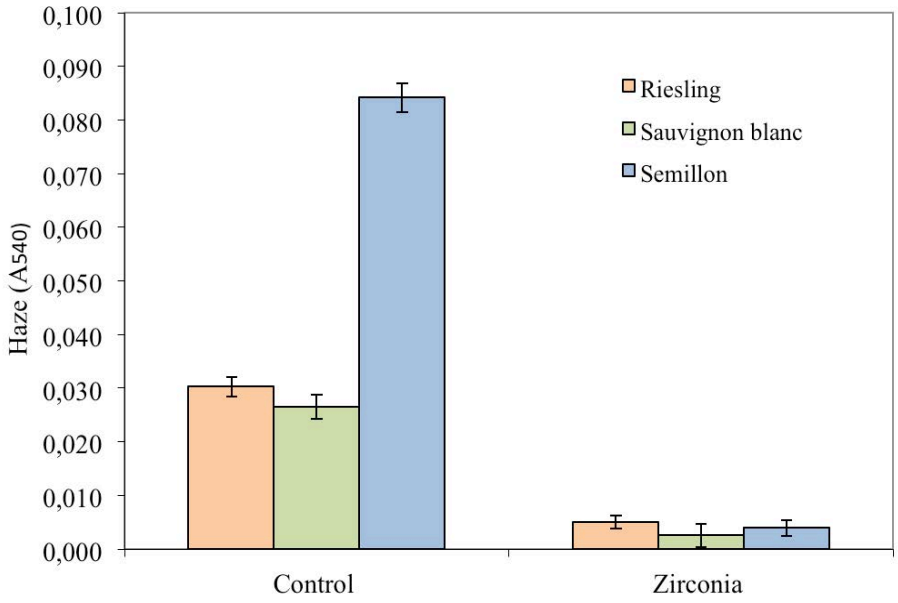

Figure 7. Haze formed by the heat test of the three wines obtained from the fermentation of juices without (Control) and with 25 g/L zirconia (Zirconia). The haze developed was measured by spectrophotometry at 540 nm. Each point is the average of at least three replicates (n = 3).

Each wine gave a haze below 2 NTU and 0.02 of absorbance at 540nm (Figure 6 and 7), hence they were fully stabilized by the zirconia treatment.

In a previous work it was demonstrated that the application of 25 g/L of zirconia could fully or partly stabilize finished wines (Marangon *et al.* 2011). The application of zirconia during fermentation seems to be much more effective and rapid in removing proteins, as demonstrated here. It is then likely that the dosage used can be sensibly decreased. This would result in a slower removal of proteins, but since with 25 g/L of pellets the adsorption action was mostly concluded in 2 days, using less zirconia for a longer time could be an advantage.

4.4 Zirconia regeneration

The same zirconia pellets regenerated for 11 times were able to stabilize a Chardonnay wine without loss of performance, as showed in figure 8 and 9 where the stabilising effect of the regenerated zirconia pellets is shown in comparison with the untreated control wine.

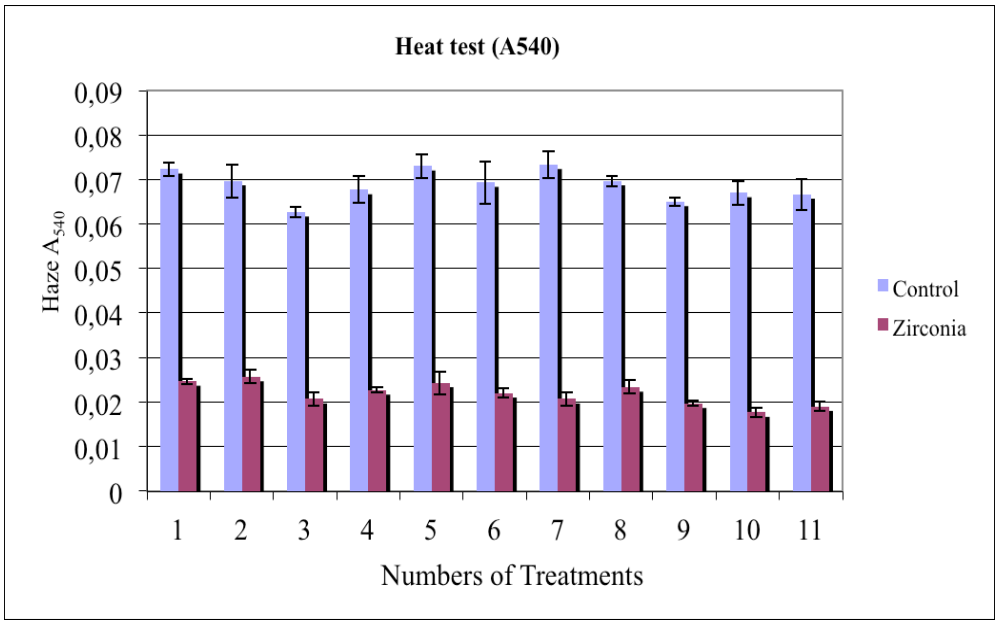

Figure 8. Haze developed in Chardonnay wines treated 11 times with the same regenerated zirconia pellets. The zirconia treatment was always comparated with a control (wine only stirred). The haze developed was measured by reading the absorbance of he samples at 540 nm.

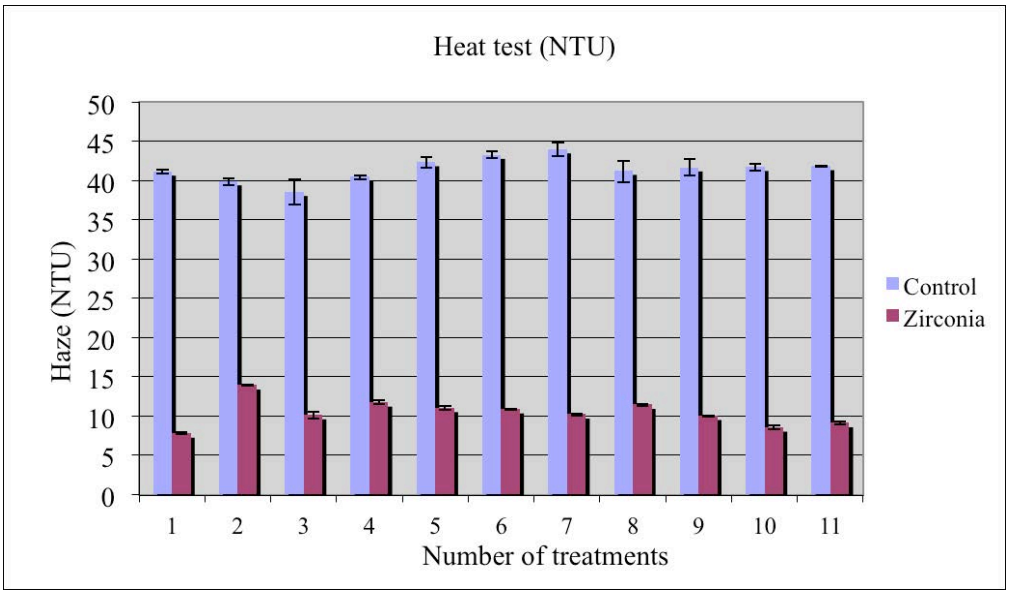

Figure 9 Haze developed in Chardonnay wines treated 11 times with the same regenerated zirconia pellets. The zirconia treatment was always comparated with a control (wine only stirred). The haze developed was measured by nephelometry and results expressed in nephelometric turbidity unit (NTU).

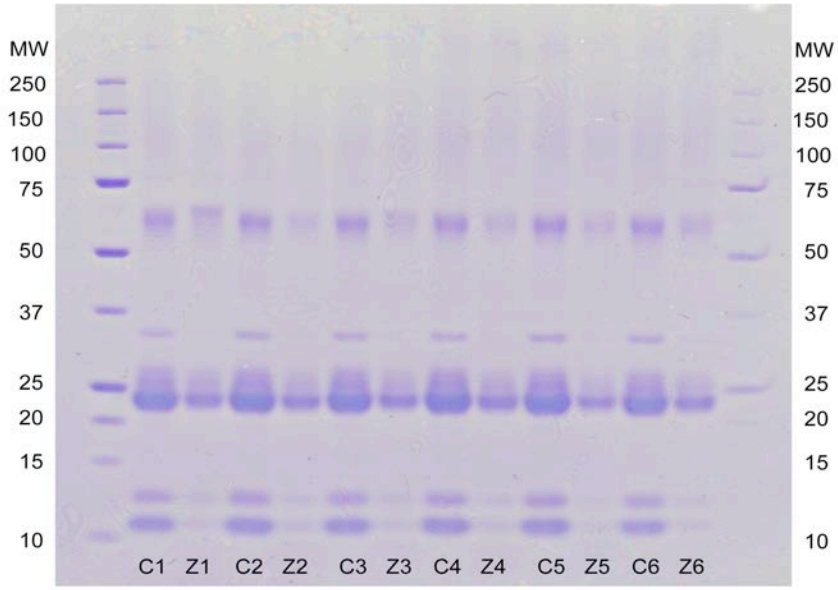

Figure 10. Effect of treatments with regenerated zirconia pellets on the protein composition of a Chardonnay as detected by NuPAGE. Proteins from 100 µL of wine were loaded per lane. Z: treated wines; C: controls. The numbers refer to the number of regenerations made on the same pellets Molecular weights in kDa are on the right (MW).

Also SDS-PAGE analysis showed the continuative action of the regenerated zirconia pellets on protein removal from wine (figure 10).
Similar results were obtained when the regenerated Zirconia pellets were used during fermentation (data not shown), indicating the effectiveness of the regeneration procedure here developed.

4.5 Conclusions

Zirconia in pellet form enclosed in a metallic cage confirmed its potential to become a viable alternative to the bentonite treatments used in winemaking to prevent protein haze formation in white wines. An improvement from a previously proposed use of zirconia as a wine protein adsorbent (Marangon *et al.*, 2011) lies on the fact that with its application during fermentation there is no need for stirring since the fermenting must is naturally mixed by the carbon dioxide released by the yeast. This fact has been proved to be essential for an efficient protein removal. Other key advantages are that with this application the zirconia dosage can be reduced, and, most importantly, that the wines produced are fully heat stable. Therefore the use of zirconia during fermentation potentially has a much lower cost than the previously proposed application on finished wines. These benefits, along with the notion that this material can be easily regenerated for a virtually indefinite number of times indicate this material as a potential additional tool for the wine industry for protein stabilization of white wines.

Acknowledgements

The author would like to thank Elizabeth J. Waters of the Grape and Wine Research and Development Corporation and Matteo Marangon and Kenneth F. Pocock of the Australian Wine Research Institute for the valuable discussions about this work and for the revision of this chapter.

This work was supported financially by Australia's Grapegrowers and winemakers through their investment body the Grape and Wine Research and Development Corporation, with matching funds from the Australian Government. The AWRI is a member of the Wine Innovation Cluster located in Adelaide. The work is part of the research activity of the "Dottorato di Ricerca in Viticoltura, Enologia e Marketing delle imprese vitivinicole" supported by Provincia di Treviso (Italy) and it has been supported in part by the Fondazione Ing. Aldo Gini (Padova, Italy).

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5. Comparison of zirconia and titania in protein stabilization of Manzoni bianco and Catarratto bianco wines

It has been observed that pellets of zirconia and titania (zirconium dioxide and titanium dioxide respectively) are able to remove the unstable proteins from both musts during alcoholic fermentation and finished wines, suggesting the possibility to use these materials for white wine stabilization.

Pellets of both the metal oxides confined in stain steel nets were inserted in the must at the beginning of the fermentation. In the case of the treatment in wine the mass should be mixed during the contact with metal oxides, but this is not necessary when the treatment is done during fermentation because the natural movement of the fermenting must can be exploited.

The analysis of the wines obtained after treatments with metal oxide pellets showed a strong reduction of protein content and, consequently, a reduction of protein haze formation as well. Zirconia and titania treatments slightly reduced the total acidy of the wine, affecting in particular the tartaric acid content. Thus also a reduction of the tartaric instability of the wine could be obtained. Furthermore, the pellets slightly accelerated the fermentation process.

Sensory analysis demonstrated no organoleptic differences among wines treated with zirconia, titania and bentonite. The recommended dosage of titania was established to be between 5 and 20 g/L, depending on the protein content of the must. The recommended dosage of zirconia was instead between 10 and 25 g/L, also depending on protein content of the must.

Zirconia and titania pellets can be easily regenerated by treatments with a sodium hydroxide solution followed by a citric acid solution and the cleaning process can be improved by raising the temperature. The material regenerated in this way can be used for a potentially indefinite number of times.

5.1 Introduction

5.1.1 Zirconium dioxide (zirconia)

The caracteristics of zirconia and its use in winemaling have been reported in the intruduction to the previous chapter.

5.1.2 Titanium dioxide (titania)

Titanium dioxide is a material with physical chemical characteristics similar to those of zirconia. Therefore also titania could have a potential in adsorbing the unstable proteins from white grape juices and wines. Titania is the naturally occurring oxide of titanium with the chemical formula TiO_2. In nature, titania is present in three crystalline forms: rutile, anatase and brookite, which are coloured because of the impurities present in the crystals. Titania has particular uses in different sectors, from medicine to photochemistry (Diebold *et al*., 2002). In 1999, Sunny *et al.* studied the interactions between Titanium dioxide and proteins in a model solution. Titanium dioxide is used in different applications in the biomedical sector and in particular in those where materials with elevate compatibility with blood are required. The elevate capability of titania to adsorb the proteins was clearly confirmed by the literature (Sunny *et al.,* 1999). Considering this ability, Omoniy *et al.* (2008) performed a research on the adsorption of white wine proteins in white wine using activated Titanium oxide. The authors found TiO_2 to be able to remove the proteins from wines, in particular those with isoelectric point between pH 3.0 and 7.0.

5.2 Materials and Methods

5.2.1 Materials

The experiments were carried out using the following juices (vintage 2010):

- Manzoni bianco provided by the company Lucchetta Marcello Soc. Agr. (Conegliano – Italy).
- Catarratto bianco provided by the company Laudicina Michele Azienda Agricola (Marsala – Italy).

The fermentations were performed in 500 mL flasks with 400 mL of juice for each sample, using the yeast DV10 (Lallemand), accurately hydrated and inoculated at 18-20˚C.

Metal cages of stain steal were constructed for metal oxide confination.

The following metal oxide samples (MO) were gently supplied by the company Saint Gobain – NorPro (USA):

- Zirconium dioxide SZ 61152, 3 mm pellets (hereafter Zirconia V1), surface 156 m^2/g, pore diameter 3-100 nm;
- Zirconium dioxide SZ 31140, 3 mm pellets (hereafter Zirconia V2), surface 80 m^2/g, pore diameter 11-121 nm, mixed with 40% of Titanium Oxide;
- Titanium dioxide ST 61120, 3 mm pellets (hereafter Titania), surface 150 m^2/g, pores diameter 15 nm.

The MO were introduced in the must immediately after yeasts inoculation. The fermentation was monitored by measuring the soluble solids in solution using the refractometer PR-101 (ATAGO CO. LTD). In the middle of fermentation 300 mg/L of diammonium phosphate (Sigma-Aldrich) was added as a nutrient for yeasts. At the end of fermentation the wine samples were put at 4 °C for 7 days, then filtered (0,45 μm) and added of sulphur dioxide (30 mg/L).

5.2.2 Microvinifications to evaluate the tartaric stability

The experiments were carried out using the following juices:

- Manzoni bianco obtained from grapes (vintage 2010) provided by the company Lucchetta Marcello Soc. Agr. (Conegliano – Italy).
- Glera bianco obtained from grapes (vintage 2010) provided by the company Lucchetta Marcello Soc. Agr. (Conegliano – Italy).

The fermentations were performed in 500 mL flasks with 400 mL of juice for each sample, using the yeast DV10 (Lallemand) accurately hydrated and inoculated at 18-20˚C.

Metal cages of stain steal were constructed for metal oxide confination.

The Zirconia sample was gently supplied by the company Saint Gobain – NorPro (USA): Zirconium dioxide SZ 61152, 3 mm pellets, surface 156 m^2/g, diameter pores 3-100 nm.

The Zirconia was introduced in the must immediately after yeasts inoculation. The fermentation was monitored by measuring the solids in solution using the refractometer PR-101 (ATAGO CO. LTD). In the middle of fermentation 300 mg/L of diammonium phosphate (Sigma-Aldrich) was added as a nutrient for yeasts. At the end of fermentation the wine samples were putted at 4 °C for 7 days, then filtered (0,45 μm) and added of sulphur dioxide (30 mg/L).

5.2.3 Regeneration of Metals Oxides

After being used in the fermentation must, the MO pellets (enclosed in stain steal cage) were regenerated by two washings with a 3 M NaOH solution at 50 °C for 2 hours. Then they were washed with a 5% citric acid solution for 30 minutes and finally with distilled water (three times) before to use them.

5.2.4 Determination pH and Total Acidity

pH was measured by pHmeter MICRO pH 2001 (CRISON – Spain) and total acidity by titration with 0.1 N NaOH, expressing values in g/L of Tartaric acid.

5.2.5 Heat Test

Glass tubes containing the wine samples were incubated at 80 °C for 2 hours and then cooled in ice for 2 hours. Following the equilibration at room temperature for 30 minutes, samples were transferred into quartz cuvette (1 mL capacity) and haze was quantified by measuring the absorption at 540 nm (Waters *et al.* 1992) using a spectrophotometer (Shimadzu UV 6010). The haze was also quantified by nephelometry in NTU (Pocock & Rankine, 1973) using a turbidimeter (2100P, HACH). To each obtained value the value measured for the same sample before heating was subtracted.

5.2.6 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoretic analyses were performed according to Laemmli (1970). Samples to be analysed were dissolved in a 0.5 M Tris-HCl pH 6.8 buffer containing 15% (v/v) glycerol (Sigma) and 1.5% (w/v) SDS (Bio-Rad) (loading buffer) and heated at 100 °C for 5 minutes before loading. For SDS-PAGE in reducing conditions, 3% (v/v) of 2-mercaptoethanol (Sigma) was also added to the loading buffer. Electrophoresis was performed in a Mini-Protean III apparatus (Bio-Rad) in 14% polyacrylamide (acrylamide-N, N' metylen-bisacrylamide 29:1; Fluka) gels. The molecular weight standard proteins were Myosin (200,000 Da), β-galactosidase (116,250 Da), Phosphorylase b (97,400 Da), Bovine Serum Albumin (66,200 Da), Ovalbumin (45,000 Da), Carbonic

anhydrase (31,000 Da), Trypsin inhibitor (21,500 Da), Lysozyme (14,400 Da) and Aprotinin (6,500 Da) (Broad Range Molecular Weight Markers, Biorad). After electrophoresis, gels were stained for 18 h with Coomassie brilliant blue R-250 (Sigma) and then destained with 7% acetic acid for 24 h (Koenig *et al.* 1970).

5.2.7 Chromatographic Analyses

Chromatographic separations were performed by HPLC (Waters 1525) fitted with a *Dual λ Absorbance Detector* (Waters 2487) and a *Refractive index detector* (Waters 2414). Data were analysed with Waters Breeze TM Chromatography software (Version 3.30).

5.2.8 Protein analysis by Reverse Phase (RP)-HPLC

The protein composition and amount of wine samples was determined by HPLC, according to the method proposed by Peng *et al.* (1997). 100 μL of sample was loaded at 1 mL/min onto a Vydac 218 MS 54 4.6 x 250 mm (Grace Davison Discovery Science^{TM}) column equilibrated in a mixture of 83% (v/v) solvent A [0.1% trifluoroacetic acid (TFA) in 8% Acetonitrile] and 17% solvent B [80% Acetonitrile, 0.1% (v/v) TFA] at 35 ºC. Proteins were eluted by a gradient of solvent A from 17% to 49% in the first 7 minutes, 49% to 57% from 7 to 15 minutes, 57% to 65% from 15 to 16 minutes, 65% to 81% from 16 to 30 minutes and than held at 81% for 5 minutes before re-equilibrating the column in the starting conditions for 6 more minutes (Tab 1). Peaks were detected at 220 nm. Commercial thaumatin from *Thaumatococcus daniellii* (Sigma-Aldrich) was used as standard.

Time (min)	% A	%B
0	83	17
7	51	49
15	43	57
16	35	65
30	19	81
31	0	100
36	0	100
37	83	17
42	83	17

Table 1 – Proteins elution gradient with Vydac 218 MS 54.

5.2.9 Determination of the Polysaccharide content

Polysaccharides were precipitated adding absolute ethanol to the wine samples to be analysed. This operation also allows to eliminate many compounds that could interfere with the analyses.

500 μL absolute ethanol were added to 100 μL of sample. After 18 hours at room temperature samples were centrifuged at 14000 rpm for 30 minutes. The supernatant was eliminated and the pellets (polysaccharides) re-suspended in ethanol and this procedure was repeated three times. The pellet was dissolved in 500 μL of distilled water and 200 μL were put into glass tubes, added of 210

μL of a 4% (p/p) phenol solution and 1 mL of concentrated sulphuric acid. After 30 minutes samples were read with a spectrophotometer (Shimadzu UV 6010) at 490 nm.

The calibration curve was built using glucose in water as the standard, at concentrations between 0 and 200 μg/mL. The glucose solution was added of 210 μL of 4% (p/p) phenol solution and 1 mL of sulphuric acid; after 30 minutes the glucose samples were read at 490 nm.

5.2.10 Total Polyphenols content determination

The phenolic content of the samples was determined colometrically with the method of Singleton and Rossi (1965), optimised for small sample volumes by Waterhouse (2002). 200 μL of sample diluted with water (1:10 v/v) were added of 1 mL of 2N Folin-Ciocalteau reagent (Sigma) diluted in water (1:10 v/v) 800 μL of 7.5% (w/v) Na_2CO_3 (Merck) solution were then added to the samples, which were then incubated for 30 min at 40°C. The absorbance at 725 nm was then measured. The calibration curve was prepared by using serial dilution of gallic acid (GAE, Fluka) in water.

5.2.11 Metal analysis

Metal contents in wines were determined by Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES), according with the standard procedure used for wines (Eschnauer H. *et al.*, 1989).

5.2.12 Tartaric stability determination

The tartaric stability analysis was performed by using the Tartarcheck (Ing. Castore Bullio, Torino – Italy). The mini-contact test measuring the conductivity (expressed in μS) decrease after addition of potassium hydrogentartrate (KHT) in cooled wine was carried out. The scale of tartaric stability provided from the manufacturer gives the follow indications: <30 μS very stable; 30 – 50 μS stable; 50 – 70 μS risk; > 70 μS instable. The saturation temperature of the wine (T_{sat}) was also measured by using a temperature maximum (T_{max}) of 25 °C and temperature minimum (T_{min}) of 5 °C.

5.2.13 Bentonite Treatment

An amount of the wines obtained by the micro-vinifications was used for bentonite treatment in order to reach protein stability. To evaluate the optimal bentonite quantity to be used in each case, preliminary tests were carried out increasing the bentonite dosage from 0.2 to 1.0 g/L. The bentonite (E-Benthon Extra, Pall) was hydrated in distilled water (1/10) for 12 hours before the use. After the treatment the wine samples were filtered (0.2 μm) and analysed by the Heat test to evaluate the protein instability. The results from the tests showed the following bentonite doses as optimal to stabilize the wines.

- First fermentation experiment (Manzoni bianco): 0.6 g/L;
- Second fermentation experiment (Manzoni bianco): 0.9 g/L.

The bentonite treated wine was stocked in closed bottle before the sensory analysis.

5.2.14 Sensory data analysis elaboration

The wines were evaluated by sensory analysis using a panel composed of expert wine tasters trained by CIRVE, Università degli Studi di Padova – Conegliano (TV). The wines were analysed by 2 preference tests for each micro-vinification experiment. In the first test the samples treated with bentonite and zirconia were compared. In the second test the samples treated with bentonite and Titanium Oxide (dosages of 10 g/L and 25 g/L) were compared. The wine tasters needed to order the wine following the preference for colour, olfaction, negative aromas intensity, taste, negative tastes intensity and general impression. The statistical elaboration of the preferences results were performed by the Friedman Test (Barillere and Benard, 1986) in order to find the minimum difference between the ranks.

5.2.15 Experimental design

The effects of the treatment with metal oxides were evaluated during fermentation in Manzoni bianco juice (from Conegliano – Italy) and Catarratto bianco (from Marsala - Italy). In detail, pellets of metal oxides were added at the beginning of fermentation. The wines obtained from the treatment were analysed in order to check protein instability and other different oenological parameters. Additional microvinifications were performed in order to evaluate the capability of the zirconia to influence the tartaric stability in Glera and Manzoni wines.

5.3 Results and Discussion

5.3.1 Kinetic of fermentation and analytical results

Microvinifications were performed in order to imitate the normal winemaking process. The fermentation was monitored by refractometry, following the decrease of solid soluble solid substances (% w/v).

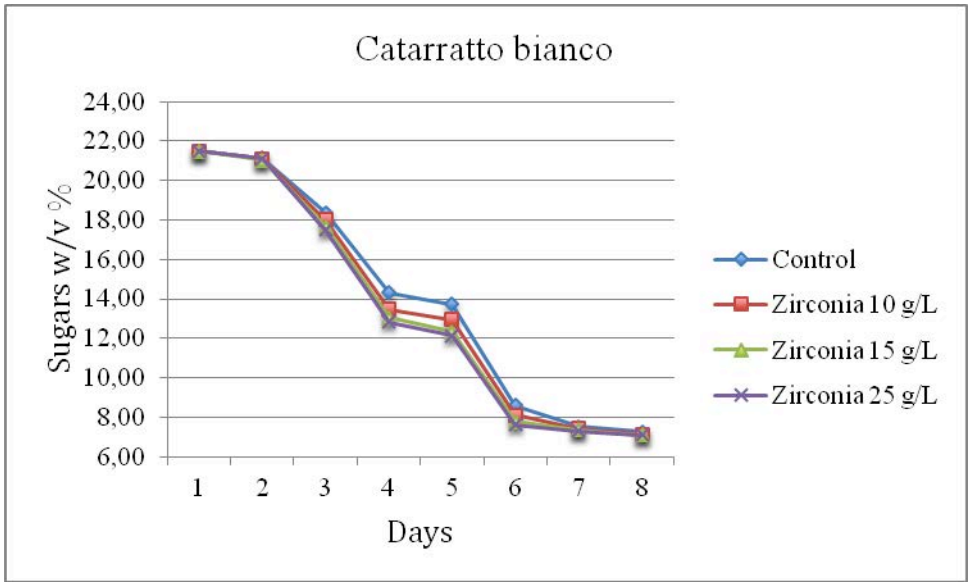

Figure 3. Catarratto bianco microvinifications. The kinetic of fermentation was monitored by refractometry measuring the soluble solid substances (% w/v).

The fermentation kinetic showed a regular development and it was completed in 8 days (figure 3). During fermentation 300 mg/L of DAP (Diammonium Phosphate) were added between the fourth and the fifth day. The DAP addition increased the fermentation rate until the sixth day. The addition of 25 g/L of Zirconium oxide increased the fermentation rate (of about one day) compared to the control (figure 3).

Total acidity and pH values of the samples treated with increasing doses of zirconia are showed in table 2.

	pH BF	pH EF	Total Acidity BF	Total Acidity EF
Control	3.60	3.27	4.60	5.80
Zirconia 10 g/L	3.60	3.35	4.60	5.20
Zirconia 15 g/L	3.60	3.07	4.60	5.00
Zirconia 25 g/L	3.60	3.53	4.60	4.55

Table 2. pH and Total acidity values (espressed in g/L tartaric acid) before and after fermentation of Catarratto bianco. BF: measure at the beginning of fermentation; EF: measure at the end of fermentation

The pH values were slightly influenced by the zirconia treatment. A little diminution occurred in the treatment with 10 g/L, while a slight increase occurred in 25 g/L zirconia treatment. Compared to that measured before fermentation, the total acidity after fermentation increased probably because the release of free organic acids by the yest that produces succinic and acetic acid (Arikawa *et al*., 1998). At the end of fermentation, the samples treated with increasing doses of zirconia showed a gradual decrease of the total acidity values.

The fermentation kinetic for the Manzoni bianco wines, treated with titania and two different types of zirconia pellets (Zirconia V1 and Zirconia V2), is shown in Figure 4.

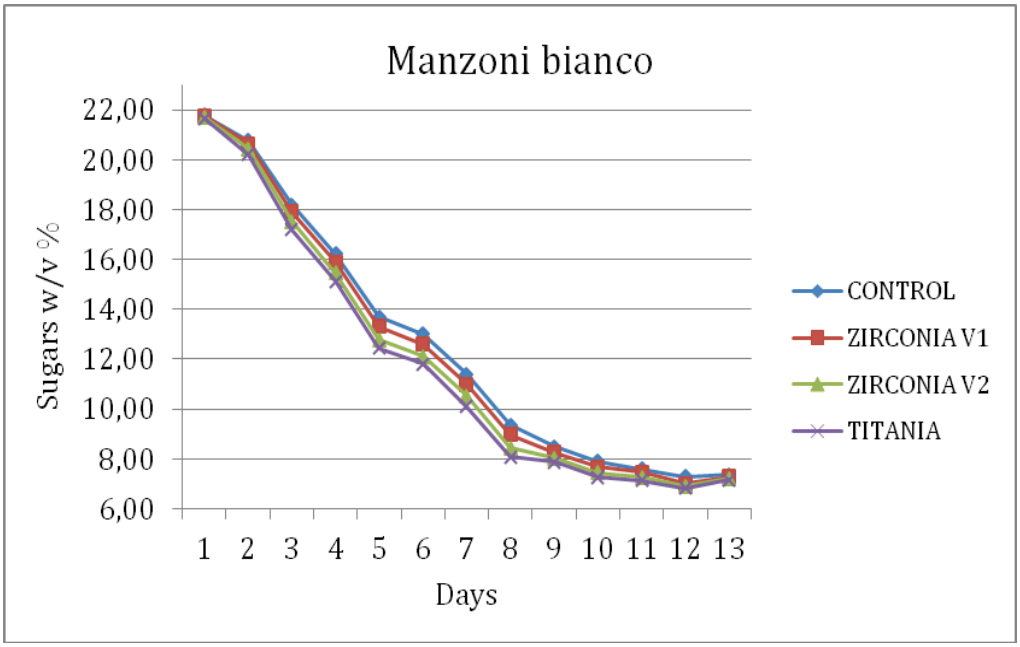

Figure 4. Manzoni bianco microvinifications. The kinetic of fermentation was monitored by refractometry measuring the soluble solid substances (% w/v).

The fermentation curve of the Manzoni bianco musts (figure 4) were very similar in the two microvinification experiments performed with titania and the two different zirconia pellet types. The zirconia treatment slightly increased the fermentation rate as observed for Catarratto samples. Both zirconia and titania treatments accelerated the fermentation process probably due to their action as a physical support for the yeast cells.

pH and acidity values for Manzoni bianco treated with titania and the two types of zirconia are shown in table 3. Also in these microvinification is possible to see an increase of total acidy in the wines with respect to the corresponding juices as observed before. The wines form musts treated with metal oxides showed a slightly reduction of total acidity when compared to the untreated controls.

	pH BF	pH EF	Total Acidity BF	Total Acidity EF
Control	3.08	2.94	4.30	7.10
Zirconia V1 25 g/L	3.08	3.11	4.30	6.80
Zirconia V2 25 g/L	3.08	2.92	4.30	6.90
Titania 25 g/L	3.08	3.00	4.30	6.80

Tab. 3 – pH and Total Acidity values (expressed in g/L of tartaric acid)in Manzoni bianco samples using different metal oxides. BF: measure at the beginning of fermentation; EF: measure at the end of fermentation

pH and acidity values for Manzoni bianco treated with three levels of titania are shown in table 4. Total acidity decreased progressively with the dosage of titania used and the pH values slightly increased. The total acidity decreased more than 1 g/L (expressed in tartaric acid) from control to 25 g/L titania treatment.

	pH BF	pH EF	Total Acidity BF	Total Acidity EF
Control	3.09	2.95	4.40	7.00
Titania 5 g/L	3.09	3.00	4.40	6.50
Titania 10 g/L	3.09	3.25	4.40	6.10
Titania 25 g/L	3.09	3.26	4.40	5.80

Table 4 - pH and Total Acidity (expressed in g/L of tartaric acid) values in Manzoni bianco samples with different dosages of Titanium oxide. BF: measure at the beginning of fermentation; EF: measure at the end of fermentation

5.3.2 Heat Test Results

In order to assesses the effects of the treatments with metal oxides on the potential wine protein instability, heat test were performed.

The results for Catarratto wines deriving from musta treated with zirconia, expressed in both NTU (figure 5) and absorbance (figure 6) indicated that the treatment was effective in reducing the potential heat instability of the samples.

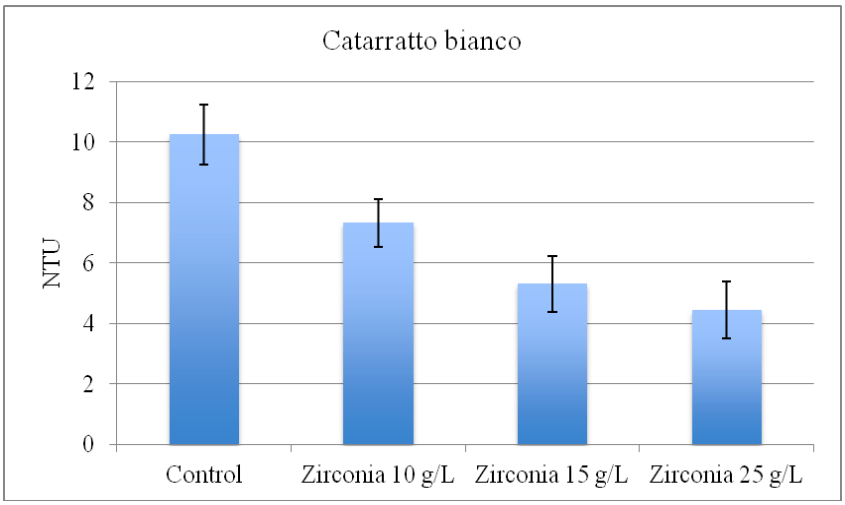

Figure 5. Heat test of Catarratto bianco wines. The haze developed was measured by nephelometry and results expressed in nephelometric turbidity unit (NTU).

As expected, the level of haze formation after the heat test decreased according to the dosage of zirconia used. The treatment with 25 g/L of zirconia pellets was able to stabilize the wine although a residual turbidity was detected with net values around 4 NTU. Indeed, the wines produced in Mediterranean regions are considered stable when giving net turbidities after the heat test lower than 5 NTU (Meistermann *et al.,* 2010).

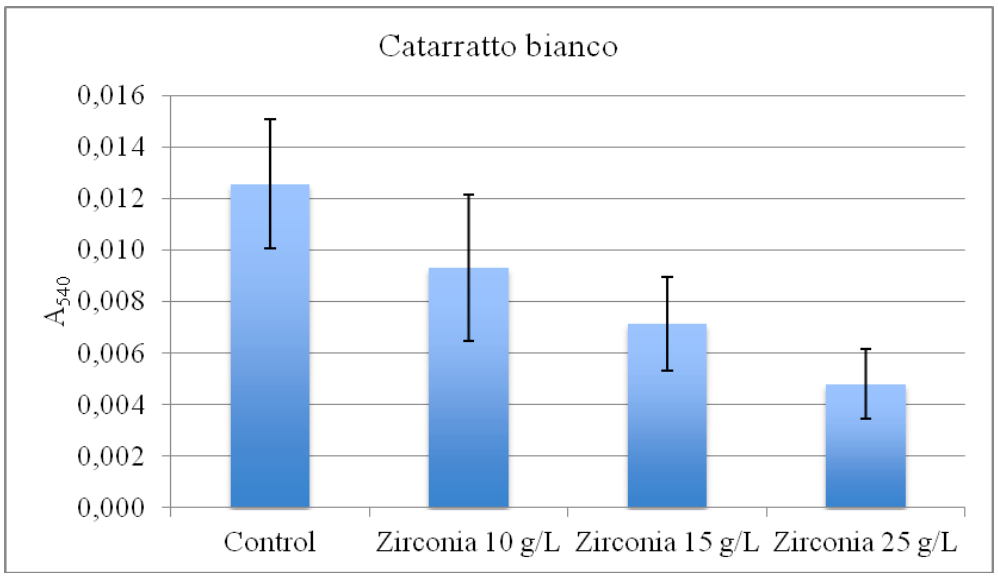

Figure 6. Heat Test of Catarratto bianco wines. The haze developed was measured by reading the absorbance of he samples at 540 nm.

When Manzoni bianco wines, produced from the samples treated with the two types of zirconia and with titania, was considered, all the trials showed a dramatic decrease of protein instability measured in both NTU (figure 7) and absorbance values (figure 8), with achievement of complete stability after the treatments with the highest dosages

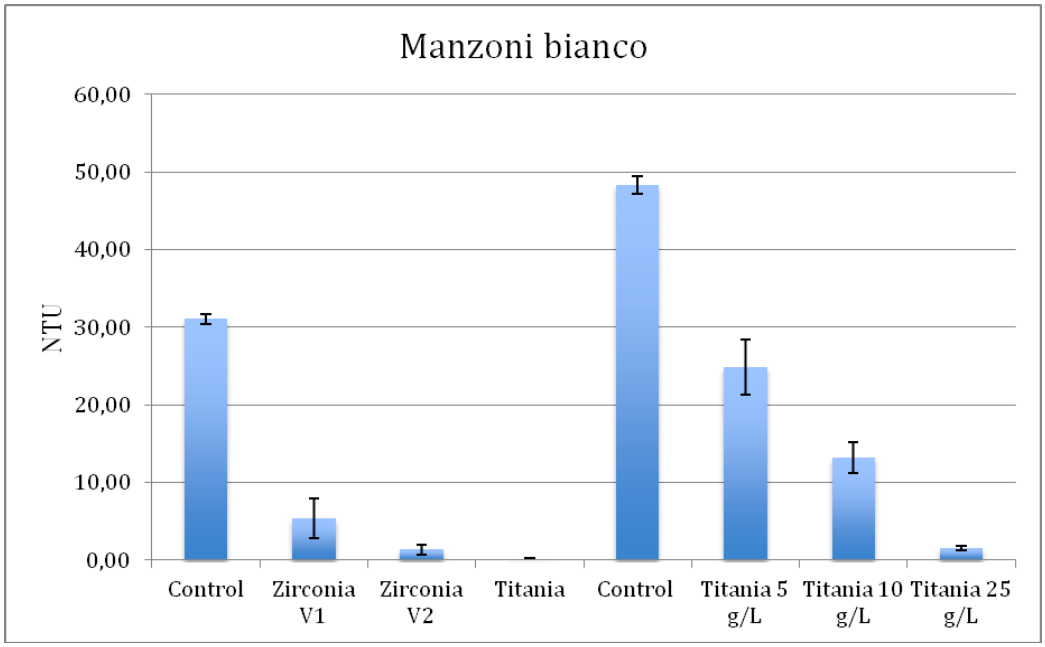

Figure 7. Heat Test results for Manzoni bianco wines. At the left side the results for the first microvinification experiment for the two different types of zirconia and titania (used at the same dosage of 25 g/L) are shown. At the right side are results of the second microvinification experiment using different dosages of titania. The haze developed was measured by nephelometry and the results expressed in nephelometric turbidity unit (NTU).

It must be noted that the two different types of zirconia pellets (used at the same dosage) showed to have a slightly different effect on wine stability, probably due to a different effectiveness in adsorbing the unstable protein components. Instead titania pellets were able to decrease more effectively the protein instability compared to those of zirconia used at the same dose. Actually with a dose of titania between 10 g/L and 25 g/L it was possible to reach the complete protein stability in Manzoni bianco wine, indicating a strong capacity of this metal oxide to remove the unstable grape proteins.

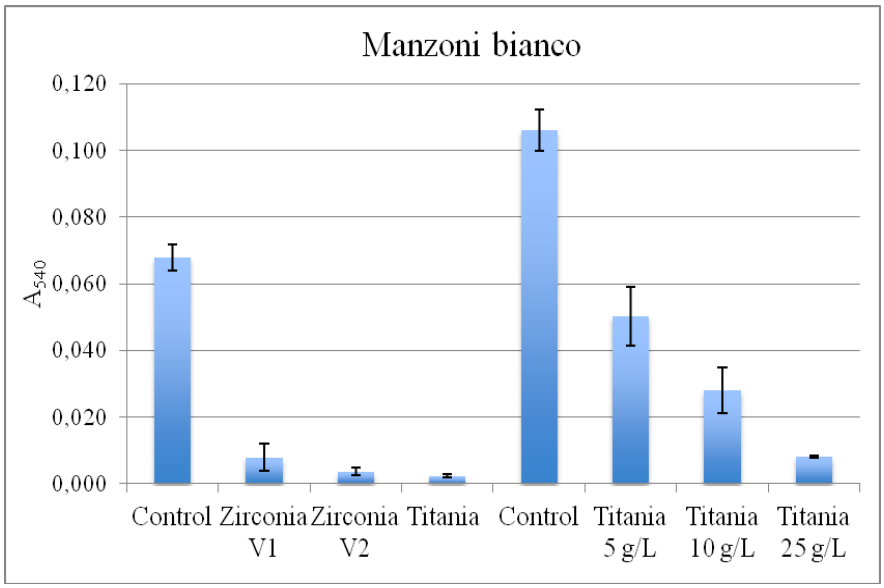

Figure 8. Heat Test results for Manzoni bianco wines. At the left side the results for the first microvinification experiment for the two different types of zirconia and titania (used at the same dosage of 25 g/L) are shown. At the right side are results of the second microvinification experiment using different dosages of titania. The haze developed was measured by absorbance at 540 nm.

These results are also supported from SDS-PAGE and RP-HPLC protein analysis (see below).

5.3.3 SDS-PAGE Analysis

SDS-PAGE allows to visualize the protein pattern in juice/wines providing information about the quantity and the quality of the proteins present in the medium. The stained bands correspond to specific molecular weights and their staining intensity can be related to the protein content of the analysed sample. The main protein bands normally detected by SDS-PAGE in wines are found at 20-25 kDa, (corresponding to Thaumatin like proteins, TLP), 30-32 kDa

(corresponding to chitinases) and 60-67 kDa (corresponding to the grape invertase).

By using SDS-PAGE, a diminution of the intensity of the protein bands in Manzoni bianco wine after treatments with metal oxides can be observed (figure 9 and 10). In particular, compared to the control and to the other samples, titania at 25g/L removed the largest quantity of proteins. However it must be noted that, in all cases, protein removal does not seem to be specific for particular bands, which, by increasing the dosages of the pellets, tend to disappear with a trend that can be related to their initial relative quantity in the untreated wine.

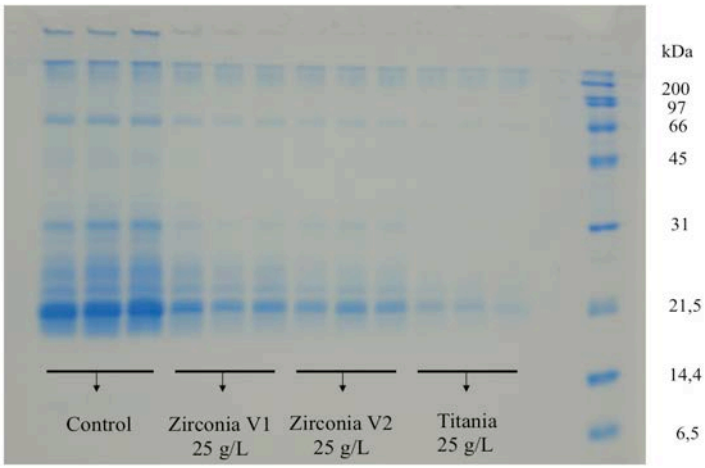

Figure 9. SDS-PAGE (T=14 %) analysis of Manzoni bianco samples, before (control) and after treatment with different metal oxides, used at the same dosage of 25 g/L. Proteins were recovered for the wine samples by precipitation with 4 volumes of cold ethanol. The MW standards are indicated on the right.

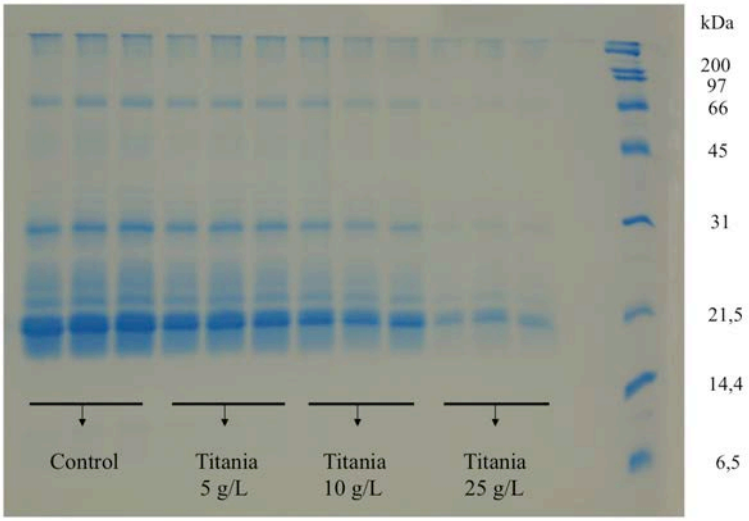

Figure 10. SDS-PAGE analysis (T=14 %) of Manzoni bianco samples before (control) and after treatment with different dosages of titania. Proteins were recovered for the wine samples by precipitation with 4 volumes of cold ethanol. The MW standards are indicated on the right.

The results obtained for Manzoni bianco samples confirm that the stabilisation effects observed after the heat tests (figures 7 and 8) are due to removal of grape proteins by the metal oxide treatments.

5.3.4 Reverse Phase (RP)-HPLC analysis

RP-HPLC analysis was performed on both Catarratto bianco and Manzoni bianco samples in order to detect the differences in both protein content (calculated from the sum of the areas of the chromagraphic peaks) and protein type (determined on the basis of the retention time) (Peng *et al*., 1997 and Marangon *et al.*, 2009) deriving from the treatment with the metal oxide pellets.

Catarratto bianco

For the samples of Catarratto bianco, a reduction in the sum of the areas of the RP-HPLC protein peaks in the wines treated with metal oxides was observed. Compared to the control, the wines treated with 10 g/L of zirconia showed a reduction of 26 %, of the protein content, which, expressed in mg/L of (commercial) Thaumatin, varied from 109,47 mg/L to 80,38 mg/L (figure 11), whereas the 15 g/L and 25 g/L treatments decreased the protein content of 38% and 41 % respectively .

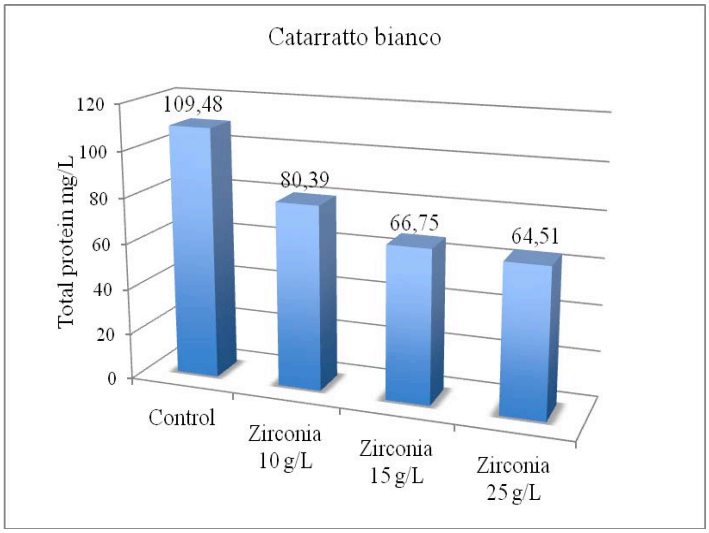

Figure 11 – Total protein content in Catarratto bianco wines calculated by RP-HPLC. The protein quantity was expressed in mg/L of Thaumatin.

The RP-HPLC profile showed five main peaks. According to the results reported by Peng *et al.* (1997) and Marangon *et al.* (2009) three of these peaks can be ascribable to isoforms of TL proteins (retention times between 9.00 and 12.00 minutes). Chitinases were less abundant than TL proteins and only two peaks corresponding to this protein type (retention time 19.00 and 24.500 minutes) (Peng *et al.,* 1997; Marangon *et al.,* 2009) were detected. However, by calculating the areas of the peaks, chitinases seemed to be removed by zirconia pellets at a higher extent compared to TL proteins (figure 12), indicating a

different affinity for the two types of proteins. Taking into account that chitinases are considered as the most instable proteins in wines (Waters *et al*., 1998), this difference can be seen as an advantage for wine protein stabilisation by using zirconia.

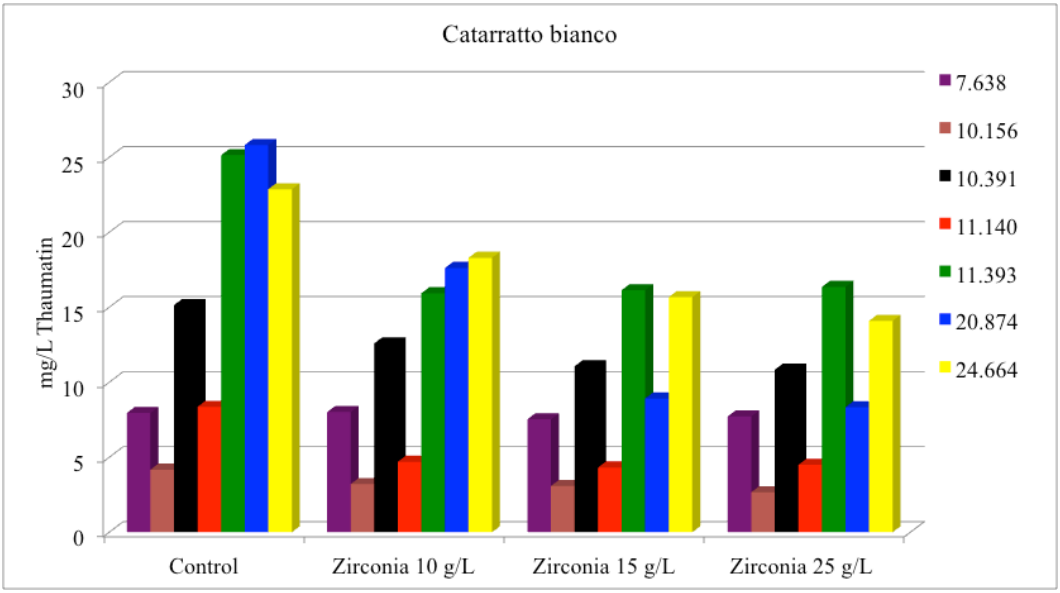

Figure 12. Variation in the quantity of the main RP-HPLC protein peaks of Catarratto bianco wines before (control) and after treatment with increasing doses of zirconia. RT (on the right) between 9.00 and 12.00. correspond to TL proteins and those between 19.00 and 24.50 to chitinases (Peng *et al*., 1997; Marangon *et al*., 2009).Protein quantity was expressed in mg/L of Thaumatin.

The overall RP-HPLC results obtained for Catarratto confirm that the treatment with zirconia results in the removal of protein in a dose-dependent manner, explaining the results of the heat tests.

Manzoni bianco

The Manzoni bianco samples obtained by treatments with the two types of zirconia and with titania at 25 g/L (first microvinification experiment) showed a

dramatic reduction of the protein content compared to the control, the diminution reaching 66% for the treatment with Titania (figure 13).

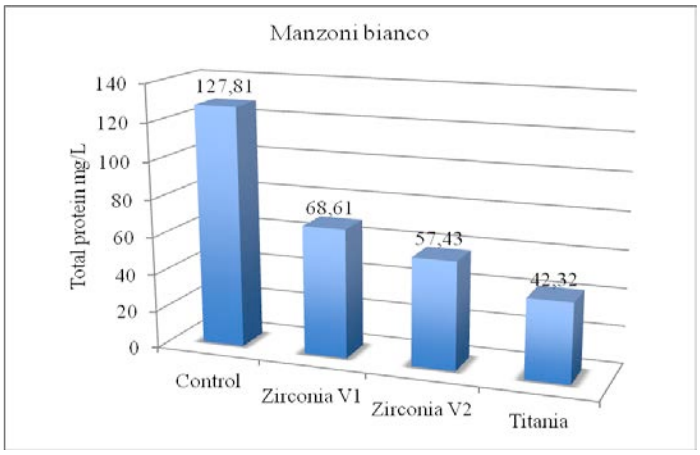

Figure 13. Protein content in Manzoni bianco samples before (control) and after the treatment with metal oxides (first micro-vinification experiment) as calculated by RP-HPLC. The protein quantity was expressed in mg/L of Thaumatin.

In this case only a small difference between the two types of zirconia was observed. The treatment with titania was the most effective, and therefore the general the results of RP-HPLC analysis confirmed those obtained by SDS-PAGE. The RP-HPLC profile of Manzoni bianco showed the presence a main peak ascribable at isoforms of TL proteins (RT 9.55), whereas three peaks corresponded to chitinases (RT 19.852 – 20.630 – 24.743). By calculating the variations of the different peak areas, a strong decrease was found for the peak with RT of 20.630 min, corresponding to a chitinase (figure 14). After titania treatment, the quantity of this protein decreased of 86 %, i. e. from 68 mg/L of the control to 9 mg/L of the treated sample (values expressed in Thaumatin equivalents). Therefore also in this case, the removal of the most instable protein components can be achieved by using metal oxides. Titania resulted most effective in this removal and its presence (40 %) in zirconia V2 enhanced

the material performance. The proteins ascribable to chitinases were more represented than TL proteins and proportionally they were the most removed.

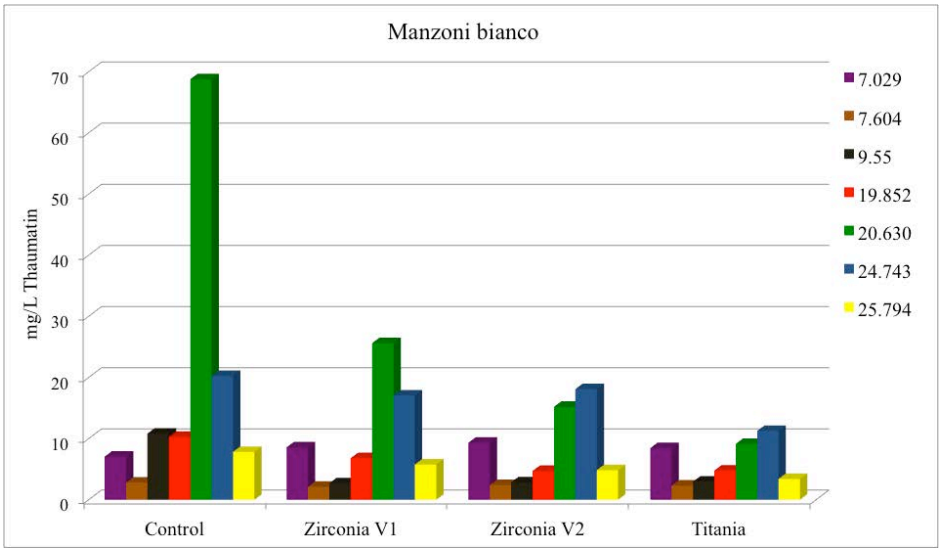

Figure 14. Variation in the quantity of the main RP-HPLC protein peaks of Manzoni bianco wines before (control) and after treatment with metal oxides used at 25 g/L. RT (on the right) between 9.0 and 12.00 min correspond to TL proteins and those between 18.00 and 24.50 min to chitinases (Peng *et al*., 1997; Marangon *et al*., 2009). Protein quantity was expressed in mg/L of Thaumatin.

When Manzoni bianco wine was treated with increasing doses of titania, (second microvinification experiment) a dose-dependent reduction in protein content was observed.

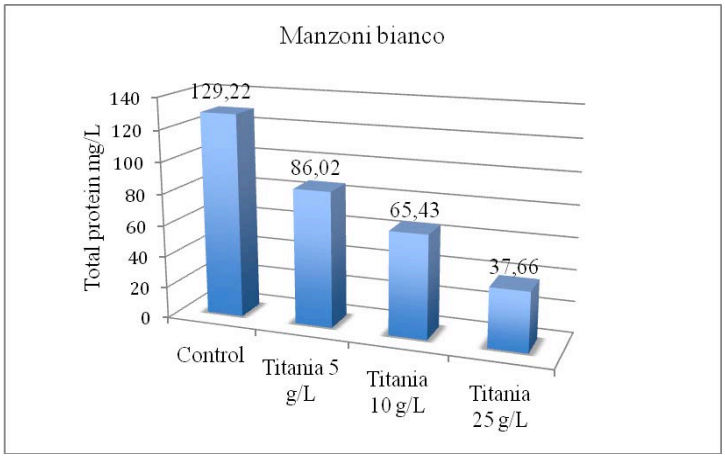

Fig.15 –Protein content in Manzoni bianco samples before (control) and after the treatment with titania (second micro-vinification experiment) as calculated by RP-HPLC. The protein quantity was expressed in mg/L of Thaumatin.

As observed before (for the first microvinification experiment) RP-HPLC analysis showed a main peak ascribable to TL proteins and three to chitinases (not shown). However, when the reduction of the areas of the single peaks was calculated a different protein composition was observed.

In the first experiment there was a major content of chitinases (the control contained 99,3 mg/L of chitinases and only 10,8 mg/L of TL proteins), while in the samples of the second experiment TL proteins prevailed (figure 16). In this wine the quantity of proteins ascribable to chitinases corresponded at 70 mg/L of Thaumatin equivalents and that of TL proteins to 27 mg/L.

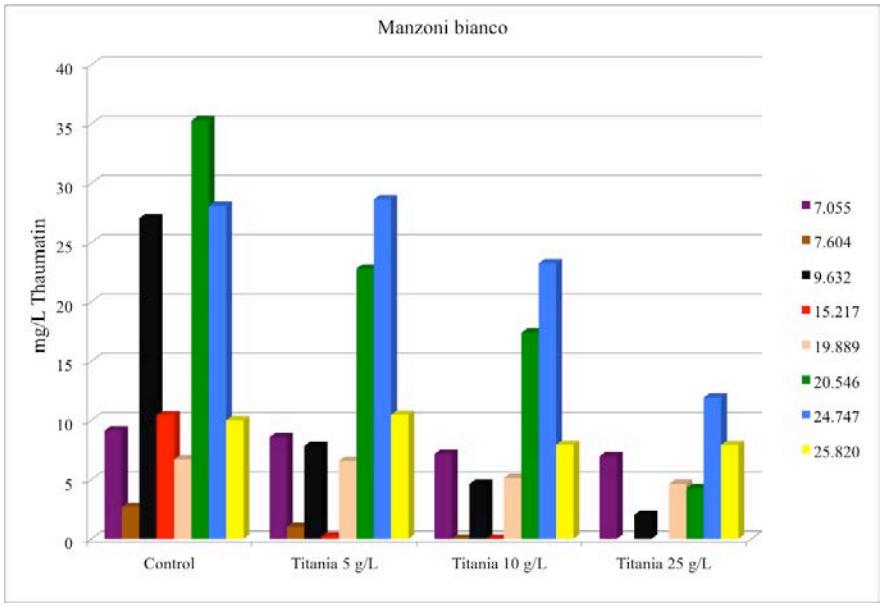

Figure 16. Variation in the quantity of the RP-HPLC protein peaks of Manzoni bianco wines before (control) and after treatment with increasing doses of titania. RT (on the right) between 9.0 and 12.00 min correspond to TL proteins and those between 19.00 and 24.50 min to chitinases (Peng *et al*., 1997; Marangon *et al.*, 2009). Protein quantity was expressed in mg/L of Thaumatin.

5.3.5 Determination of the Total Polysaccharide content

In order to determine the effects of metal oxide treatments on the content of other macromolecules that can be important for wine quality (Waters *et al*., 1994; Moreno-Arribas *et al.*, 2000), total polysaccharides were quantified in the different wine samples. In general, the results indicated variations also in the content of these compounds, although the quantity present in untreated Catarratto bianco was much higher than of Manzoni bianco (figure 17). This initial difference, that can be due to different genotypic and geographic

provenience of the grapes probably affected the results obtained for these compounds after the treatments with metal oxides.

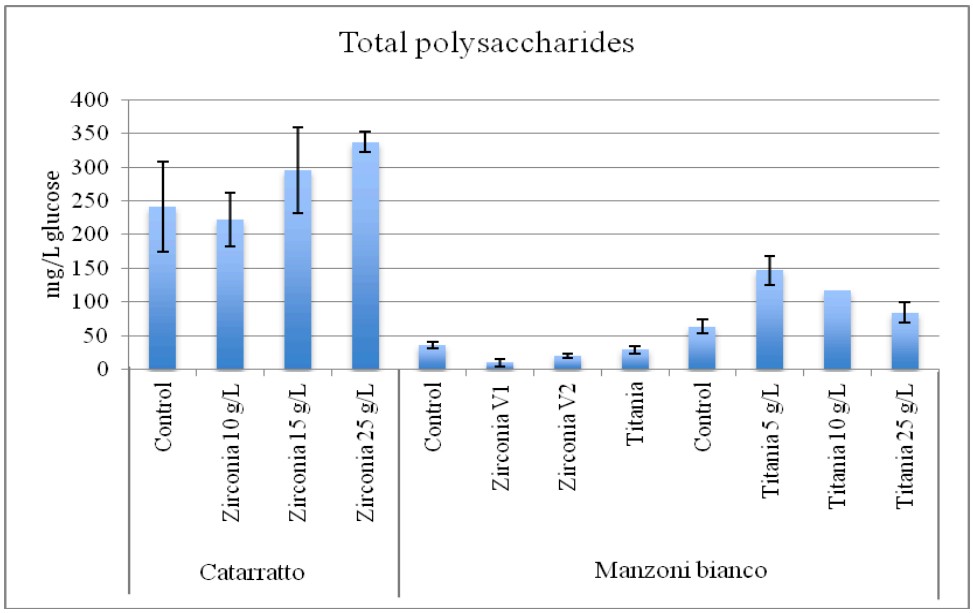

Figure 17. Total polysaccharides content in the different samples. The results are expressed in mg/L of glucose.

In Catarratto bianco an increase of polysaccharides content after the treatment with 25 g/L zirconia was observed, where the other treatments (with 10 – 15 g/L of zirconia) showed a slightly diminution. *Saccharomyces cerevisiae* during fermentation releases a variable amount of polysaccaharides as glucans and mannoproteins (Yokotsuka *et al*., 1997; Beltran *et al.*, 2004; Marks *et al.*, 2008). Especially the mannoproteins are indicated as important quality factor (Waters *et al.*, 1993; Caridi, 2006). The presence of Zirconia pellets during fermentation could improve the production of polysaccharides by *S. cerevisiae*.
A different situation occurred in Manzoni bianco where the samples treated with zirconia showed a diminution in polysaccharide content. However, in samples of Manzoni bianco treated with titania the polysaccharides content

increased respect the control. The phenomenon could related to the activity of *S. cerevisiae* as previously mentioned.

In general the utilization of metal oxides (especially titania) pellets during fermentation tends to increase the polysaccharides content in the wines. The reasons for this result should be better investigated.

5.3.6 Determination of the total Polyphenols content

Total polyphenols determination by the with Folin-Ciocalteau assay was performed on the different samples. In general, the metal oxide treatments determined a certain diminution of the polyphenols content, which, also in this case, was dose-dependent (figure 18). However, in Catarratto bianco treated with 25 g/L zirconia an increased quantity of polyphenols was observed.

In wine some polyphenols are bound to proteins (Asano *et al.,* 1982; Marangon *et al*., 2010) and then the treatment with metal oxide, by removing the proteins could remove also the polyphenols which are part of the complexes with proteins, confirming the results reported previously for bentonite treatments (Puigdeu *et al.,* 1996). Moreover, the surface properties of the metal oxides permitted to adsorb positive and negative compounds as well (for example organic acids). Consequently this material could also be adsorb part of negatively charged polyphenols.

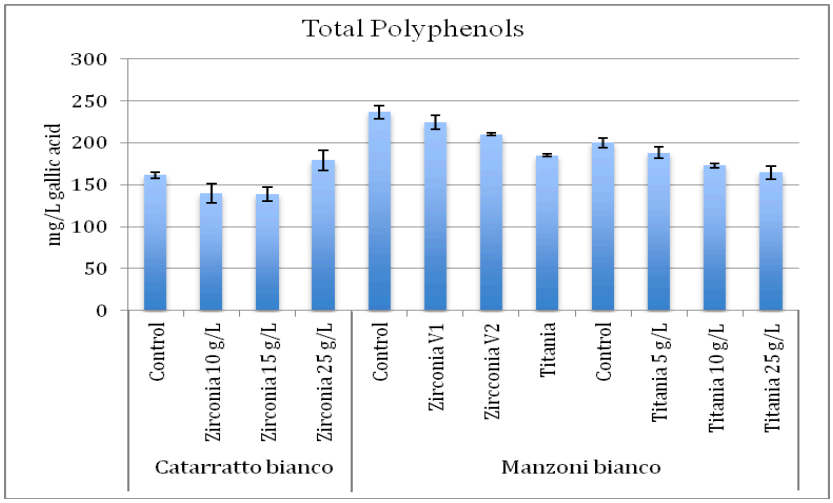

Figure 18 – Total polyphenols in Catarratto and Manzoni bianco samples before (control) and after the treatments with metal oxides. The results are expressed in mg/L of gallic acid.

5.3.7 Determination of the ions content

The ion content in was measured in the samples by inductively coupled plasma atomic emission spectroscopy (I.C.P. - O.E.S.). 24 elements including heavy metals were determined (table 5) to check the effects of the different materials added to the wines.

The results showed similar effects for the wines treated with the two types of zirconia, with a diminution of calcium, copper, phosphorus, magnesium and zinc, while iron and sodium increased with respect to the control. Titania treatment affected the same elements but its action resulted more intensive compared with that of zirconia at the same dosage. A slightly increase of zirconium and titanium in some samples treated with metal oxides was also observed.

	Catarratto bianco		Manzoni bianco			
ppm	**Control**	**Zirconia 25 g/L**	**Control 2**	**Zirconia V1 25 g/L**	**Zirconia V2 25 g/L**	**Titania 25 g/L**
Al	0.448	0.439	0.13	0.164	0.12	0.119
As	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
B	6.73	6.34	1.97	1.73	1.84	1.86
Br	<10	<10	<10	<10	<10	<10
Ca	69.3	48.6	56	47.5	44.9	42.8
Cd	< 0.003	< 0.003	< 0.003	< 0.003	< 0.003	< 0.003
Co	< 0.003	< 0.003	< 0.003	< 0.003	< 0.003	< 0.003
Cr	< 0.003	< 0.003	< 0.003	< 0.003	< 0.003	< 0.003
Cu	0.636	0.313	0.064	0.036	0.031	0.024
Fe	0.319	0.39	0.133	0.095	0.282	0.771
Hg	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
K	875	914	339	330	374	337
Mg	95.1	85.3	68.4	62.2	60.8	59.5
Mn	0.521	0.38	1.3	0.969	1	0.931
Mo	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03
Na	32,9	367	8.00	75.9	8.62	9.06
Ni	< 0.007	0.011	0.017	0.014	0.034	0.033
P	131	57.1	152	49.3	69.4	61.4
Pb	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
S	104	93.5	129	127	135	165
Sn	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Ti	< 0.003	< 0.003	< 0.003	0.025	3.42	0.25
Zn	0.161	0.108	0.297	0.143	0.154	0.131
Zr	< 0.003	2.43	< 0.003	2.28	0.226	0.03

Tab. 5 – Elements determination in samples. The determination was performed by inductively coupled plasma atomic emission spectroscopy.

Catarratto bianco and Manzoni bianco treated with 25 g/L of zirconia showed an increase of sodium content of 11 and 9 times respectively. This phenomenon did not occur after the other treatments. Perhaps zirconia pellet contain a certain quantity of Na and the high quantity of zirconia used caused the release of detectable amounts of this ion.

The removal of copper could favour reduction phenomena in wines. Indeed, the copper is indicated as an important element in determining the reduction potential of wines that affect in particular the olfaction sensations (Cacho *et al*., 1995; Danilewicz, 2003). The copper level in Catarratto bianco treated with Zirconia (25 g/L) decreased more than 50%.

Phosphorus was strongly removed in all treatments (between 55% and 70%). Moreover, metal oxides influenced the calcium content that decreased between 16% and 30% in all wines treated. This aspect has particular importance for the tartaric stability of the wines. Before bottling the wine are normally stabilized to avoid tartaric precipitation caused by potassium bitartrate and calcium tartrate. Decreasing calcium content could then diminish the tartaric instability (Berg and Keefer, 1959; Mckinnon *et al*., 1995).

5.3.8 Tartaric stability

The capacity of the metal oxides to partially remove the organic acids and also calcium ions suggested to evaluate the possibility to increase the tartaric stability in white wines by using these adsorbent materials. Indeed, when the juices were treated with zirconia the tartaric stability of the wines showed to improve.

Microvinification experiments performed by treating with zirconia a Glera must allowed to obtain wines with reduced tartrate instability compared to the control, the difference of conductivity decreasing from 103 µS (determined in the control) to 87 µS after treatment with 12 g/L of Zirconia pellets (figure 19).

Consequently the wine resulted not completely stablised needing a further tartrate stabilization process performed with the standard methods. However the energy necessary to fully stabilize the wines can be reduced after Zirconia treatment of the musts.

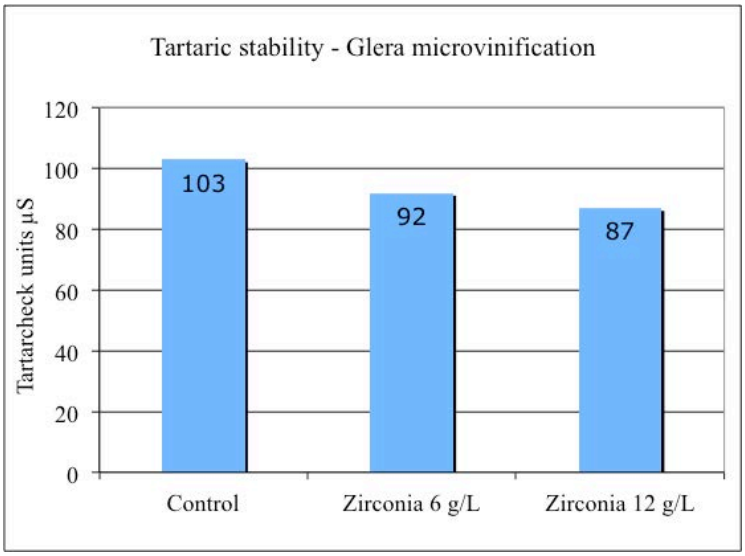

Figure 19. Tartaric stability in Glera wines deriving from a must not treated (control) and treted with 2 doses of Zirconia pellets. The analysis was performed by the mini-contact test, which measure the conductivity (expressed in µS) drop after addition of potassium hydrogentartrate (KHT) to the wine. The scale of tartaric stability provided from manufacturer gives the follow indications: <30 µS very stable; 30 – 50 µS stable; 50 – 70 µS risk; > 70 µS unstable.

Differently from Glera, microvinification experiments with Manzoni bianco showed that the treatment with zirconia greatly reduced the tartaric instability of the wines to a point to be considered stable (figure 20). Indeed all wines treated with Zirconia, used at different doses, resulted stable or very stable with values between 25 and 35 µS, much lower than that of the control (92 µS). These results were also confirmed by determination of the saturation temperature (Tsat) that decreased from 19.6 °C (control wine) to 16.1 °C (wine treated with 25 g/L of Zirconia) (not shown)

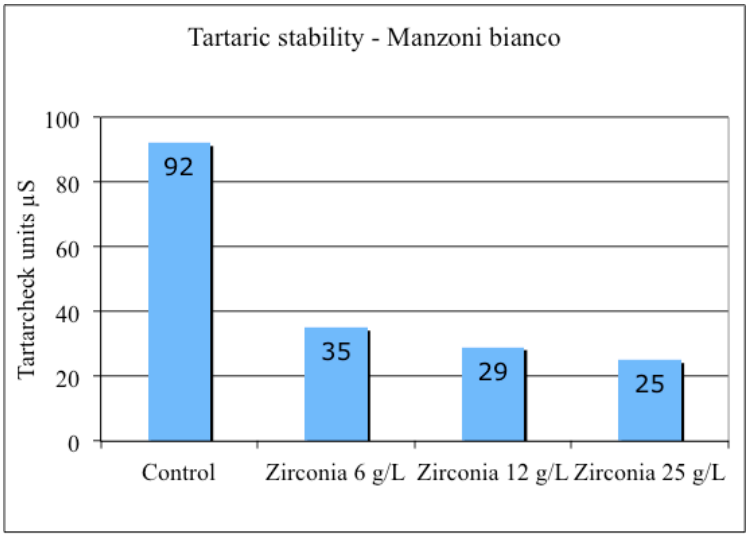

Figure 20. Tartaric stability in Manzoni bianco wines deriving from musts deriving from musts not treated (control) and treated with 3 doses of Zirconia pellets. The analysis was performed by the mini-contact test, which measure the conductivity (expressed in µS) drop after addition of potassium hydrogentartrate (KHT) to the wine. The scale of tartaric stability provided from manufacturer gives the follow indications: <30 µS very stable; 30 – 50 µS stable; 50 – 70 µS risk; > 70 µS unstable.

These preliminary results show a strong effect of the zirconia treatment on the tartaric stability of the Manzoni wine which was rendered fully stable. However, due to the different behaviour of Glera and Manzoni bianco, the possibility to increase tartaric stability in wines by the use of zirconia should be better investigated in other varieties. Moreover also the effect of titania on tartrate stability remain to be established.

5.3.9 Sensorial analysis

Manzoni bianco wines obtained from musts treated with zirconia (type V2) were compared by sensorial analysis to the wines stabilised by the classical bentonite treatment. No statistically significant differences in all the parameters considered (colour, olfaction, taste, defects and general impression) among the

various wines considered were detected., indicating that protein stabilization with zirconia does not results in an impairment of the organoleptic characteristics of the wines.

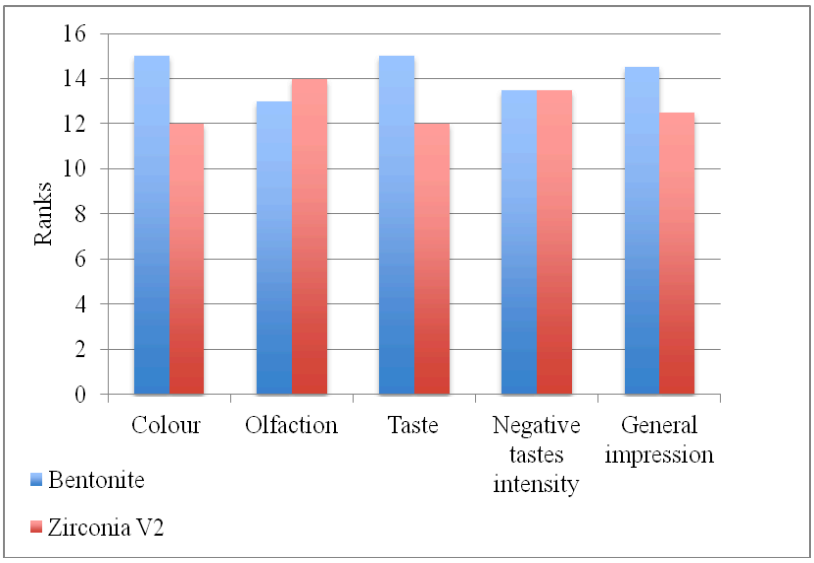

Figure 21. Results of the sensorial analysis of Manzoni bianco wines stabilised by bentonite (0.9 g/L) and zirconia V2 (25 g/L). Freidman Test was used for statistics Lower values indicate higher preference.

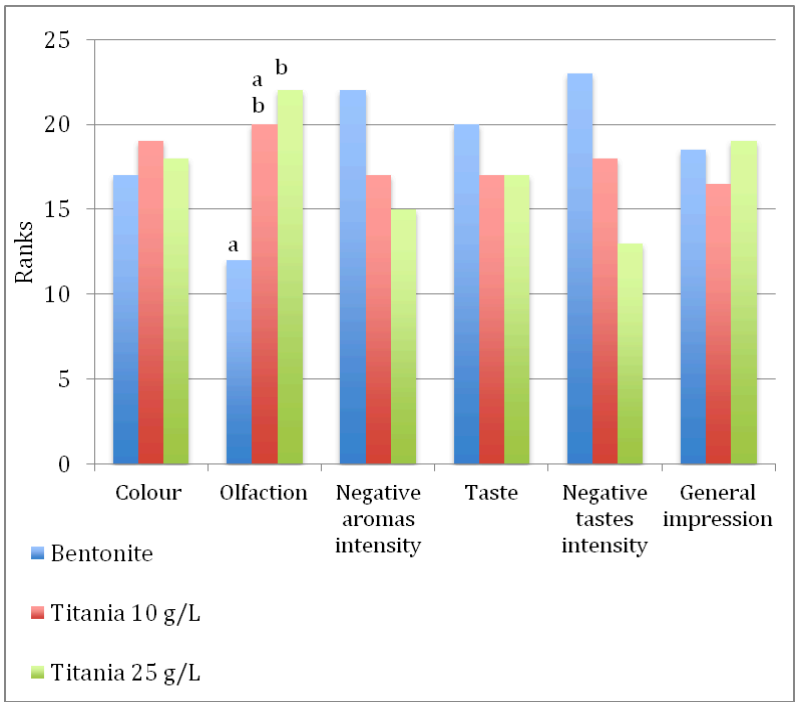

Figure 20. Statistical elaboration with Freidman Test in Manzoni bianco (second microvinification experiment). Comparison between the wines stabilized with Bentonite (0.6 g/L) and titania (10 g/L and 25 g/L). Lower values indicate major preference.

Also the treatment of the must with titania at 2 different dosages did not affect the sensorial characteristics of the wine when compared to wines treated with bentonite as regards colour, taste, defects and general impression. However when the parameter "olfaction" was considered, the wine treated with bentonite was preferred (figure 20).

From these results it can be concluded that, in general, stabilisation with zirconia and titania do not alter the sensorial characteristics of the wines, at least in comparison with wines stabilised by bentonite treatment.

5.3.10 Rigeneration of the adsorbents

After fermentation the pellets of zirconia and titania were regenerated and used again in succession for other microvinifications. The protocol proposed was able to completely regenerate the materials. The protein profiles of Manzoni bianco wine samples treated with regenerated Zirconia V2 demonstrated that the treatment was still able to reduce the grape proteins in solution (figure 21). Moreover, the heat test of the wine samples produced after treatments with the regenerated pellets of Zirconia V2 showed results comparable with those obtained after treatments with the pellets used for the first time (data not shown).

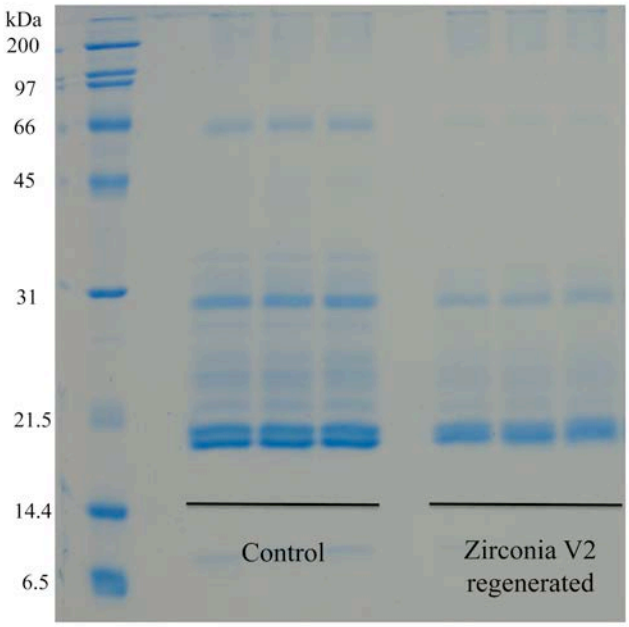

Figure 21. SDS-PAGE analysis (T=14 %) of Manzoni bianco samples before (control) and after treatment with regenerated Zirconia V2 pellets (25 g/L). Proteins were recovered for the wine by precipitation with 4 volumes of cold ethanol. The MW standards are indicated on the right.

However, the next step for optimisation of the regeneration protocol should be that to find the way to minimise the quantity of sodium hydroxide because its dangerousness and its cost as well.

5.4 Conclusions

The results of this work showed that is it possible to completely stabilize white wines by using zirconia and titania pellets as protein adsorbent materials during fermentation. The metal oxides, used as pellets, have been shown to effectively adsorb the grape proteins responsible for wine instability. The application of zirconia for wine stabilization have been already described showing the potentiality of this material for protein removal from white wines (Pachova *et al*., 2002; Pashova *et al*., 2004a-b; Salazar *et al*., 2006, 2007). However, the method here described, which uses metal oxide pellets confined in steel cages during fermentation allows a great simplification of the system. Indeed, compared to procedures previously developed, the system here proposed does not need of complex and expensive equipment and can be virtually used in all wineries with a simple and cheap approach. Adding pellets of metal oxides enclosed in metal cages at the beginning of the fermentation allows to exploit the natural movement of the fermenting must, thus enhancing protein removal and wine stabilisation. At the end of fermentation the metal cages with the pellets can be removed and it is possible to proceed with the normal winemaking operations (cold stabilization, filtering, bottling).

Also the improvement of the tartrate stability by treatment with zirconia, which partially remove the tartaric acid in excess, as preliminarily indicated here, seems to be promising as a tool to reduce the energy costs normally needed for tartrate stabilisation.

Moreover, also the method for the regeneration of the adsorbents here proposed is simple and inexpensive, allowing to use the same material for a virtually unlimited number of cycles, thus reducing the overall costs for protein stabilisation and also the quantity of waste material. This possibility to regenerate the materials also justifies the employment of relatively high doses

of pellets (i.e. 10-25/g /L) that can be necessary to achieve wine protein stabilisation.

The comparison between two types of zirconia gave similar results for the wines tested. Although different behaviours can not be excluded for the different types of zirconia pellets that can be found on the market, this is an indication of a general applicability of this material in white wine stabilisation.

Also titania pellets, tested here on wines for the first time, showed to be suitable for protein removal form wines, the action of this material being even stronger than that of zirconia.

The treatment of the musts with metal oxides resulted in minimal modification of the content of some elements of the wines, but slightly modified their total acidity and pH. These modifications however did not affected the sensorial characteristics of the wines stabilised with metal oxides, that were statistically similar to wines treated with bentonite.

In conclusion the use of metal oxides seems to be a promising alternative to that of bentonite to stabilise white wines in terms of protein haze formation However, the study of their effects on an industrial scale appears to be necessary to definitely assess their applicability as a new tool in white winemaking.

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6. Carrageenan and Pectin as tools to reduce the protein instability in white wines

Carrageenan and Pectin could be a valid alternative to bentonite in protein stabilization of white wines. The capacity of carrageenan to remove the unstable proteins from both a wine model solution and a Riesling grape juice was studied. Winemaking trials were carried out in order to evaluate the effectiveness of carrageen and pectin addition alone or in combination before or after cold settling and racking of a Chardonnay juice. All treatments decreased wine protein instability and carrageenan resulted the most effective agent to remove unstable proteins.

6.1 Introduction

In the winemaking process, it is important to remove the unstable grape proteins that could remain in finished wines and to provoke haze during wine storage (Bayly and Berg, 1967). Bentonite (a clay acting as cation exchanger) has been used widely in oenology as a fining agent for its established efficacy as well as its low cost. However, bentonite fining has some drawbacks such as significant wine volume losses (Waters *et al.,* 2005), impairment of the organoleptic properties (Lubbers *et al.,* 1993; Ribéreau-Gayon*et et al.*, 2000) and interference with membrane-based winemaking technologies (Waters *et al*., 2005; Salazar *et al*., 2007).

For these reasons, several researchers have investigated alternative procedures, including the use of other adsorbents (Sarmento *et al*., 2000; Vincenzi *et al.,* 2005), and immobilized tannic acid (Weetall*et al.,* 1984) or proanthocynidins

(Powers *et al.,* 1988), ultrafiltration (Hsu *et al.*, 1987), and proteases (Waters *et al.,* 1992), but no alternative has proven sufficiently effective.

Cabello-Pasini and colleagues (2005) have been proposed the addition of polysaccharides from seaweeds (carrageenan, agar and alginic acid) as a tool for protein removal from white wines.

Carrageenan is a generic name for a family of natural and water soluble polysaccharides extracted from red seaweeds. Carrageenan is widely used as high quality ingredient in food and cosmetics (Renn D., 1997; Rudolph B., 2000). This polysaccharides is mainly constituted of linear chains of alternating β-(1→3)-D-galactose and α-(1→4)-D-galactose units (Cosson *et al.,* 1995). Carrageenan has a strong negative charge over a wide pH range due of its great number of sulfate groups (Mackie and Preston, 1974). This characteristic allows its interaction with positively charged beer and wine proteins (Cabello-Pasini*et al.*, 2005).

Pectin is an anionic heteropolysaccharide of partially esterified α-1,4 linked D-galacturonides, containing varying amounts of covalently attached rhamnose and branches of L-arabinose, D-galactose, D-xylose, and L-rhamnose (Thakur *et al.,* 1997). Some pectins are used for stabilizing effect in acidified dairy drinks and emulsions due to its interactions with the cationic groups of proteins (Pereyra*et al.*, 1997; Syrbe *et al.,* 1998).

Preliminary experiments were carried out in order to evaluate the capacity of carrageenan to remove Thaumatin in model wine and unstable proteins in Riesling juice. Then, a microvinification experiment was performed with a Chardonnay juice in order to evaluate the capability of carrageenan and pectin to remove unstable grape proteins and to reduce the protein instability of the wines.

6.2 Materials and Methods

6.2.1 Materials

The pectinase used was Ultrazyme CPL (Novozyme, Bagsvaerd, Denmark). The yeast strain was EC1118 (Lallemand, Canada). A solution of 2% carrageenan (Genuvisco®, CPKelco, Denmark) in distilled water was prepared iust before use. The pectin (Genu®, CPKelco, Denmark) was used in powder form.

6.2.2 Treatment of model wine and juice with Carrageenan

Different carrageenan dosages (150 mg/L, 300 mg/L and 1000 mg/L) were added to a model wine solution (12 % alcohol, 5 g/L tartaric acid, pH 3.20) containing 500 mg/L Thaumatin from *Thaumatococcus daniellii* (Sigma) and to a filtered Riesling grape juice. The samples were mixed overnight at room temperature and then filtered (0.45 μm).

6.2.3 Treatment with Carrageenan and Pectin during wine making

A Chardonnay must (400 L, vintage 2009) from South Australia was used to perform an experimental microvinification. Twenty fermenters (20 L each) were prepared. After addition of pectinase (30 mg/L), juices were cold settled for 24 h at 0 ºC and then racked.

Pectin and carrageenan were added singularly or in combination before or after cold settling and racking of the juice (Table 1). In pre-racking treatments, pectin and/or carrageenan were added to unclarified juice by mixing the ingredients into the juice prior to cold settling and racking off juice lees. In

post-racking treatments the pectin and/or carrageenan were added to the clarified juice after cold settling and racking off juice solids and prior to the addition of yeast. Pectin was added at 2 g/L in three of the six treatments. The 2% carrageenan stock solution was added to obtain a final concentration of 0.25 g/L in four of the six treatments.

After racking, yeast was properly re- hydrated and inoculated in according to manufacturer's recommendations at 200 mg/L. Fermentations were conducted between 15 and 18 °C. When residual sugar reached < 2 g/L, samples were chilled to 0 °C and SO_2 levels adjusted. Wines were deemed cold stable using the three days cold stability test (absence of crystals after incubation at - 4 °C for 72 h). Wines were filtered (0.45 µm) and bottled in 750 mL bottle about 4 months after the end of fermentation.

Number of Fermenters	**No. 001-003**	**No. 004**	**No. 005-007**	**No. 008-010**	**No. 011-014**	**No. 015-017**	**No. 018-020**
Pectin (pre-racking)	_	_	2.0 g/L	_	_	2.0g/L	_
Carrageenan (pre-racking)	_	_	0.25g/L	0.25g/L	_	_	_
Pectin (post-racking)	2.0g/L	_	_	_	_	_	_
Carrageenan (post-racking)	_	_	_	_	_	0.25g/L	0.25g/L
yeast	0.20g/L	0.20g/L	0.20g/L	0.20g/L	0.20g/L	0.20g/L	0.20g/L

Table 1. Scheme of carrageenan/pectin addition at different times of the wine making process.

6.2.4 Analytical Methods

Alcohol, specific gravity, pH, titratable acidity, glucose/fructose and volatile acidity analysis were performed by the Commercial Service of The AWRI using a Foss WineScan FT 120 as described by the manufacturer (Foss, Hillerød, Denmark). Free and total SO_2 were measured by the aspiration method (Rankine and Pocock, 1970). °Brix were measured by refractometry and °Baumé by densitometry.

6.2.5 Protein content determination

Protein content was determined by EZQ® protein quantitation kit (Molecular probes®, Australia) following the manufacturer's instructions. The calibration curve was built using serial dilution from 0 to 250 mg/L of Thaumatin from *Thaumatococcus daniellii* (Sigma-Aldrich, Australia). Fluorescence measurements were taken using excitation/emission settings of 485/590 nm with a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, California, USA).

6.2.6 Protein content determination by HPLC

Protein concentration and composition were determined by reverse-phase (RP) HPLC with a Vydac 2.1 x 250 mm C8 column (208TP52 Grace Davison Discovery Sciences, Australia) on an Agilent 1200 system according to the method of Marangon *et al*. (2009) with modifications as suggested by Van Sluyter *et al*. (2009). Injection volumes were 25 µL. From the 210 nm chromatogram, protein identity was assigned by comparison of the retention

times (RT) to those of purified grape PR proteins (Peng *et al*., 1997; Marangon *et al*., 2009) as follows: peaks with a RT between 9.00 and 12.00 min were assigned to the TL protein classes, whereas peaks eluted from 19.00 and 24.50 min were assumed to be chitinases. Thaumatin from *Thaumatococcus daniellii* (Sigma-Aldrich, Australia) was used as the standard for protein quantification.

6.2.7 Heat Test

Glass tubes containing the wine samples were incubated at 80 °C for 2 hours and then cooled in ice for 2 hours. Following equilibration at room temperature for 30 minutes, samples were transferred into quartz cuvette (1 mL capacity) and haze was quantified by measuring the absorption at 540 nm (Waters *et al.,* 1992) using a spectrophotometer (Shimadzu UV 6010). The haze was also quantified by nephelometry in NTU (Pocock and Rankine, 1973) using a turbidimeter (2100P, HACH). To each obtained value the value measured for the same sample before heating was subtracted.

6.2.8 Determination of the Polysaccharide content

Polysaccharides were precipitated by adding absolute ethanol to the wine samples to be analysed. This operation also allowed eliminating many compounds that could interfere with the analyses.

500 µL absolute ethanol was added to 100 µL of sample. After 18 hours at room temperature samples were centrifuged at 14000 rpm for 30 minutes. The supernatant was eliminated and the pellets (polysaccharides) re-suspended in ethanol and this procedure was repeated three times. The pellet was dissolved in 500 µL of distilled water and 200 µL were put into glass tubes, added of 210 µL of a 4% (p/p) phenol solution and 1 mL of concentrated sulphuric acid.

After 30 minutes samples were read with a spectrophotometer (Shimadzu UV 6010) at 490 nm.

The calibration curve was built using glucose in water as the standard, at concentrations between 0 and 200 μg/mL. The glucose solution was added of 210 μL of 4% (p/p) phenol solution and 1 mL of sulphuric acid; after 30 minutes the glucose samples were read at 490 nm.

6.2.9 Statistical analyses

Data were analysed by one-way completely randomized ANOVA with CoHort Software (CoStat version 6.311, Monterey, CA) and data significance assessed by Student-Newman-Keuls test. Each measure was the result of at least three replicates unless otherwise stated.

6.3 Results and Discussion

6.3.1 Treatment of model wine and juice with Carrageenan

The experiment carried out in model wine and filtered Riesling grape juice demonstrated the capacity of carrageenan to remove unstable proteins (figure 1, 2 and 3). In model wine 1000 mg/L of carrageenan were able to remove completely the Thaumatin added.

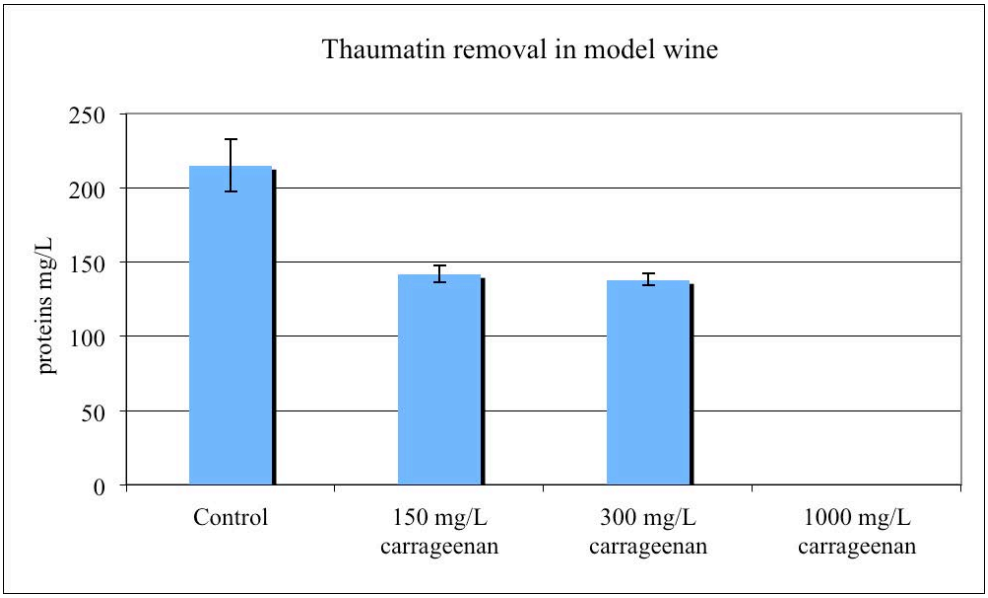

Figure 1. Thaumatin content in model wine treated with differ dosages of carrageenan. The protein content was measured by EZQ® method and expressed in mg/L of Thaumatin.

However, model wine is a simple solution compared to wine and many variables could influence the carrageenan action in a complex matrix. This reason could explain the different kinetic of protein removal observed in the grape juice where the amount of protein removed was proportional to the quantity of added carrageenan (figure 2).

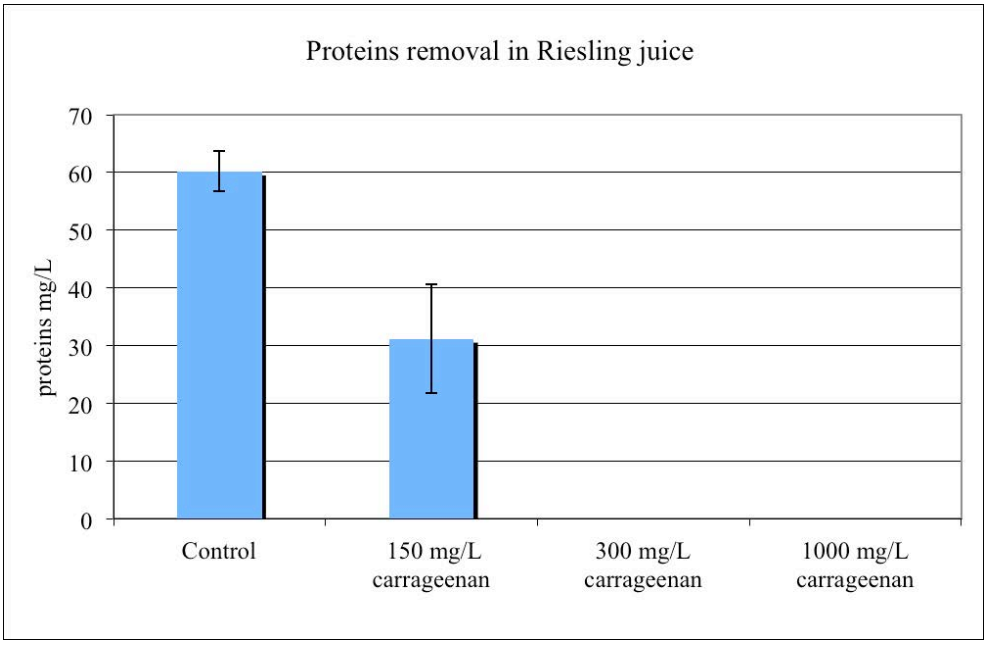

Figure 2. Protein content of Riesling juice treated with different dosages of carrageenan. The protein content was measured by EZQ® method and expressed in mg/L of Thaumatin

The juice treated with 300 mg/L of carrageen showed a complete absence of proteins whereas the 150 mg/L dose halved the protein content. RP-HPLC analysis of treated juices showed that protein removal was not specific, the TL protein with RT of 9.7 being the most affected component (figure 3).

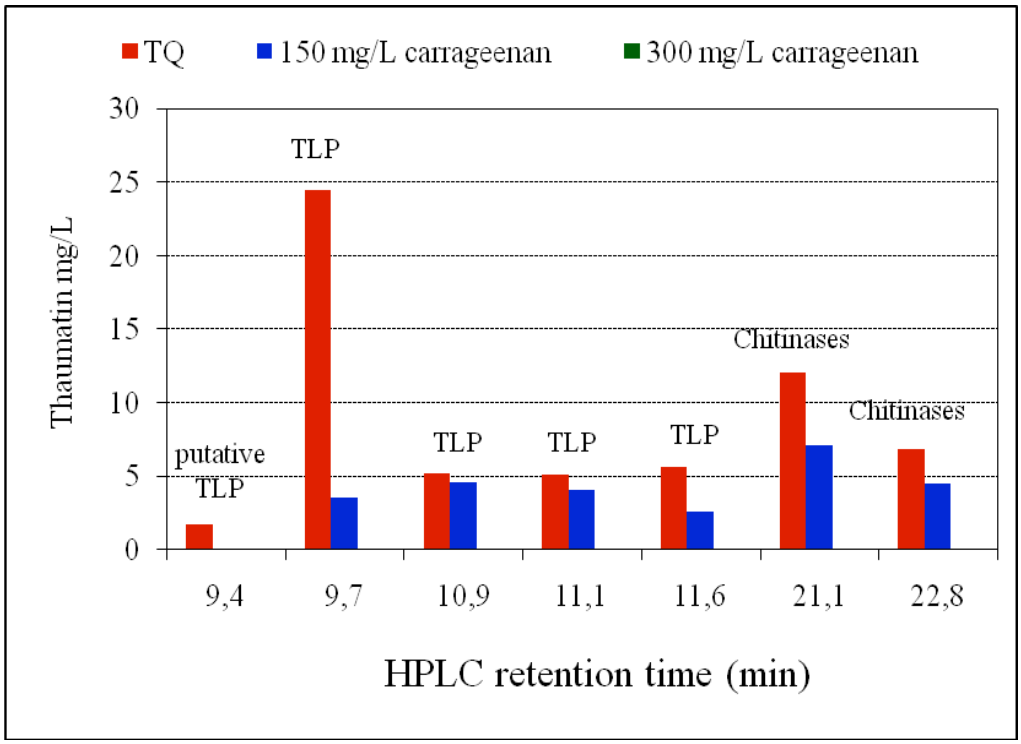

Figure 3.Variation in the quantity of the main RP-HPLC protein peaks of Riesling juice treated with different dosages of carrageenan. RT between 9.00 and 12.00 min correspond to TL proteins and those between 19.00 and 24.50 min to chitinases (Peng *et al*., 1997; Marangon *et al.,* 2009). Protein quantity was measured in AU and expressed in mg/L of Thaumatin.

These preliminary results demonstrated the capacity of carrageenan to remove the unstable grape proteins from the juice.

6.3.2 Treatment with Carrageenan and Pectin during wine making

A decrease of the fermentation rate during microvinification in the presence of carrageenan was observed, while pectin alone did not affect the fermentation rate. The length of fermentation in carrageenan trials was 3-11 days longer that

observed in the controls (table 2). Moreover in carrageenan trials a certain formation of foam (figure 4 and 5) during fermentation was seen.

Figure 4. Microvinification experiment: 1-3 Pectin pre-racking, 4 Control, 5-7 Pectin pre-racking Carrageenan pre-racking, 8-10 carrageenan pre-racking, 11-14 Control, 15-17 Pectin pre-racking Carrageenan post-racking, 18-20 Carrageenan post-racking.

1 Pectin pre-racking | 2 Pectin pre-racking | 3 Pectin pre-racking | 4 Control | 5 Pectin pre-racking Carrageenan pre-racking

6 Pectin pre-racking Carrageenan pre-racking | 7 Pectin pre-racking Carrageenan pre-racking | 8 Carrageenan pre-racking | 9 Carrageenan pre-racking | 10 Carrageenan pre-racking

11 Control | 12 Control | 13 Control | 14 Control | 15 Pectin pre-racking Carrageenan post-racking

16 Pectin pre-racking Carrageenan post-racking | 17 Pectin pre-racking Carrageenan post-racking | 18 Carrageenan post-racking | 19 Carrageenan post-racking | 20 Carrageenan Post-racking

Figure 5. Microvinification experiment in the middle fermentation. It was observed the formation of foam in carrageenan trials.

Analysis of the bottled wines (at 138 days after the treatments) did not demonstrate differences for parameters such as free and total SO_2, specific gravity and residual sugars (glucose + fructose) (data not shown). The ethanol content in carrageenan treatments resulted significantly lower (0.15 % v/v) than that of all the other treatments. That difference did not correspond to a larger quantity of residual sugars and can be explained by a possible interaction of carrageenan directly to ethanol or indirectly by influencing the yeast metabolism. The samples treated with pectin showed a total acidity decrease (around 8%) and a slightly increase of pH values. In contrast, the carrageenan treatments did not affect acidity.

All treatments reduced the protein content especially when pectin and carrageenan were used together (figure 6). The carrageenan resulted to have a stronger action than pectin, in particular when additions were made after racking. The protein removal by carrageenan occurred immediately after the treatment. This aspect indicates a quick reaction of the carrageenan with the grape proteins. Successive analysis showed that this reduction persisted in the carrageenan post racking treatment with or without pectin (figure 6). Pectin treatment showed a partial protein reduction one day after the treatment, but that reduction improved 138 days after the treatment.

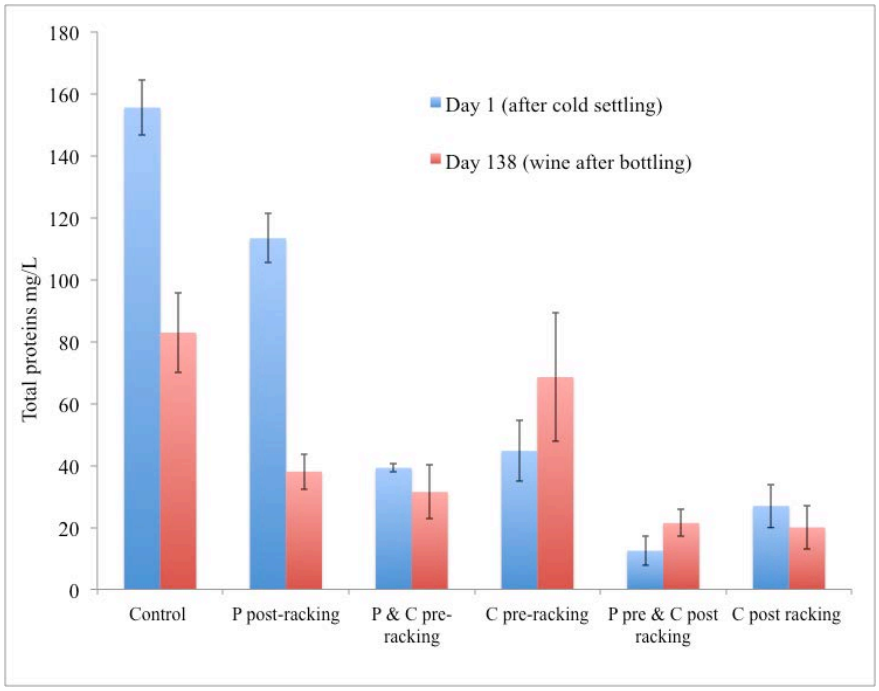

Figure 6. Total protein content of the musts after addition of carrageenan and/or pectin (day 1) and in the corresponding bottled wines (day 138). P: pectin; C: carrageenan. protein was measured by the EZQ® method and expressed in mg/L of Thaumatin. The 20 samples were analysed in triplicate.

The protein stability of the wines obtained after the different treatments was then determined by the heat test. The pectin and carrageenan treatments reduced protein instability (table 2) but did not stabilize completely the wines. Among the treatments, that with carrageenan post-racking coupled with pectin pre-racking produced the most stable wines with a 70 % reduction of the instability (from 41.4 NTU of the control to 11.8 NTU). In general these results can be related to the capability of carrageen to remove proteins as observed in figure 6. The treatment with pectin alone resulted the less effective to decrease the protein instability measured by the heat test, although this was a treatment that reduced dramatically the proteins content of the bottled wine (figure 6). Perhaps the residual proteins were more instable than the proteins removed.

Ferment ID	Treatment	Fermentation time (days)	Polysaccharide (mg/L)		Net haze after heat test (NTU)
			Day 12	Day 138	
004, 011-014	Control	7.8 [a]	606 [b]	325 [ab]	41.4 [a]
001-003	Pectin post racking	8.0 [a]	820 [a]	417 [a]	20.3 [b]
005-007	Pectin - Carrageenan pre racking	16.3 [a]	876 [a]	355 [ab]	17.5 [b]
008-010	Carrageenan pre racking	11.3 [a]	767 [a]	358 [ab]	12.4 [b]
015-017	Pectin pre racking - Carrageenan post racking	10.8 [a]	835 [a]	299 [b]	11.8 [b]
018-020	Carrageenan post racking	19.5 [a]	771 [a]	358 [ab]	14.5 [b]

Table 2. Effects of the treatments on wine polysaccharide content before (day 12) and after bottlins (day 138) and heat stability after bottling (day 138). Mean values are shown ($n \geq 3$). Values were analysed by the Student-Newman-Keuls test ($P = 0.05$), where a, b and c indicate statistically different values.

After 12 days, the content of polysaccharides of the wines increased in all treatments with respect to the control, whereas in the bottled wine (day 138) only that produced with coupled pectin pre-racking and carrageenan post racking showed a statistically lower polysaccharide content. As reported by many athors, a high quantity of polysaccharides can affect the physical, chemical and organoleptic properties of the wine (Waters *et al.,* 1993; Dupin *et al.,* 2000; Vidal *et al.*, 2004). Consequently the protein stability could be

increased for both proteins removal and an increased polysaccharides content which should act as haze protective factors (Waters *et al.*, 1993).

6.4 Conclusions

Carrageenan and Pectin demonstrated the capacity to remove unstable proteins and to decrease the protein instability in a Chardonnay juice treated before fermentation. Carrageenan resulted more affective in protein removal than pectin. The carrageenan action was also demonstrated in experiments using model wine and a Riesling grape juice. However, carrageenan slow down the fermentation rate and the wine resulted to have a lower alcohol content compared to the wines obtained with the others treatments. Pectin treatments decreased the total acidity content and slightly increase the pH values of the wines.

In conclusion the addition of carrageenan in musts could be a potential tool for increasing the protein stability in white wines because of its ability to remove proteins from the grape juice

Acknowledgements

The author would like to thank Elizabeth J. Waters of the Grape and Wine Research and Development Corporation, Matteo Marangon and Kenneth F. Pocock of the Australian Wine Research Institute, for the valuable discussions about this work and Gemma West of WIC Winemaking for experimental winemaking.

This work was financially supported by Australia's Grapegrowers and Winemakers through their investment body “The Grape and Wine Research and Development Corporation”, with matching funds from the Australian Government. The AWRI is a member of the Wine Innovation Cluster located in Adelaide. The work is part of the research activity of the "Dottorato di Ricerca

in Viticoltura, Enologia e Marketing delleimpresevitivinicole" supported by Provincia di Treviso (Italy) and it has been supported in part by the Fondazione Ing. Aldo Gini (Padova, Italy). Fosters Wines, Orlando Wines, Yalumba Wine Company and Casella Wines are also specifically thanked for funding the small scale winemaking part of this project.

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7. Conclusions

The aims of this thesis were to find a new technicques or methods capable to remove and/or degrade the grape proteins in order to increase protein stability in white wines with the final goal to substitute bentonite or decrease its amount for wine stabilization.

The first approach was based on the isolation and purification of proteases from phytopathogenic fungi. *Sclerotinia minor* and *Botrytis cinerea* were chosen for their capability to produce active proteases. A culture medium with hydrolyzed casein as nitrogen source increased the production of an aspartyl protease produced by *S. minor*. The partially purified protease was able to degrade the grape proteins *in vitro* tests and to decrease the protein instability in microvinification trials. Juices of Glera and Manzoni bianco treated 24 hours with the protease of *S. minor* produced stable wines. However the protease production by the fungus as well as its efficacy were inconstant and seemed to be related to the residual laccase activity found in the culture of the fungus.

B. cinerea was not able to remove efficacy the grape proteins in contrast with what was previously reported (Marchal *et al.,* 1998; Cilindre *et al*., 2007). A partially purified protease from *Botrytis cinerea* was actually unable to degrade the grape proteins *in vitro* and in microvinification trials. The capacity of this fungus to eliminate the grape proteins was explained by an oxidative mechanism involving the enzyme laccase, which was found to give rise to oxidation products of polyphenols, by reacting with grape proteins, caused their insolubilisation.

The second approach was based on the application of pellets of metal oxides, including zirconium dioxide (zirconia), titanium dioxide (titania) and a material produced mixing zirconia and titania, as adsorbents for grape proteins in white wines and musts during fermentation. The pellets of these metal oxides were enclosed in a metallic cage and introduced in wine or juice. The stirring

resulted an important factor in the kinetics of protein absorption as reported in the literature (Pachova *et al.,* 2002; Pashova *et al.,* 2004a; Pashova *et al.,* 2004b; Salazar *et al.,* 2006; Salazar *et al.*, 2007; Marangon *et al.*, 2011). To achieve effective stirring, the natural movement caused by the fermentation process was exploited. The *Saccharomyces cerevisiae* activity was not affected by the presence of metal oxides pellets; indeed the juices treated with these pellets showed a kinetic of fermentation a little faster compared the control. Different juices and wines, including those particularly rich in proteins as Manzoni bianco and Sauvignon, were completely stabilized after being treated with dosages from 15 to 25 g/L of metal oxides. Titania demonstrated a stronger action than zirconia to remove grape proteins. The wines obtained showed a reduction of the total acidity between 5 and 15% and a slightly increase of pH values. Besides some elements as aluminium, calcium, copper, manganese, phosphorus and zinc decreased in wines and juices treated with metal oxides, while sodium increased.

Polysaccharides generally improved in the samples treated with metal oxides in particular with Titania, probably by affecting the polysaccharide excretion by *S. cerevisiae* during fermentation. In contrast, wine polyphenol content slightly decreased after Zirconia and Titania treatments, probably because they were bounded by the unstable proteins removed.

The capability to decrease the total acidity by removing organic acids (in particular tartaric acid) and the ability to increase the polysaccharides in the wine indicated the possibility to increase tartrate stability in wines by the use of metal oxides. Indeed, Manzoni bianco and Glera juices treated with zirconia showed a reduction of tartrate instability of the wines compared to the controls. The tartrate stabilisation by treatments with zirconia, as preliminarily indicated here, seems to be a promising as a tool to reduce the energy costs of winemaking.

Finally, sensorial comparison of wines stabilized with metal oxides and bentonite, indicated non difference among these wines.

Zirconia and titania pellets can be easily regenerated by treatments with a sodium hydroxide solution followed by a citric acid solution and the cleaning process can be improved by raising the temperature. The material regenerated in this way can be used for a virtually indefinite number of times. This possibility to regenerate the materials also justifies the employment of relatively high doses of pellets (i.e. 10-25/g /L) that can be necessary to achieve wine protein stabilisation.

The use of metal oxides seems to be a promising alternative to that of bentonite to stabilise white wines in terms of protein haze formation However, the study of their effects on an industrial scale appears to be necessary to definitely assess their applicability as a new tool in white winemaking.

The third approach in wine protein stabilisation was thet based on the use of carrageenan and pectin. Their ability to remove unstable grape proteins were evaluated in Riesling and Chardonnay juices. Carrageenan and Pectin demonstrated the capacity to remove unstable proteins and to decrease protein instability when added to to the juice before fermentation. However, carrageenan impaired the fermentation rate and reduced the alcohol content of the wine. Whereas pectin treatments decreased the total acidity content and slightly increase the pH values.

In conclusion the different approaches followed in this thesis confirm the difficulty to find proteases able to stabilise wines against protein haze formation. Instead the use of metal oxides and also that of carrageenan demonstrated to be promising techniques to reach protein stability in white wines. Before being proposed in current use, however, all these treatments need to be evaluated for their effects on an industrial winemaking scale, evaluating also their costs and environmental impact.

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8. Articles published

8.1 Articles published in scientific journal with impact factor

M. Marangon, **M. Lucchetta**, D. Duan, V.J. Stockdale, A. Hart, P.J. Rogers and E.J. Waters (2011) Protein stabilisation of Chardonnay juice by addition of carrageenan and pectin. *Article submitted to Australian Journal of Grape and Wine Research*

M. Marangon, **M. Lucchetta** and E.J. Waters (2011) Protein stabilisation of white wines using zirconium dioxide enclosed in a metallic cage. *Australian Journal of Grape and Wine Research 17: 28-35.*

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8.2 Articles published in scientific Italian journals

S. Vincenzi, D. Gazzola, **M. Lucchetta** and A. Curioni (2011) Effetto della posizione della bottiglia durante la rifermentazione e la maturazione del Prosecco Sur Lies. *Accepted in Rivista di Viticoltura e di Enologia*

ASVO AUSTRALIAN JOURNAL OF Grape and Wine Research

Protein stabilisation of Chardonnay juice by addition of carrageenan and pectin

Journal:	*Australian Journal of Grape and Wine Research*
Manuscript ID:	AJGWR-11-095
Manuscript Type:	Original Article
Date Submitted by the Author:	22-Aug-2011
Complete List of Authors:	Marangon, Matteo; The Australian Wine Research Institute Lucchetta, Marco; Università di Padova, Biotecnologie agrarie Duan, David; Foster's Group Stockdale, Vanessa; Treasury Wine Estate Hart, Allen; Foster's Group Rogers, Peter; Foster's Group; Griffith University, School of Science Waters, Elizabeth; Grape and Wine Research and Development Corporation
Keywords:	wine, protein, haze, bentonite, carrageenan, pectin

Protein stabilisation of Chardonnay juice by addition of carrageenan and pectin

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Running title: Adsorbents for protein stabilisation of wines

Abstract

Backgrounds and Aims: Bentonite is commonly added to white wines to remove the grape proteins responsible for haze formation. Despite being effective this technique has drawbacks, thus new solutions are desirable. Here we assessed the ability of carrageenan and pectin to remove heat unstable grape proteins, and the impact that such addition has on the physicochemical and sensorial profile of the wines.

Methods and results: Carrageenan and pectin were added separately or in combination to a Chardonnay juice prior to fermentation. Both adsorbents removed proteins (up to 75%) thus increasing wine protein stability. Carrageenan was more effective than pectin at increasing wine protein stability.

Conclusions: Pectin and carrageenan removed protein and partially stabilised the wines.

Significance of the Study: These findings may lead to the uptake by the wine industry of an alternative procedure for protein stabilisation of wines and give indication to winemakers on the impact of adding pectin or carrageenan to juice pre-fermentation.

Keywords: wine, protein, haze, bentonite, carrageenan, pectin.

Protein stabilisation of white wines using zirconium dioxide enclosed in a metallic cage

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Abstract

Backgrounds and Aims: White wines are stabilised by removing the heat unstable proteins through adsorption by bentonite. Bentonite fining is not an efficient wine processing step and can also remove other wine components. Alternative absorbents are thus sought; zirconium dioxide (zirconia) is recognised as a promising candidate. The aim of this work was to assess the viability of zirconia treatments to stabilise white wines, with particular attention on process development.

Methods and Results: Effective treatment was achieved by enclosing zirconia pellets into a metallic cage submerged in the wine. With this method, the wine could be treated with the adsorbent for the time required for protein stabilisation, and then removed without further manipulation. Zirconia treatments of three unstable wines partially or fully stabilised them without detectable modifications of their physicochemical parameters and colours, apart from the removal of metals and some acids, particularly when wines were treated for long times and with high dosages of the adsorbent. A simple and inexpensive zirconia regeneration method was also developed.

Conclusions: The zirconia application to wine was very effective in removing proteins, and the proposed regeneration procedure could facilitate the uptake and development of zirconia-based solutions for the wine industry.

Significance of the Study: This study confirmed the effectiveness of zirconia in removing wine proteins and demonstrated that the proposed method of application has the potential to become a viable alternative to bentonite.

Abbreviations

CIELAB Commission Internationale de l'Eclairage Lab transmission values L* a* b*; **MW** molecular weight; **NTU** nephelometric turbidity unit; **PR** pathogenesis related; **TCA** trichloroacetic acid; **TL protein** thaumatin-like protein

Keywords: *zirconium dioxide*, *protein*, *wine*, *haze*, *Sauvignon Blanc*, *Riesling*, *Chardonnay*

Introduction

The presence of residual proteins from grapes in finished white wines is undesirable because of their key role in causing haze during wine storage (Bayly and Berg 1967). Since the 1930s, bentonite (a clay cation exchanger) has been used widely in oenology as a fining agent, to stabilise wines by protein adsorption (Saywell 1934). Bentonite is still extensively used because of its established efficacy as well as its low cost. However, bentonite fining has some drawbacks such as a significant wine volume loss (3–10%) because of poor settling (Waters et al. 2005). Other bentonite-associated costs include waste disposal, occupational health and safety issues, and interference with increasingly common membrane-based winemaking technologies (Waters et al. 2005, Salazar et al. 2007). Moreover, bentonite is not a specific adsorbent and may reduce both undesirable and desirable compounds such as aroma, flavour and anthocyanin compounds (Miller et al. 1985, Voilley et al. 1990, Lubbers et al. 1993, Ribéreau-Gayon et al. 2000). For these reasons, alternative procedures for protein removal from white wine have been extensively investigated, including other adsorbents (Sarmento et al. 2000, Cabello-Pasini et al. 2005, Vincenzi et al. 2005, de Bruijn et al. 2009a,b), use of immobilised tannic acid (Weetall et al. 1984) or proanthocyanidins (Powers et al. 1988), ultrafiltration (Hsu et al. 1987), and proteases (Waters et al. 1992), but no alternative has proven sufficiently cost-effective to date.

One promising solution is represented by the adsorption of unstable proteins on the surface of zirconium oxide (Pachova et al. 2002, Pashova et al. 2004a,b, Salazar et al. 2006, 2007). Zirconium dioxide, a metal oxide commonly known as zirconia, is a material characterised by low corrosion potential, low thermal conductivity, hardness, and high thermal and mechanical resistances (Piconi and Maccauro 1999, Liu et al. 2005, Manicone et al. 2007). Because of its features, it has many applications such as a catalyst or support material, refractory material, ceramic material and biomechanical support in medical implants (Stichert and Schuth 1998, Piconi and Maccauro 1999, Rovira-Bru et al. 2001, Chevalier 2006, Mallick et al. 2006). Despite very promising results obtained with both batch addition and continuous systems for wine stabilisation treatments (Pachova et al. 2002, Pashova et al. 2004a,b, Salazar et al. 2006, 2007), fining with zirconia has not been developed into a commercial process or used commercially, and its ability to be a bentonite substitute for wines with high protein concentrations has not been established.

The aim of this work was to confirm the ability of zirconium dioxide to protein stabilise white wines, to test the impact of this

doi: 10.1111/j.1755-0238.2010.00112.x

treatment on wine quality and to develop an alternative method of its application that would be feasible as common winemaking practice.

Materials and methods

Wine samples

Three unfined wines (Chardonnay, Riesling and Semillon) from the 2007 vintage from Adelaide Hills (South Australia) were used in preliminary experiments undertaken in 2009. A further three unfined wines (Chardonnay, Riesling and Sauvignon Blanc) from the 2009 vintage from South Eastern Australia were used in the larger scale experiments in 2009. All wines were donated by commercial producers, were made using standard winemaking practices and on a commercial scale, and were stored below 10°C before the experiments were undertaken.

Materials

The zirconia used was originally in pellet form (Saint-Gobain NorPro, Staw, Ohio, USA) and was donated by Prof Francisco López. Zirconia pellets were small disks with a diameter of 3 mm and a thickness of 1 mm, a pore size of 6.2 nm, a surface area of 108.5 m^2/g and with tetragonal morphology (Salazar 2007). Zirconia powder was obtained by grinding the pellets with a mortar and pestle. The bentonite used was a sodium-calcium bentonite, Nacalit (Erbslöh, Geisenheim, Germany). Bentonite stock was prepared at 50 g/L in water, at least 24 h before use. The material used to prepare the zirconia bags was Miracloth (Calbiochem, Los Angeles, California, USA).

Protein High-Performance Liquid Chromatography (HPLC)

Protein concentration and composition was determined by reverse-phase HPLC with a Vydac 2.1 × 250 mm C8 column (208TP52 Grace Davison Discovery Sciences, Baulkham Hills, New South Wales, Australia) on an Agilent Technologies 1200 system (Santa Clara, California, USA) according to the method of Marangon et al. (2009) with modifications as suggested by Van Sluyter et al. (2009). Injection volumes were 25 µL. From the 210 nm chromatogram, protein identity was assigned by comparison with retention times of purified grape pathogenesis related (PR) proteins (Marangon et al. 2009, Van Sluyter et al. 2009) as follows: peaks with a retention time between 12 to 16 min were assigned to the thaumatin-like (TL) protein class, whereas peaks eluted from 24 to 28 min were classified as chitinases.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed with NuPage 4–12% Bis-tris, 1.5-mm thick, 15-well gels (Invitrogen, Mt Waverley, Victoria, Australia) and a XCell SureLock Mini Cell (Invitrogen) following the manufacturer's instructions. Approximately 50 mg of $Na_2S_2O_5$ were added to the top reservoir prior to running to prevent cysteine oxidation. Samples were prepared by precipitating proteins from 100 µL of wine with two volumes of cold trichloroacetic acid (TCA) (10%) in acetone. After incubating overnight at −20°C, samples were centrifuged (13 000 × *g*, 15 min, 0°C) and the pellet washed with 80% acetone to remove the TCA. After a second wash with 80% acetone (13 000 × *g*, 15 min, 0°C) pellets were dissolved in 40 µL of loading buffer (Invitrogen NuPage recipe) with 5% (v/v) 2-mercaptoethanol and boiled for 5 min. Precision Plus Protein unstained standards were from Bio-Rad (Bio-Rad Laboratories Pty.Ltd, Regents Park, New South Wales, Australia). Proteins were stained with Pierce Imperial Protein Stain (Quantum Scientific, Sydney, New South Wales, Australia) according to the manufacturer's microwave instructions with an extended incubation in the stain to increase sensitivity.

Protein content determination

Protein content was determined by EZQ® protein quantitation kit (Invitrogen) following the manufacturer's instructions. The calibration curve was built using serial dilution from 0 to 250 mg/L of thaumatin from *Thaumatococcus daniellii* (Sigma-Aldrich, Castle Hill, New South Wales, Australia). Fluorescence measurements were taken using excitation/emission settings of 485/590 nm with a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, California, USA).

Heat test

Wines were heated at 80°C for 2 h and cooled in ice for 2 h. After equilibration at ambient temperature, the haze was measured by calculating the difference in the absorbance values at 540 nm (Waters et al. 1992) or in a nephelometric turbidity unit (NTU) by means of a nephelometer (Pocock and Rankine 1973) between the heated and unheated samples.

Analytical methods

Alcohol, specific gravity, pH, titratable acidity, glucose/fructose and volatile acidity analysis were performed by the Commercial Service of The Australian Wine Research Institute using a Foss WineScan FT 120 as described by the manufacturer (Foss, Hillerød, Denmark). Free and total SO_2 were measured by the aspiration method (Rankine and Pocock 1970).

Organic acids by HPLC

The concentration of organic acids (citric, tartaric, malic, succinic and lactic) was determined by HPLC using an Aminex column (HPX-87H, 300 × 7.8 mm, Bio-Rad) fitted on an Agilent 1200 series quaternary pump LC (Agilent Technologies). The column was eluted at 65°C in isocratic mode with 5 mM H_2SO_4 at a flow rate of 0.5 mL/min. Samples were diluted 5-fold to a final content of 1.25% (v/v) isopropanol and 1 mM sulphuric acid, and centrifuged at 13 000 × *g* for 15 min at 4°C. The supernatant was held in an Agilent 1200 series High Performance temperature-controlled auto-sampler at 4°C prior to injection. Injection volumes were 10 µL. Organic acids were detected by absorbance at 210 nm by an Agilent 1200 series diode-array Detector.

Colour analyses

Wine colour was assessed by the tristimulus method CIELAB as described by Kwiatkowski et al. (2007).

Metal analysis

Metal contents in wines were determined by inductively coupled plasma – optical emission spectrometry performed by the Waite Analytical Services (Glen Osmond, South Australia, Australia http://www.adelaide.edu.au/was/), School of Agriculture, Food and Wine, University of Adelaide.

Experimental design for large scale experiments

Zirconia pellets were applied to wine samples in a stainless steel, 4 cm diameter tea infuser ball. Each of the three wines was treated with the infuser cages containing four doses of zirconia in pellet form (0, 5, 10, 25 g/L). The experiment was conducted at 18°C. Stirring with a magnetic bar (at 140 rpm) was applied throughout the experiment. A polypropylene container of 450 mL volume (Sarstedt, Nümbrecht, Germany) previously

tested for its relative non-permeability to oxygen for the duration of the experiment was filled with 430 mL of wine. The system was sealed with a high density polyethylene screw cap (Sarstedt) and Parafilm. Two syringe needles were inserted into the lid to allow both the sampling and maintenance of N_2 ullage (by means of a balloon). Each experiment was performed in triplicate, and analyses for each replicate were performed in triplicate except where indicated.

Regeneration experiment

Zirconia was regenerated by washing the pellets (enclosed in the metallic cage) twice with 3 M NaOH at 50°C for 2 h and then with 5% citric acid at room temperature for 30 min. The pellets in the cage were then rinsed three times in distilled water before use. An aliquot (100 mL) of 2009 Chardonnay was treated for 24 h with 25 g/L of zirconia pellets under the same conditions as described above. At the end of each treatment the pellets were regenerated and the procedure repeated.

Sensory assessment

An informal tasting was carried out to provide an indication of differences in aroma and flavour among the treatments and to highlight possible faults from the treatment. Six Australian Wine Research Institute (AWRI) tasters with extensive experience in wine sensory assessment, independently and in silence, assessed the 2009 Riesling and Chardonnay wines including untreated, bentonite-treated and zirconia-treated samples of each variety. Samples (30 mL) were poured in constant order across tasters, in coded International Organisation for Standardisation (ISO) tasting glasses. The tasters were asked to write free-choice notes about the wines' appearance, aroma and flavour, and also to indicate any perceived taints or faults. After the wines were tasted, a discussion was held.

Results and discussion

Preliminary experiments

Several exploratory experiments were undertaken to identify the most appropriate conditions to use to assess the efficiency and utility of this adsorbent for wine. Firstly, the protein removal efficiency of pellet and powder forms of zirconia was assessed by adding them (at 10 g/L) to a 2007 Semillon wine (preliminary results, data not shown). Results confirmed previous observations (Salazar 2007), with faster protein adsorption shown by the powder, especially in the first minutes of the treatment. Protein adsorption by the pellet had not reached a plateau even after 350 h. The slower protein adsorption shown by the pellet could be related to its protein binding sites being less accessible than those of the powder. This might be because of a different surface area and/or pore size of the two materials. Similar results were obtained with a 2007 unfined Riesling wine (data not shown). In the absence of stirring, no protein removal was observed even after 50 h (data not shown), indicating that circulation of wine was essential for the correct functioning of the adsorbent within a reasonable time period.

The addition of adsorbents to wine without physical restrictions has the disadvantage of subsequent removal of the adsorbents, usually by filtration or centrifugation, with the associated costs of doing so. We therefore assessed the performance of both the pellet and powder forms immobilised within a structure that allowed fluid exchange. The first experiments were undertaken by trapping both the powder and pellet forms of zirconia into a spherical 'bag' made from Miracloth and extending contact times for more than 2 days. The final protein removal of the two forms was more similar under these circumstances than under addition of the absorbent without physical restrictions (Table 1); indeed, the residual protein content was reduced by 42% by the powder and by 56% by the pellet. This might seem inconsistent with our preliminary results in which the powder worked better than the pellet, but it is possible that the longer contact time between the wine and the adsorbents meant that both forms reached the maximal binding capacity by the end of the contact period. Enclosing the adsorbent in a bag also has the advantage of easy recovery of wine through simply removing the bag rather than having to centrifuge, rack or filter the wine.

Another key parameter to investigate was the possible effect of temperature on adsorption. Therefore, an experiment was undertaken with pellets in bags at 2°C and 18°C and a shorter contact time of 18 h. Wines were more stable (Figure 1) and more protein was removed (data not shown) after treatment at 18°C than at 2°C for all treatments except the highest addition. It is possible that heat stabilisation could be achieved at 2°C with longer contact time and that the delay is because of mixing and access to the protein binding sites of the adsorbent.

We also examined whether the shape of the structure enclosing the pellets affected adsorption (Figure 2a). Over the 18-h test period, wine stabilisation (Figure 2b) occurred slightly more rapidly in a long narrow ('sausage-like') bag compared with a spherical shape, even though they contained the same amount of zirconia. This is likely because the geometry of the 'sausage' is allowing easier access of wine to all the pellets relative to a sphere. However, with contact times of 18 h, the differences in haze between the treatments disappeared. It is noteworthy that wine haze (Figure 2b) decreased more quickly when a metallic cage was used, probably because of the combined effect of having a mass of pellet through which the wine passes slowly, but a higher turnover of wine through the bigger pores of the cage compared with those of the fabric bag.

Overall, these results suggest that parameters that influence accessibility and contact between wine and the pellet are important because they drive protein adsorption. These preliminary results were used to design the operational conditions for a larger scale experiment carried out in the second part of this work.

Large scale experiment

Protein removal and heat stability. Three unfined wines from the 2009 vintage (Riesling, Chardonnay and Sauvignon Blanc) were treated with zirconia pellets enclosed in tea infusers at three different rates (see Figure 2a, shape 3). The wines were chosen because of their relatively high total protein concentration, heat instability and bentonite requirement (Table 2). To our knowledge, zirconia has never been used in wines with a protein concentration higher than 31 mg/L (Pashova et al. 2004a, Salazar 2007), that is values several fold lower than the wines under investigation in this study.

There were clear effects of dosage of zirconia and contact time on protein removal as had been observed in the preliminary experiments. The increased removal of protein with increasing dose was particularly evident at the maximal contact time for each of the wines (Figure 3). The kinetics of protein removal seemed to be wine-dependent. In fact, protein removal reached a plateau in Riesling after 48 h and in Sauvignon Blanc after only 6 h, whereas it did not appear to do so in the Chardonnay wine during the 72 h of treatment even though this latter wine contained the lowest initial total protein concentration. While the wines were similar in most of the standard compositional parameters (Table 3) it is possible that differences in colloidal components such as polysaccharides (not measured) or the proportions of the types of wine proteins present influenced the adsorption

Table 1. Effect of 65 h of contact time (with stirring) of a Semillon 2007 unfined wine treated with 10 g/L of pellet/powder enclosed in a bag made of Miracloth.

Sample	Change in turbidity after heat test (in mAU at 540 nm)	Protein content (in mg/L of thaumatin)
Semillon wine	60	102.0
Semillon wine treated with pellet	21	44.8
Semillon wine treated with powder	30	58.4

Heat test net values lower than 20 mAU indicate stability.

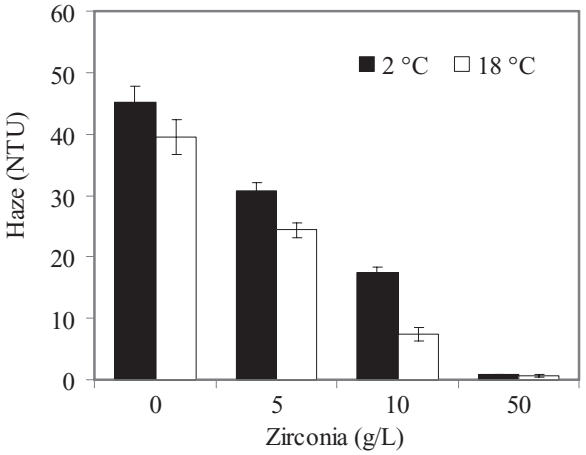

Figure 1. Effect of temperature and zirconia dose on the haze potential of a 2007 Riesling wine after 18 h of treatment with stirring. (NTU, nephelometric turbidity unit.)

Table 2. Parameters involved in wine heat instability.

	Chardonnay	Riesling	Sauvignon blanc
Initial protein content (mg/L)	100.6	195.6	138.2
Bentonite requirement (g/L)	0.9	1.6	1.7
Haze potential (NTU)†	53.4	46.3	27.4

† Change in turbidity (in NTU) following a heat test. (NTU, nephelometric turbidity unit.)

kinetics. The Chardonnay wine contained the lowest percentage of chitinases in comparison with the total proteins in the control and Sauvignon Blanc the highest (measured by HPLC, Figure 4a), yet the Sauvignon Blanc wine reached maximum protein removal (plateau) the quickest and Chardonnay the slowest (Figure 3). These results could indicate a higher affinity of zirconia towards TL proteins than chitinases. This was explored by determining protein composition of the wines by HPLC and SDS-PAGE. There was no clear evidence of preferential removal of TL proteins from the HPLC chromatogram (Figure 4a). The SDS-PAGE profiles of the wines (see data for Sauvignon Blanc in Figure 4b) confirmed that protein removal was general rather than preferential. The 20–40 mg/L of protein still found in wines

Table 3. Physicochemical parameters of wines after treatment with zirconia.

Parameter†	Chardonnay (72 h treatment)					Riesling (192 h treatment)					Sauvignon Blanc (72 h treatment)				
	Untreated wine	Control	5 g/L Zirconia	10 g/L Zirconia	25 g/L Zirconia	Untreated wine	Control	5 g/L Zirconia	10 g/L Zirconia	25 g/L Zirconia	Untreated wine	Control	5 g/L Zirconia	10 g/L Zirconia	25 g/L Zirconia
Alcohol (% v/v)	12.6	12.6	12.5	12.6	12.5	11.2	11.2	11.2	11.2	11.1	12.4	12.6	12.5	12.6	12.5
Specific gravity	0.9912	0.9912	0.9912	0.9909	0.9906	0.9933	0.9932	0.9931	0.993	0.9927	0.9909	0.9909	0.9908	0.9906	0.9903
pH	3.27	3.24	3.26	3.27	3.33	3.08	3.08	3.12	3.15	3.21	3.22	3.22	3.25	3.26	3.33
Titratable acid pH 8.2 (g/L)	6.4	6.4	6.2	6.0	5.4	6.8	6.7	6.6	6.3	5.9	6.0	6.0	5.8	5.6	5.0
Titratable acid pH 7.0 (g/L)	6.0	6.2	5.9	5.7	5.2	6.4	6.4	6.3	6.0	5.5	5.5	5.5	5.3	5.1	4.6
Glucose + fructose (g/L)	0.6	0.6	0.6	0.6	0.7	<0.27	0.4	<0.27	<0.27	0.4	0.4	0.6	0.5	0.5	0.6
Volatile acidity as acetic acid (g/L)	0.30	0.32	0.33	0.31	0.31	0.24	0.25	0.21	0.23	0.23	0.22	0.23	0.21	0.22	0.2
Sulphur dioxide (free) (mg/L)	47	42	41	40	34	21	20	19	18	16	<4	4	4	4	4
Sulphur dioxide (total) (mg/L)	145	139	137	136	129	73	76	75	72	71	41	42	42	42	40
Bentonite requirement (g/L)	0.9	0.9	0.6	0.3	0	1.6	1.6	0.9	0.5	0	1.7	1.7	1.2	0.8	0.2
Haze (NTU)	54.7 ± 0.4	53.4 ± 0.5	33.2 ± 0.8	20.5 ± 1.0	2.1 ± 0.2	52.2 ± 0.2	46.2 ± 0.9	32.7 ± 0.7	18.5 ± 0.8	1.8 ± 0.4	27.4 ± 0.4	24.0 ± 1.0	22.8 ± 0.8	21.0 ± 0.6	11.4 ± 0.3

† Data from single determinations except for haze that was done in triplicate. (NTU, nephelometric turbidity unit)

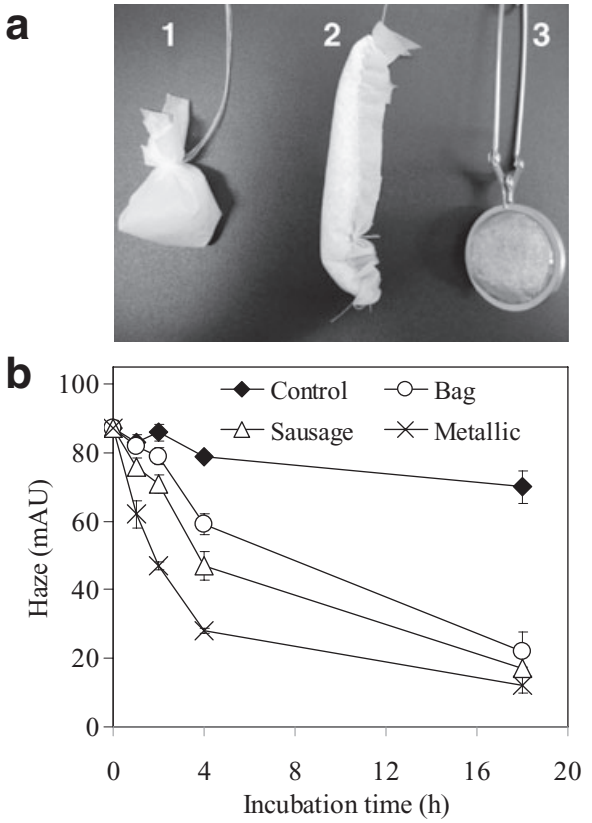

Figure 2. (a) Three bag shapes used in the experiments: (1) Miracloth spherical bag; (2) Miracloth long/narrow bag ('sausage'); and (3) metallic cage. Each bag contains the same amount of zirconia pellets. (b) Effect of bag shape and contact time on the haze potential of a 2007 Riesling wine. Wines were held at 18°C and stirred during the experiment. Each point is the average of at least three replicates ($n = 3$).

at the end of the contact time mainly had a molecular weight of 22–25 kDa, consistent with these proteins being both TL proteins and chitinases (Marangon et al. 2009, Van Sluyter et al. 2009).

A reduction in protein levels is usually accompanied by a reduction in haze potential and thus an increase in wine stability (Mesquita et al. 2001), but there have been exceptions to this observed by others (Bayly and Berg 1967, Pashova et al. 2004a). Therefore, the heat stability of wines after treatments was further assessed by subjecting the wine to a heat test and measuring changes in turbidity, while the bentonite required to stabilise the wines after the zirconia treatment was also determined (Table 3). Two of the three wines were fully stabilised with 25 g/L of zirconia. These wines contained residual protein concentrations below 21 mg/L whereas the Sauvignon Blanc wine that did not achieve stability still contained 43 mg/L protein (Figure 3). Despite not achieving stability, the 25 g/L treatment of the Sauvignon Blanc wine resulted in a decrease in the amount of bentonite required for stability from 1.7 g/L to 0.2 g/L (Table 3). These results confirm that protein removal from wines and the resulting increase in stability and decrease in bentonite requirement was proportional to the quantity of pellets used. The results also further confirmed the non-specific adsorption of proteins by this material.

Physico-chemical and metal analysis. Investigations by others have suggested that zirconia treatments have small effects on the chemical and sensorial properties of wine (Salazar et al. 2006). However, these studies relate to contact times

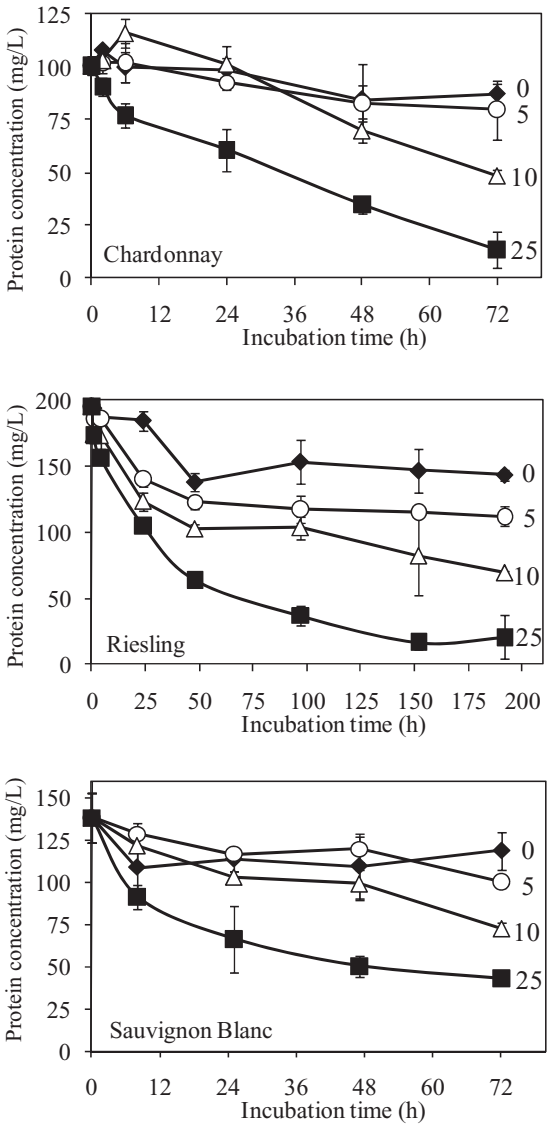

Figure 3. The effect of zirconia dose and contact time on the protein concentration of three wines. Protein removal measured by EZQ kit. Zirconia addition rates: C, 0 g/L; 5, 5 g/L; 10, 10 g/L; 25, 25 g/L. Wines were held at 18°C and stirred at 140 rpm during the experiment. Each point is the average of at least three replicates ($n = 3$). Note: incubation times and initial protein contents differ between the three wines.

between wine and zirconia that were shorter than those under investigation here. Hence, common wine compositional parameters were determined to check if other wine components were removed along with protein. In addition, metal analysis was also undertaken to determine if the zirconia released metals into the wine, even though it was high purity grade and was washed with distilled water and rinsed with wine before each experiment. The data show that only wine acidity (Table 3) and levels of Cu, Fe and Al (Table 4) were affected, and in a dose-dependant manner. In particular, a slight increase in pH and decrease in total acidity were noted, in a trend that was consistent with the increasing dosage of zirconia used.

HPLC was used to quantify organic acids in the three wines treated with increasing dosages of zirconia (Table 5). Results suggest that the reduction in total acidity (see Table 3), was probably attributable to the diminution of three acids: citric, tartaric and malic, while no differences were noticed in the levels of succinic and lactic acids. It is likely that the drop in total

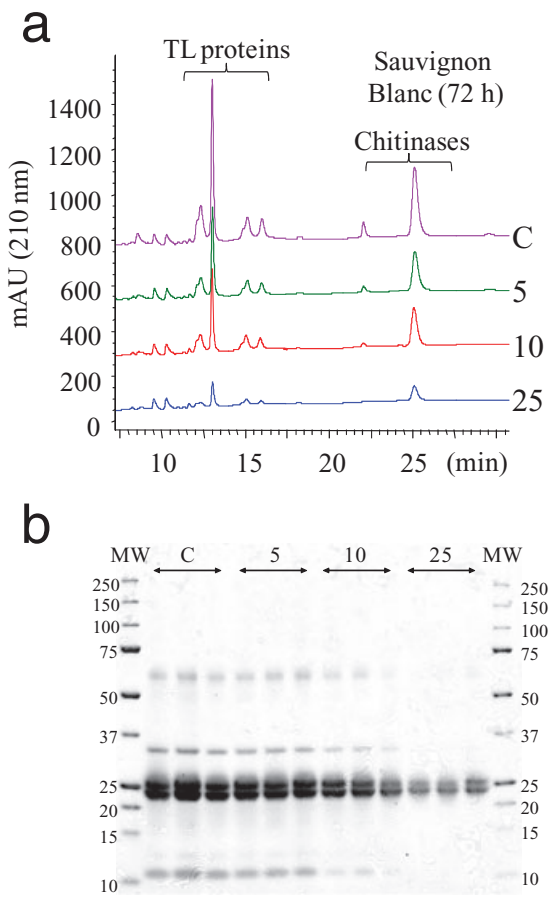

Figure 4. (a) The effect of zirconia dose on the protein composition and concentration of Sauvignon Blanc shown by reverse phase (C8) high-performance liquid chromatography chromatogram. The contact time is given in the figures. Wines were held at 18°C and stirred during the experiment. (b) The effect of zirconia dose on the protein composition of Sauvignon Blanc wine after 72 h of zirconia treatment shown by NuPAGE. Proteins from 100 µL of wine were loaded per lane. The three replicates made for each of the treatments are shown. Wines were held at 18°C and stirred during the experiment. Zirconia addition rates: C, 0 g/L; 5, 5 g/L; 10, 10 g/L; 25, 25 g/L. (MW, molecular weight in kDa.)

acidity is mainly because of the diminished content of tartaric acid, even if, in terms of percentage, it is the citric acid that is removed most.

Wine colour differences because of the treatment were determined by CIELAB. The values ΔE^*_{ab}, a measure of differences in colour between samples was calculated for pairs of treatments (Table 6). Pairs with values of ΔE^*_{ab} greater than 1 are likely to be detected as different by the human eye (Kwiatkowski et al. 2007). The treatments with increasing dosages of zirconia modified the colour in a way that seems proportional to the dosage utilised, but none of the treated wines could potentially be detected as different from the respective controls by the human eye (Table 6).

Altogether, these results demonstrate that long contact times with high doses can lower the acidity of the wine and affect the metal ion composition. Preliminary results indicate that shorter treatments would result in less drastic acidity diminutions (not shown).

Sensory assessment. An informal sensory evaluation of zirconia-treated Riesling and Chardonnay wines was conducted to assess whether the zirconia treatment had any sensory effect.

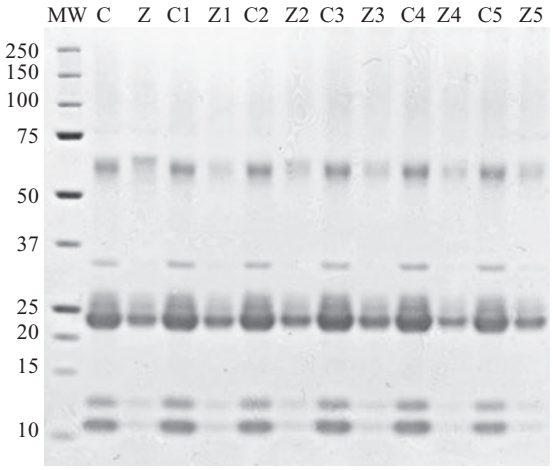

Figure 5. The effect of zirconia regeneration on the protein content of treated wines (Z) versus controls (C), as shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The numbers refer to the number of regenerations the pellets had undergone. (MW, molecular weight in kDa.)

Previous studies (Salazar et al. 2006, Salazar 2007) have indicated that the treatment had no influence on the sensory properties of the treated wine, but the contact times between wine and zirconia were shorter than those under investigation here.

For both wines several tasters commented on lower perceived acidity for the zirconia-treated samples, consistent with the approximately 1 g/L difference in titratable acidity between the control and zirconia treated wines.

For the Chardonnay wine, the judges were not able to easily discriminate among the wines. There was some indication of a low-level rubbery, sulfide-related aroma for this sample. The Riesling wine treated with zirconia exhibited a stronger sulfide character, described as cabbagey or rubbery. The sulfide-related aroma does not appear to be because of zirconia itself adding a taint to the wine as analysis of model wines left in contact with zirconia showed an absence of thiol and other sulfur compounds commonly associated with sulfide off-flavour in wines (data not shown). The development of these sulfide characters in wines is often associated with reductive conditions (Kwiatkowski et al. 2007, Lopes et al. 2009) and it is possible that the removal of copper and iron (see Table 5) by the treatment and subsequent storage of the wine in anaerobic conditions could have resulted in an environment in which these characters were more likely to develop.

Zirconia regeneration. One particularly interesting characteristic of zirconia as an adsorbent is its ability to be regenerated. It was previously reported that regeneration could be achieved by heating at 500°C for 12 h (Salazar et al. 2007), but this type of procedure is unlikely to be commercially applicable. We therefore assessed the ability of cleaning products commonly available in most cellars and wineries, such as citric acid and NaOH, to regenerate the material.

Zirconia pellets enclosed in tea infusers were repeatedly exposed to wine and then regenerated by treatment with NaOH and citric acid. Results showed that the pellets maintained their ability to remove protein for at least 11 regenerations. Protein composition in treated wines for pellets regenerated six times is shown in Figure 5. The ability of the zirconia to be regenerated was confirmed by the heat stability of wines treated with zirconia that had undergone up to 11 regenerations without a

Table 4. Metal concentration of wines† after treatment with zirconia

Metal (mg/L)‡	Chardonnay 72 h				Riesling 192 h				Sauvignon Blanc 72 h			
	Control	5 g/L Zirconia	10 g/L Zirconia	25 g/L Zirconia	Control	5 g/L Zirconia	10 g/L Zirconia	25 g/L Zirconia	Control	5 g/L Zirconia	10 g/L Zirconia	25 g/L Zirconia
Fe	0.58	0.58	0.08	0.03	1.20	0.52	0.15	0.55	0.34	0.13	0.11	0.04
Mn	0.73	0.73	0.64	0.55	1.60	1.64	1.60	1.30	0.50	0.45	0.43	0.36
B	6.00	5.97	5.89	5.40	4.90	4.94	4.86	4.40	2.52	2.45	2.42	2.28
Cu	0.11	0.11	0.07	0.03	0.12	0.10	0.16	<0.03	0.08	0.06	0.05	<0.03
Zn	0.51	0.51	0.48	0.42	1.30	1.27	1.31	1.00	1.16	1.08	1.02	0.85
Ca	60.00	59.86	61.14	60.00	89.00	91.02	85.96	87.00	61.97	63.20	61.58	61.03
Mg	107.00	107.32	108.83	105.00	95.00	96.51	93.23	93.00	94.65	95.69	96.04	94.43
Na	68.00	67.80	76.32	84.00	41.00	42.57	40.63	47.00	21.15	24.59	26.30	37.96
K	400.00	400.00	400.00	390.00	410.00	420.00	430.00	400.00	490.00	500.00	500.00	490.00
P	114.00	113.65	69.71	32.00	135.00	109.03	133.95	32.00	157.24	132.05	107.42	53.60
S	84.00	84.07	83.94	79.00	111.00	112.15	115.71	105.00	59.86	58.04	57.19	55.08
Al	0.08	0.08	0.03	0.03	0.09	0.04	0.09	0.03	0.06	0.03	0.02	0.01
Cr	<0.04	<0.04	<0.04	<0.04	0.08	0.05	<0.04	0.09	<0.04	<0.04	<0.04	<0.04

† Data from single determinations. ‡Mo, Co, Ni, Ti, Cd, Pb and Se were not included in the table because their contents were below the detection limit (respectively 0.06, 0.06, 0.07, 0.009, 0.04, 0.2, 0.6 mg/L).

Table 5. Organic acid concentration† by HPLC of wines after treatment with zirconia.

	Zirconia (g/L)	Citric (g/L)	Tartaric (g/L)	Malic (g/L)	Succinic (g/L)	Lactic (g/L)	Total Acidity (g/L)
Chardonnay after 72 h	0	0.19	2.92	2.32	2.07	0.14	7.65
	5	0.19	2.96	2.39	2.14	0.14	7.83
	10	0.15	2.82	2.31	2.10	0.14	7.53
	25	0.13	2.61	2.23	2.11	0.15	7.24
Riesling after 197 h	0	0.11	4.17	1.46	2.43	0.25	8.43
	5	0.11	4.08	1.45	2.42	0.25	8.30
	10	0.10	3.96	1.35	2.34	0.24	7.99
	25	0.09	3.71	1.36	2.41	0.25	7.82
Sauvignon Blanc after 72 h	0	0.20	3.98	1.40	2.26	0.27	8.11
	5	0.19	3.66	1.35	2.11	0.24	7.55
	10	0.19	3.41	1.38	2.17	0.24	7.39
	25	0.17	3.03	1.32	2.18	0.25	6.95

† Data from single determinations.

Table 6. Mean CIELAB ΔE^*_{ab} values for wines treated with increasing dosages of zirconia.

Treatments	ΔE^*_{ab}†		
	Chardonnay	Riesling	Sauvignon Blanc
Control vs 5 g/L zirconia	0.3	0.3	0.3
Control vs 10 g/L zirconia	0.5	0.7	0.4
Control vs 25 g/L zirconia	0.7	0.9	0.8

† $\Delta E_{ab}^* = \sqrt{\left[(L1-L2)^2 + (a1-a2)^2 + (b1-b2)^2\right]}$.

noticeable decrease in efficiency (the average NTU value of 11 regenerations was 10.5 and the value of the last regeneration was 9.1 NTU). It is possible that the pellets may have continued to perform after further regeneration, because no evidence of reduced protein absorption capacity was detected up to 11 regenerations. However, small physical losses of the material could eventually result in reduced performance.

Conclusions

Zirconia was confirmed to be an excellent candidate for protein adsorption from wines. The proposed modification of its application to wine (pellets enclosed in a metallic cage) represents a step forward in the adoption of this material commercially. Other immobilisation possibilities may be more suitable to larger scale wine production than the method used here, and this should be further explored. This study has also demonstrated that regeneration of the material can be relatively simple. Its ability to reduce acidity and metal ion concentration may be an additional advantage for wine production and could also be potentially exploited by other industry sectors.

One of the main drawbacks of the zirconia treatment seems to be the necessity of stirring and relatively high dosage levels. The easy regeneration process somewhat reduces the negative aspects of the requirements for high doses, because the material can be reused many times. However, the physical properties of

the zirconia used in this study were not optimised. It is possible that, if optimised, the addition rates and contact times could be significantly reduced. This issue of the need for stirring could be overcome by treating the wines when agitation occurs for other reasons, such as during fermentation. Preliminary results indicate this solution to be both feasible and promising.

Acknowledgements

This work was supported financially by Australia's grapegrowers and winemakers through their investment body the Grape and Wine Research and Development Corporation, with matching funds from the Australian Government. The AWRI is a member of the Wine Innovation Cluster located in Adelaide. The work of Marco Lucchetta is part of the research activity of the 'Dottorato di Ricerca in Viticoltura, Enologia e Marketing delle imprese vitivinicole' supported by Provincia di Treviso (Italy) and it has been supported in part by the Fondazione Ing. Aldo Gini. Leigh Francis, Patricia Osidacz and Belinda Bramley are thanked for their help with the sensory evaluation and the sensory panellists for assessing the wines. We thank Maurizio Ugliano and Tracey Siebert for sulphur compounds analysis, Steve Van Sluyter for helpful discussions regarding this work and Antonio Garcia Cordente and Cristian Varela for assistance with organic acid analysis. Francisco López, Universitat Rovira i Virgili (Spain) is thanked for donating the zirconia and Vanessa Stockdale, Foster's Group, Australia for donating wines.

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Manuscript received: 11 January 2010
Revised manuscript received: 15 July 2010
Accepted: 3 August 2010

Analytica Chimica Acta 660 (2010) 110–118

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Heating and reduction affect the reaction with tannins of wine protein fractions differing in hydrophobicity

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ARTICLE INFO

Article history:
Received 18 August 2009
Received in revised form 14 October 2009
Accepted 19 October 2009
Available online 27 October 2009

Keywords:
Wine
Thaumatin-like proteins
Chitinases
Haze
Tannin
Hydrophobicity

ABSTRACT

During the storage, bottled white wines can manifest haziness due to the insolubilisation of the grape proteins that may 'survive' in the fermentation process. Although the exact mechanism of this occurrence is not fully understood, proteins and tannins are considered two of the key factors involved in wine hazing, since their aggregation leads to the formation of insoluble particles. To better understand this complex interaction, proteins and tannins from the same unfined Pinot grigio wine were separated. Wine proteins were then fractionated by hydrophobic interaction chromatography (HIC). A significant correlation between hydrophobicity of the wine protein fractions and the haze formed after reacting with wine tannins was found, with the most reactive fractions revealing (by SDS-PAGE and RP-HPLC analyses) the predominant presence of thaumatin-like proteins. Moreover, the effects of both protein heating and disulfide bonds reduction (with dithiotreithol) on haze formation in the presence of tannins were assessed. These treatments generally resulted in an improved reactivity with tannins, and this phenomenon was related to both the surface hydrophobicity and composition of the protein fractions. Therefore, haze formation in wines seems to be related to hydrophobic interactions occurring among proteins and tannins. These interactions should occur on hydrophobic tannin-binding sites, whose exposition on the proteins can depend on both protein heating and reduction.

1. Introduction

Winemakers constantly need to deal with the possible appearance of turbidity during the storage of white wines after bottling. This occurrence can be caused by the insolubilisation of the grape proteins which remain in wine after the fermentation process [1,2]. These proteins are pathogenesis-related (PR) proteins, specifically thaumatin-like proteins and chitinases [3] that tend to aggregate during wine storage, resulting in the formation of light-dispersing particles that, above certain dimensions, can be visually detected as haze [1,2]. Turbid wines are not dangerous for consumption. However they are unattractive for consumers that tend to reject them. This has important economic consequences for the producers. Nowadays, the mechanism of protein haze formation is not fully understood despite the big efforts done worldwide on this topic. It is known that the protein hazing is a process dependent on the presence of both proteins and non-proteinaceous wine components (factor X), among which the sulfate anion has been proposed as the most likely candidate for the missing essential factor [4]. It is believed that a slow denaturation leads to wine protein aggregation, with their consequent flocculation into a hazy suspension and, finally, formation of precipitates [5,6].

The native condensed tannins of wine (flavan-3-ol oligomers and polymers) play a relevant role in this process. Tannins are water-soluble phenolic compounds with molecular weights of 500–3000 Da, showing a high tendency to bind proteins, especially those with high proline content [7–9]. Because of their ability to precipitate salivary proteins [10–12], tannins are responsible for wine astringency [13]. In addition, tannins are responsible for several phenomena occurring, including protein haze formation in alcoholic beverages [14,15] and this feature has been tentatively exploited as a tool for the protein removal from wines [16,17].

Wine protein reactivity with endogenous grape tannins has been extensively studied, although a precise characterisation of the behaviour of the single wine protein components is scant [14,18]. Several studies suggest that hydrophobic bonding may be the major mode of interaction between condensed tannins and proteins. Oh

Abbreviations: TL-proteins, thaumatin-like proteins; PR-proteins, pathogenesis-related proteins; HIC, hydrophobic interaction chromatography; DTT, DL-dithiotreithol; PAS, Periodic Acid-Schiff stain; MW, molecular weight; MWCO, molecular weight cut off; *Mr*, relative mobility.

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doi:10.1016/j.aca.2009.10.038

et al. [19] studied the interaction in tannin–protein complexes, concluding that the dominant mode was the hydrophobic interaction rather than hydrogen bonding as previously supposed. Siebert et al. [20] confirmed this statement, showing that hydrogen bonding is not as important as hydrophobic interactions are in determining the reactivity between proteins and polyphenols.

To assess if hydrophobic interactions are involved in protein–tannin reactivity in wine, and thus haze formation, in this work wine proteins have been fractionated according to their hydrophobicity and the obtained fractions analyzed in order to elucidate the relation between wine protein hydrophobicity and their aptitude to develop haze in the presence of tannins. Moreover, some of the conditions which can be involved in the exposure of hydrophobic tannin-binding sites on wine protein were investigated by studying the effect of both proteins heating and reduction on haze formation in the presence of wine tannins.

2. Experimental

2.1. Materials

Proteins were purified from (unfined) Pinot grigio wine (vintage 2006) produced in Conegliano (Italy). The model wine was prepared with 5 g L^{-1} tartaric acid (Baker, Milan), 12% ethanol buffered to pH 3.20 with NaOH. The reducing agent was DL-dithiotreithol (DTT, Fluka, Milan).

2.2. Wine total macromolecules preparation

After wine sterile filtration with cellulose acetate filters (pore size of 0.20 μm, Millipore, Milan), wine macromolecules were concentrated by ultrafiltration (Stirred Cell System, Amicon, Milan) through 3 kDa MWCO membranes. Retentates were dialyzed against distilled water in 3.5 kDa dialysis bags (Spectra/Por3, Spectrum, Los Angeles) and passed on Solid Phase Extraction (SPE) C-18 cartridges (1 mL resin, Supelco, Bellefonte) to remove residual polyphenols from the protein extract [5]. Obtained preparations were then frozen and freeze dried.

2.3. Wine total tannin preparation

Total wine polyphenols were purified by passing the 3 kDa ultrafiltered wine (obtained during the macromolecules concentration step), formerly diluted (1:1) with distilled water acidified with 0.1% (v/v) trifluoroacetic acid, on SPE C-18 cartridge (1 mL resin, Supelco) and eluted with methanol. After solvent evaporation, the sample was dissolved in water before being freeze dried. The obtained powder was re-dissolved in 80% ethanol and tannins purified through a passage onto a glass column (400 mm × 24 mm) containing 30 g of Sephadex LH-20 resin (Sigma, Milan). After washing the column with 2 column volumes of 80% ethanol, tannins were eluted with 60% acetone. Acetone was then evaporated from the tannin solution (at 40 °C by Büchi Rotavapor R-114), and the resulting solution frozen and freeze dried.

2.4. Protein content determination

Proteins were precipitated from 1 mL of wine using the KDS method [21]. The pellets were then dissolved in 1 mL of distilled water and quantified using a BCA-200 protein assay kit (Pierce, Rockford) according to the manufacturer's instructions. The calibration curve was prepared by using a serial dilution of bovine serum albumin (BSA, Sigma) in water.

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoretic analyses were performed according to Laemmli [22] in a Mini-Protean III apparatus (Bio-Rad, Milan) with T = 14% (acrylamide/N,N′-metylen-bisacrylamide 29/1; Fluka) gels. Gels were alternatively stained with the Silver stain [23] or Periodic Acid-Schiff (PAS) stain procedures [24].

2.6. Heat test

Solutions in ultrafiltered (3.5 kDa MWCO) and model wine of both the total proteins (at 75 mg L^{-1}) and HIC fractions (at 75 mg L^{-1}) were heated at 80 °C for 6 h and placed at 4 °C for 18 h. After equilibration with the room temperature (25 °C), the haze was measured by calculating the difference between absorbance values at 540 nm obtained after and before heating (A_{540} after − A_{540} before) [6,25].

2.7. Total polysaccharide content determination

The polysaccharide content was determined colorimetrically according to Segarra et al. [26].

2.8. Total tannins content determination

The total tannins content was determined colorimetrically according to Bate-Smith [27] and Bourzeix [28].

2.9. Chromatography

Chromatographic separations were performed with both a low pressure system and an analytical HPLC. The low pressure system was an ÄKTA purifier FPLC (GE-Healthcare, Milan) fitted with an UV detector. Data were processed by the Unicorn 5.11 software (GE-Healthcare). For analytical HPLC separations, a Waters 1525 HPLC (Waters, Milan) equipped with a Dual λ Absorbance Detector (Waters 2487; Waters) and a Refractive Index detector (Waters 2414; Waters) was used. Data were analysed by the Waters Breeze™ Chromatography Software (Version 3.30).

2.9.1. Anion exchange chromatography (AEC)

In order to separate proteins from neutral polysaccharides, the total Pinot grigio wine macromolecules (≈500 mg of freeze dried wine macromolecules) were dissolved in 20 mM Tris–HCl pH 8.5 and loaded onto a Resource™ Q column (Amersham Biosciences, Uppsala) equilibrated with the same buffer at a flow rate of 1 mL min^{-1}. Bound proteins were eluted with a step gradient of NaCl (0–1 M) in 1 min. Unbound and total protein peaks were collected, dialyzed against water and freeze dried.

2.9.2. Analysis and quantification of proteins by RP-HPLC

The protein composition and concentration of wine AEC-FPLC fractions was determined by reversed phase (RP) HPLC. Samples (100 μL) were loaded at 1 mL min^{-1} onto a C18 column (4.6 mm × 250 mm, Vydac 218 MS 54, Hesperia, CA, USA) fitted with a C18 guard column (Vydac 218 MS 54, 4.6 mm × 5 mm, Hesperia, CA, USA) equilibrated in a mixture of 83% (v/v) solvent B [8% acetonitrile, 0.1% trifluoroacetic acid (TFA)] and 17% solvent A [80% acetonitrile, 0.1% (v/v) TFA] and held at 35 °C. Proteins were eluted by a gradient of solvent A from 17% to 49% in the first 7 min, 49% to 57% from 7 to 15 min, 57% to 65% from 15 to 16 min, 65% to 81% from 16 to 30 min and then held at 81% for 5 min before re-equilibrating the column in the starting conditions for 6 min [29,30]. Protein elution was followed by absorbance at 220 nm. Peaks with a retention time between 8.9 and 10.7 min were

assigned to the TL-protein classes, whereas peaks eluted from 19.5 and 24.5 min were assumed to be chitinases [29,30].

2.10. Hydrophobic interaction chromatography (HIC)

Proteins obtained by AEC were fractionated according to surface hydrophobicity [30]. The protein fraction obtained by AEC was dissolved in eluant A (50 mM sodium phosphate, 1.25 M ammonium sulfate, pH 5.0) and loaded at 0.7 mL min^{-1} into a BioSuite™ Phenyl 10 μm HIC 7.5 mm × 75 mm column (Waters). Eluant B was 50 mM sodium phosphate, pH 5.0. After sample loading, proteins were eluted in 30 min by a linear gradient of decreasing ammonium sulfate (from 100% eluant A to 100% eluant B). The conductivity of the buffer at elution's time of each peak was used as an index for protein surface hydrophobicity. In fact, the more hydrophobic the molecule, the less salt needed to elute the proteins. Collected fractions were pooled, dialyzed and freeze dried.

2.11. Statistical analyses

Data were analyzed by one-way completely randomized ANOVA with CoHort Software (CoStat, version 6.311) and data significance assessed by Student–Newman–Keuls test. Each measure was the result of at least three replicates.

2.12. Experimental design

Total Pinot grigio molecules >3 kDa were concentrated by ultrafiltration and total proteins recovered by AEC. Afterwards, total proteins were fractionated by HIC in six main fractions. The reactivity of tannins with proteins was assessed by dissolving the total and the 6 (HIC) protein fractions in model or ultrafiltered (on 3 kDa MWCO membrane) Pinot grigio wine at the same concentration (75 mg L^{-1}). Each experiment was conducted by measuring the turbidity (A_{540}) formed following different treatments immediately after the addition of tannins (at 50 mg L^{-1} of final concentration).

3. Results and discussion

3.1. Protein fractionation

Total Pinot grigio wine macromolecules were fractionated by AEC (Fig. 1) in an unbound fraction (flow through) containing 6.20% (w/w) of protein, and a retained fraction composed of 82.65% (w/w) of protein.

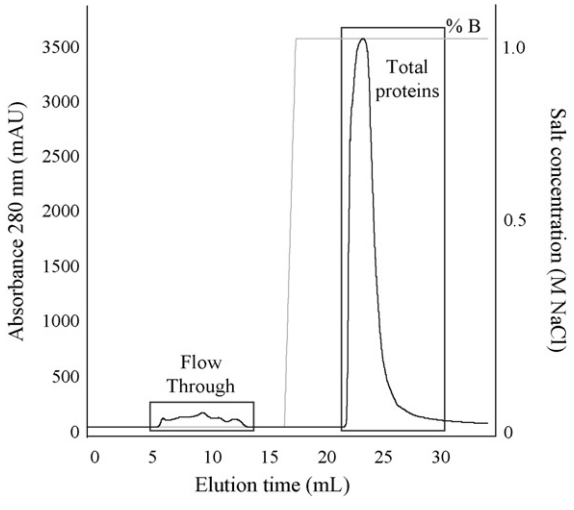

Fig. 1. Fractionation of the wine macromolecules (>3 kDa) (≈500 mg of freeze dried powder) by anion exchange chromatography (AEC).

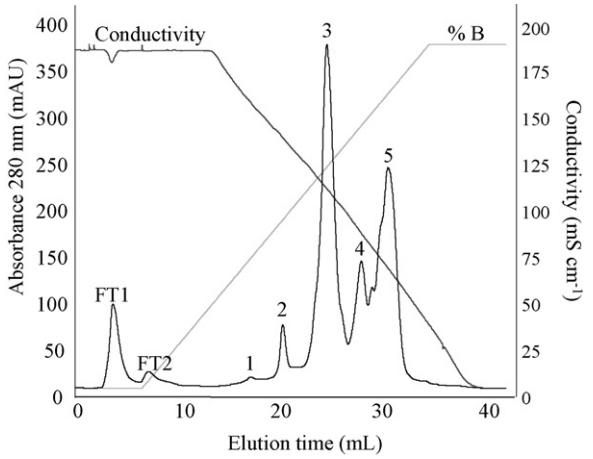

Fig. 2. Fractionation by hydrophobic interaction chromatography (HIC) of the protein peak (50 mg of freeze dried powder) obtained by AEC of wine macromolecules (see Fig. 1). Numbers indicate the collected fractions.

Table 1
Percentage on the total peak area and conductivity (used as a putative index of protein surface hydrophobicity) of the fractions obtained by HIC of wine proteins. FT1, FT2, 1–5 indicate protein fractions as obtained by HIC separation shown in Fig. 2.

HIC fraction	Peak area (% on the total)	Conductivity (mS cm^{-1})	Putative protein surface hydrophobicity
FT1	7.91	181.7	Low
FT2	1.93	187.8	Low
1	0.94	161.1	Low-medium
2	5.05	142.9	Low-medium
3	40.59	115.6	Medium
4	13.24	92.2	Medium-high
5	30.34	72.9	High

This latter peak was subjected to hydrophobic interaction chromatography (HIC), thus obtaining protein fractions differing in surface hydrophobicity (Fig. 2 and Table 1). By combining the corresponding peaks obtained from 8 chromatographic runs (each starting from 50 mg of protein), sufficient amounts of (freeze dried) proteins to characterize protein–tannin reactivity were obtained (Table 2). The protein concentration of the HIC fractions was tentatively calculated by subtracting the polysaccharide content from the total weight of each fraction (Fig. 3). Residual polysaccharides were mainly located in the unbound fraction FT1. This result indicated the presence of charged polysaccharides, previously retained by the AEC column. Because of the almost complete absence of protein in fraction FT1, this fraction was not tested in the following experiments. In contrast, the protein fractions obtained during the application of the HIC gradient were almost devoid of polysaccharides (apart for small residues at the beginning of the gradient),

Table 2
Weight distribution (in mg of dry weight and percentage) of the freeze dried powders deriving from the fractions obtained by HIC of wine proteins. FT1, FT2, 1–5 indicate protein fractions as obtained by HIC separation shown in Fig. 2.

HIC fraction	Total weight (mg)	% on the total weight
FT1	101	59.0
FT2	7.2	4.2
1	8.8	5.1
2	6.6	3.9
3	24.5	14.3
4	5.2	3.0
5	18	10.5
Total	171.3	100.0

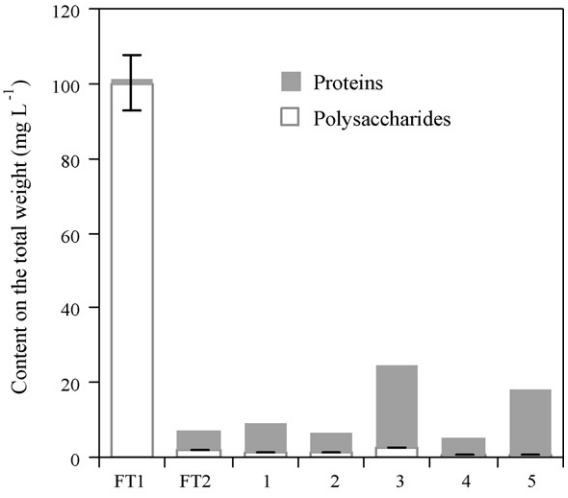

Fig. 3. Protein and polysaccharide distribution on the total weight of each HIC fraction. FT1, FT2, 1–5 indicate protein fractions as obtained by HIC separation shown in Fig. 2.

allowing to exclude the possible interference of polysaccharides in the study of haze formation.

HIC fractions were analysed by SDS-PAGE (Fig. 4). PAS staining (Fig. 4b) confirmed that fraction FT1 was the richest in polysaccharides, with residual amount of high MW sugars detectable also in fractions FT2, 1 and 2.

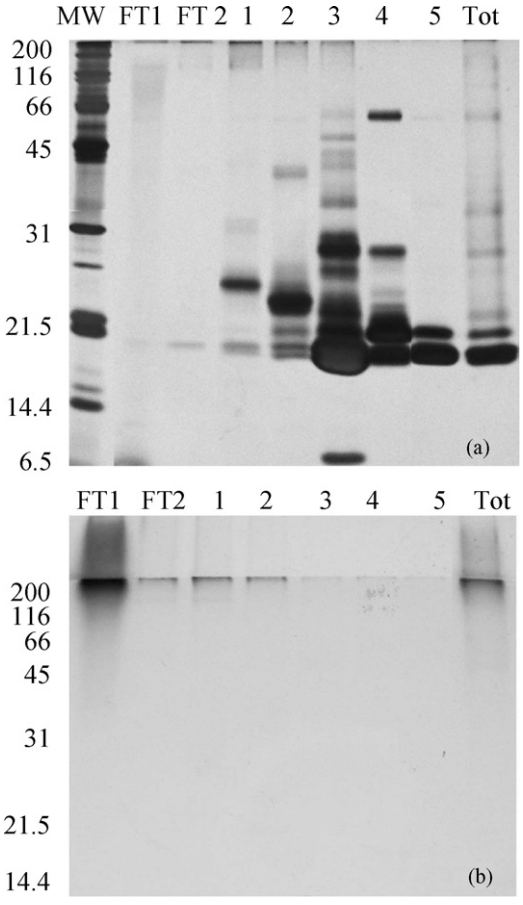

Fig. 4. SDS-PAGE (T = 14%, C = 3%) in non-reducing conditions of HIC fractions. Gels were stained for total protein (a) and sugars (b). MW standard proteins are on the left of each gel (lanes MW). Non-fractionated wine proteins are on the right lanes of each gel (lanes Tot). FT1, FT2, 1–5 indicate protein fractions as obtained by HIC separation shown in Fig. 2.

While FT1 and FT2 almost lacked protein in silver stained gels, all the other HIC fractions were shown to contain several protein bands, differing in both relative mobility (*Mr*) and staining intensity. A protein with apparent MW of ≈65 kDa (possibly invertase) was detected only in fraction 4, while other protein bands with similar *Mr* were present in more than one fraction (Fig. 4a). In particular, bands with apparent MWs between 24 and 30 kDa were detectable in fractions 1–4. The *Mr*s of these bands corresponded to those of the grape chitinases [31,32]. Moreover, with the exception of FT1, all fractions (but mainly fractions 3–5) comprised bands at 18–21 kDa, which were the sole proteins present in fraction 5. It is generally recognised that the wine proteins with these MWs belong to the thaumatin-like (TL) proteins of grapes, which can be present in different isoforms [29,30,33]. The general trend observed for protein elution from the HIC column confirmed that TL-proteins are the most hydrophobic among wine proteins [30]. Moreover, the presence of TL-proteins in fractions with different hydrophobicity, corresponding to differences in HIC retention times, indicated the possibility of different functional properties of the individual TL-protein isoforms.

The results of SDS-PAGE were confirmed by RP-HPLC analysis of the HIC fractions (Table 3), which showed that the peaks matching to different TL-proteins isoforms (eluted from 8.9 to 10.7 min) [29,30] were contained in the three most hydrophobic fractions (3–5). Moreover, RP-HPLC results showed that chitinases were mainly contained in HIC fraction 1 and 2, while quantities of this class of proteins were detected also in fractions 3 and 4 (Table 3).

3.2. Reactivity between wine tannins and protein fractions differing in hydrophobicity

To study the reaction among proteins and tannins taking place during the storage of white wines, and in particular to establish the relationship existing between protein hydrophobicity and reactivity with tannins, a series of experiment were set up using proteins and tannins purified from the same Pinot grigio wine. The protein–tannin ratio was selected on the basis of literature data [34] and preliminary tests. A 'realistic' dose of 50 mg L^{-1} of total wine tannins was used in each experiment. By testing this dosage with total wine proteins (at 75 mg L^{-1}), a detectable haze was achieved in both ultrafiltered and model wines.

Protein–tannin reactivity of the different HIC fractions was then studied. Total and fractionated proteins did not give haze when heat-tested alone in model wine (Fig. S-1), confirming the necessity of other "factors" to allow haze formation [4].

The turbidity produced at 25 °C (Fig. 5) and after heating the mixture (heat test) (Fig. 6) was taken as a measure of protein–tannin reactivity [35]. With the amounts of proteins and tannins used, measurable haze was produced at room temperature, indicating that the aggregation of these compounds was very rapid. By comparing the behaviour of the different HIC fractions in these conditions, a linear trend indicating the increase in haze formed according to the surface hydrophobicity of the HIC fractions in model wine was noticed (Fig. 5b, grey line). In contrast, the reaction in the same conditions but in ultrafiltered wine (Fig. 5b, black line), which gave a lower haze level, did not follow a similar trend, suggesting that turbidity was unrelated to protein hydrophobicity in this case. This might be due to a change in protein–tannin reactivity in the presence of low MW (<3 kDa) compounds in the real wine.

When the reaction was done in the conditions of the heat test a relevant increase in turbidity was noted. In this case, however, the correlation between protein hydrophobicity and reactivity with tannins became significant not only for the samples dissolved in model wine, but also for those in ultrafiltered wine (Fig. 6b). Indeed, heating could modify the behaviour of proteins by exposing addi-

Table 3
Distribution of RP HPLC protein peaks of the HIC fractions (calculated as percentage of the total peak area of each fraction). FT1, FT2, 1–5 indicate protein fractions as obtained by HIC separation shown in Fig. 2.

Retention time	HIC fractions (% on total peak area)							
	Total protein	FT1	FT2	1	2	3	4	5
4.4	0.8		93.2					
4.9					32.7			
7.3	0.4	80.9			1.0			
7.7	3.0				3.6	9.1		
8.3	1.2							
TL-proteins								
8.9	50.9	5.0			13.8	86.9	32.6	1.7
9.9	2.4						11.5	4.6
10.4	11.2					0.4	41.0	20.4
10.7	24.1		2.5					72.2
Chitinases								
19.5					0.5			
20.0	6.0	10.9	3.7	81.8	42.8	3.5	14.9	1.2
22.8					0.2			
25.4		3.2	0.6	18.2	5.4			
Total (%)	100	100	100	100	100	100	100	100

tional reactive sites, initially buried in the hydrophobic protein core, and also increases the strength of hydrophobic interactions [36]. It is noteworthy that, for the three most hydrophobic fractions, the extent of the reaction in ultrafiltered wine was higher than that observed in model wine. This could be due to the effect of low MW compounds, including residual tannin molecules and the sulfate ion, already indicated as the "factor X" that might activate the mechanism of protein–tannins interaction [4].

HIC fraction 1 gave no haze and thus appeared to be stable after heating in both model and ultrafiltered wines, despite it being shown to contain proteins, identified as chitinases (Table 3 and Fig. 4), which are well known for their heat instability [37]. This event could be due to the stabilising action of residual polysaccharides detected in this fraction (Figs. 3 and 4) [38,39]. However, another possibility for the low reactivity of this fraction with tannins is related to the low surface hydrophobicity of its proteins, as determined by their early elution from the HIC column.

In contrast, the level of 0.02 AU, which is considered the threshold for wine protein instability after the heat test [3] was exceeded for the total wine proteins and HIC fractions 2–5, with an almost linear relation existing between hydrophobicity and turbidity (Fig. 6b).

Therefore, the idea that hydrophobic interactions are involved in protein–tannin complexation and that the level of protein

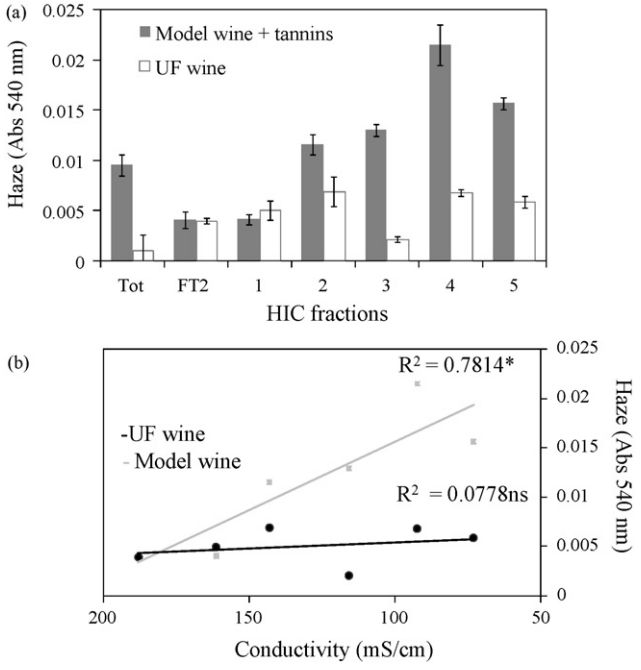

Fig. 5. (a) Turbidity (A_{540}) developed at room temperature (25 °C) immediately after mixing wine proteins (total and HIC fractions at 75 mg L^{-1} final concentration) with wine tannins (at 50 mg L^{-1}) in model (grey bars) and ultrafiltered (UF, 3 kDa, white bars) wines. Bars with different letters are significantly different at $P \leq 0.01$ (Student–Newman–Keuls test). (b) Correlation between hydrophobicity (expressed as conductivity) of the HIC fractions and the haze formed at room temperature in the presence of tannins. FT1, FT2, 1–5 indicate protein fractions as obtained by HIC separation shown in Fig. 2.

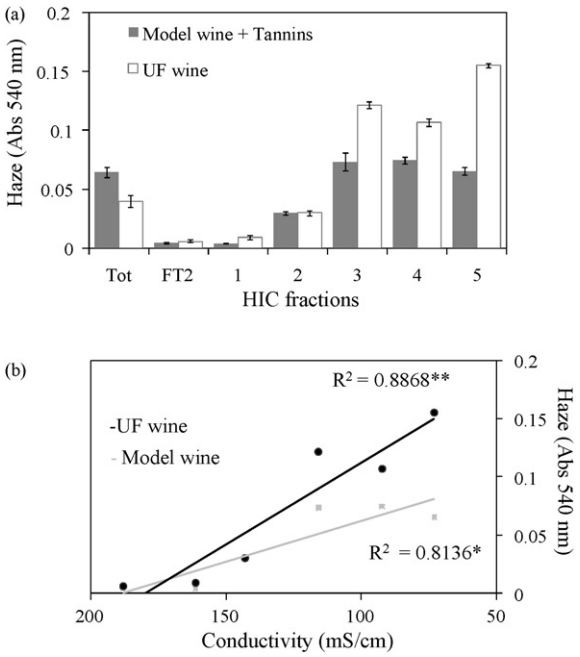

Fig. 6. (a) Turbidity (A_{540}) developed after the heat test of the same samples of Fig. 5. Bars with different letters are significantly different at $P \leq 0.01$ (Student–Newman–Keuls test). (b) Correlation between hydrophobicity (expressed as conductivity) of the HIC fractions and the haze formed after the heat test in the presence of tannins. FT1, FT2, 1–5 indicate protein fractions as obtained by HIC separation shown in Fig. 2.

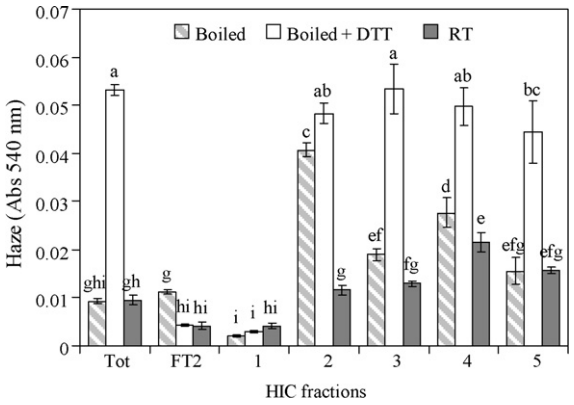

Fig. 7. Turbidity developed after the reaction at room temperature (25 °C) of wine tannins (50 mg L^{-1}) added at room temperature to mixtures of wine proteins (total and HIC fractions) dissolved at 75 mg L^{-1} in model wine. Wine proteins were not heated (■) or previously boiled (15 min, 100 °C) in the absence (▨) or presence (□) of 420 mM DTT. Bars with different letters are significantly different at $P \leq 0.01$ (Student–Newman–Keuls test). FT1, FT2, 1–5 indicate protein fractions as obtained by HIC separation shown in Fig. 2.

hydrophobicity affects the reactivity with tannins [19,20], seems to be confirmed also for the tannin–protein system of white wine.

3.3. Effect of protein heating and reduction on reactivity with tannins

Bottled white wine stability is affected by the conditions of storage, which are mainly related to temperature fluctuations. Moreover, a regular decrease of the redox potential, promoted by temperature and light [40], occurs in the bottle. A combination of a more reducing environment and heat will aid in the loss of protein tertiary structure, resulting in the exposure of additional hydrophobic sites on the proteins and increased accessibility of these sites to tannins [20], leading to turbidity [18,41].

Significant haze formation by wine proteins and tannins in model wine can be obtained only when the mixture is heated (compare the results of Figs. 5 and 6). On the contrary, heating (at 100 °C) the wine proteins before the addition of tannins, performed at room temperature (25 °C), resulted in turbidity values similar to those obtained without heating (Fig. 7). Therefore, it seems that the exposure of some additional tannin-binding sites on the wine proteins caused by heat is a temperature-dependent reversible process, involving protein unfolding (at high temperature)/refolding (after cooling down). However, the process of exposition of tannin-binding sites could be potentially hindered by the presence of intramolecular disulfide (S–S) bonds stabilising the protein structure.

These aspects were studied as follows: total and HIC-fractionated wine proteins (Fig. 2) were dissolved in model wine in the presence or absence of the reducing agent DTT and then heated. After cooling the mixtures at room temperature, purified wine tannins were added and the resulting turbidity measured (Fig. 7) in comparison with the turbidity formed in the same conditions by the unheated proteins without DTT. DTT is normally employed as a protein reductant at pHs ≥7.00. At the wine pH (3.20), however, DTT appeared to still be active, but in a less drastic way [42], which was assumed to better reproduce the reducing conditions in the bottle.

Statistical analysis indicated that the series of samples containing DTT (boiled + DTT) gave a haze significantly higher ($P \leq 0.005$) than that produced in the corresponding samples without DTT (boiled). In addition, the haze in the series in which heating was applied before tannins addition (boiled) was also significantly higher ($P \leq 0.005$) than that not heated (RT). By comparing the haze formed in the samples heated before tannin addition with that formed in the corresponding unheated samples, different behaviours were observed among HIC fractions. Generally heating the protein fractions before the addition of tannins gave more haze compared with the unheated samples, with fraction 2 showing the highest difference (Fig. 7). Fraction 2 contained a main protein band with MW ≈25 kDa (Fig. 4), which was assumed to be a chitinase, as confirmed by RP-HPLC (Table 3). Protein bands with similar MWs were not found in any other fraction (Fig. 4). This fact would explain the peculiar behaviour of fraction 2, in which heating could cause a permanent protein denaturation, resulting in the irreversible exposure of tannin-binding sites which remained available for interaction after cooling down. In contrast, for fractions 3–5, containing mainly TL-protein components (Table 3 and Fig. 4), the effect of protein heating was not as significant as it was for fraction 2 (Fig. 7). Taking into consideration the results obtained by heating the protein-tannin mixtures during the reaction, which resulted in high turbidity values for fractions 3–5 (Fig. 6), this result should indicate that the potential binding sites exposed during the thermal treatment reverted to being inaccessible for tannins due to protein refolding after cooling down, as shown in the model of Fig. 9a and b. The different behaviours noted for fraction 2 and fractions 3–5 can be ascribed to the nature of their protein components (mainly chitinases for fraction 2 and mainly TL-proteins for fractions 3–5), which would behave differently in response to temperature variations.

The presence of the reducing agent DTT led to a dramatic haze increase when the total wine proteins were added with tannins after being heated and cooled down, reaching an almost five times higher turbidity value than the control (without DTT) (Fig. 7). The same behaviour was seen also for the HIC fractions and in particular for those containing TL-proteins (fractions 3–5), whereas for the less hydrophobic fractions DTT had no significant effect (Fig. 7). Indeed, fraction 1 was unaffected by reduction, indicating that no additional tannin-binding sites were exposed in these conditions and that the low hydrophobicity of the proteins of this fraction was maintained also after denaturation. Among the other fractions, it was only for fraction 2 that the reducing conditions did not result in a large increase in turbidity compared with the heated but not reduced sample. In contrast, the three following fractions 3–5, when treated with DTT and combined with wine tannins, showed a haze level two to three times higher than those observed in the absence of DTT (Fig. 7). These different behaviours were obviously related to the characteristics of the proteins present in the different HIC fractions. In particular, for fraction 2, which mainly contained chitinases, it is likely that new tannin-binding sites were exposed on these proteins only as a consequence of the thermal denaturation, while the role of S–S bonds breakage on this exposition was minimal. Reduction was much more effective for fractions 3–5, this fact being probably attributable to their content of TL-proteins (Table 3 and Fig. 4). Taken together, the results indicated that wine TL-proteins were less affected in their reactivity with tannins by heating than chitinases. However, the reduction of the intramolecular S–S bonds of TL-proteins [43] should allow the protein structure to be destabilised thus making the protein predisposed to unfold under the heat treatment. Consequently, the exposure of the new tannin-binding sites initially buried in the protein core (Fig. 9) resulted in increased haze formation in the presence of wine tannins.

To evaluate the effect of different temperatures on protein haze formation in the presence of tannins coupled with that of the reducing conditions, the total wine proteins were dissolved in model wine and incubated at four different temperatures with or without DTT. After cooling the solutions at room temperature, wine tannins were added and the haze measured (Fig. 8). Results showed the lack of significant temperature effect on turbidity formation in the absence of DTT, confirming the results of Fig. 7. Therefore,

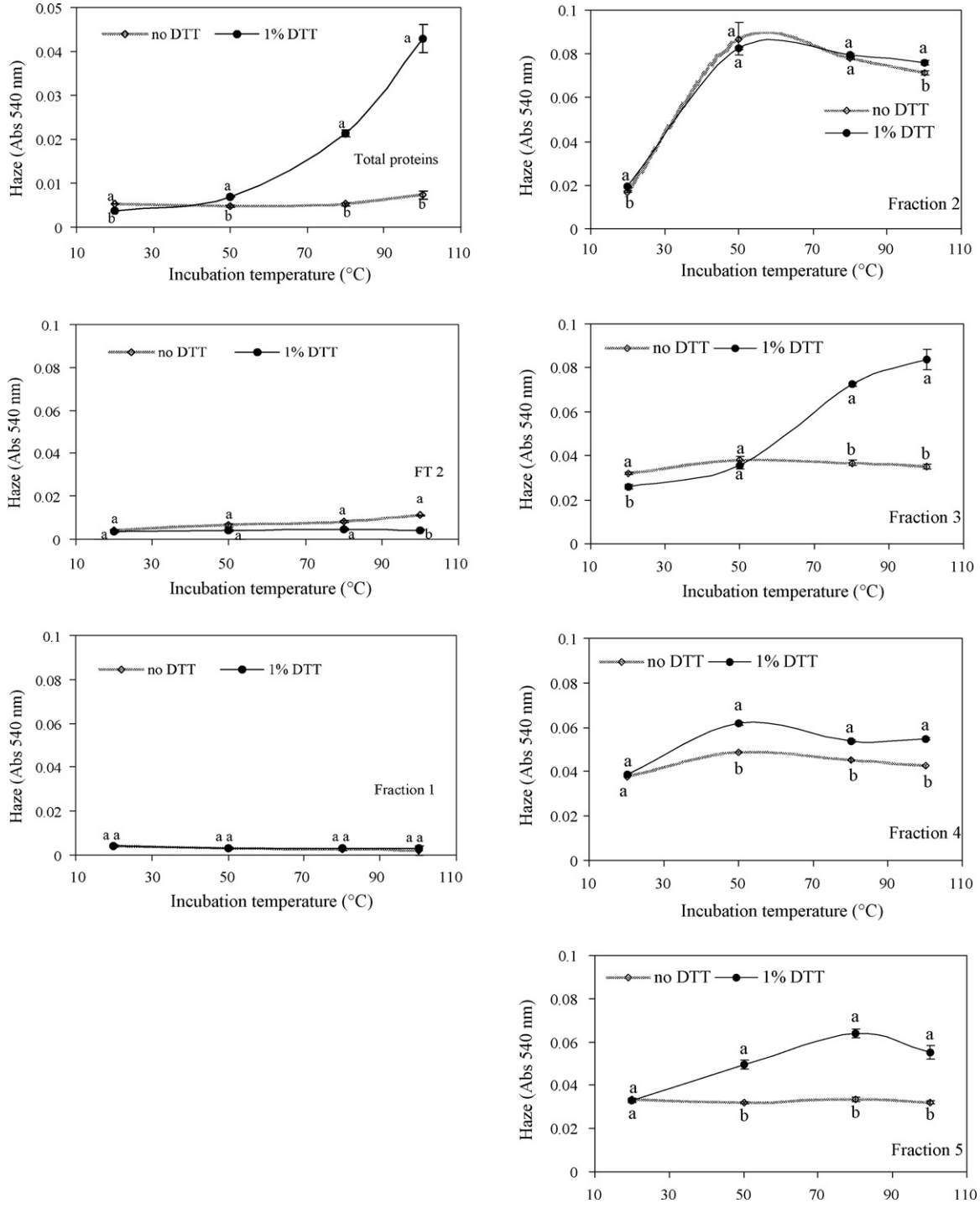

Fig. 8. Turbidity developed after the reaction at room temperature (25 °C) of wine tannins (50 mg L^{-1}) added at room temperature to mixtures of wine proteins (total and HIC fractions) dissolved at 75 mg L^{-1} in model wine. Protein samples were previously heated for 15 min at four different temperatures (20, 50, 80 and 100 °C) in the absence (◊) or presence (●) of 420 mM DTT. Bars with different letters are significantly different at $P \leq 0.01$ (Student–Newman–Keuls test).

heating the wine proteins seemed not to modify their structure at such an extent able to change their reactivity with tannins. Conversely, the reducing agent DTT confirmed its ability to render the proteins, in terms of reactivity with tannins, prone to the effects of the treatment with increasing temperatures. In particular, the level of turbidity formed in the presence of the reducing agent started to significantly increase from the samples pre-heated at 50 °C, indicating this temperature as the lower limit for the exposure of new tannin-binding sites on total wine proteins. This occurred only in the presence of DTT, indicating a major role of the reducing conditions in the formation of turbidity in the presence of tannins.

The low hydrophobicity fractions, FT2 and 1, did not show any significant haze formation after being treated at each of the temperature tested, independently from the presence of reducing conditions (Fig. 8), confirming the previous results of Fig. 7. In contrast, HIC fraction 2 reacted with wine tannins if heated at the relatively low temperatures (50 °C), this result being not affected by the presence of DTT (Fig. 8). This result confirmed that the exposure

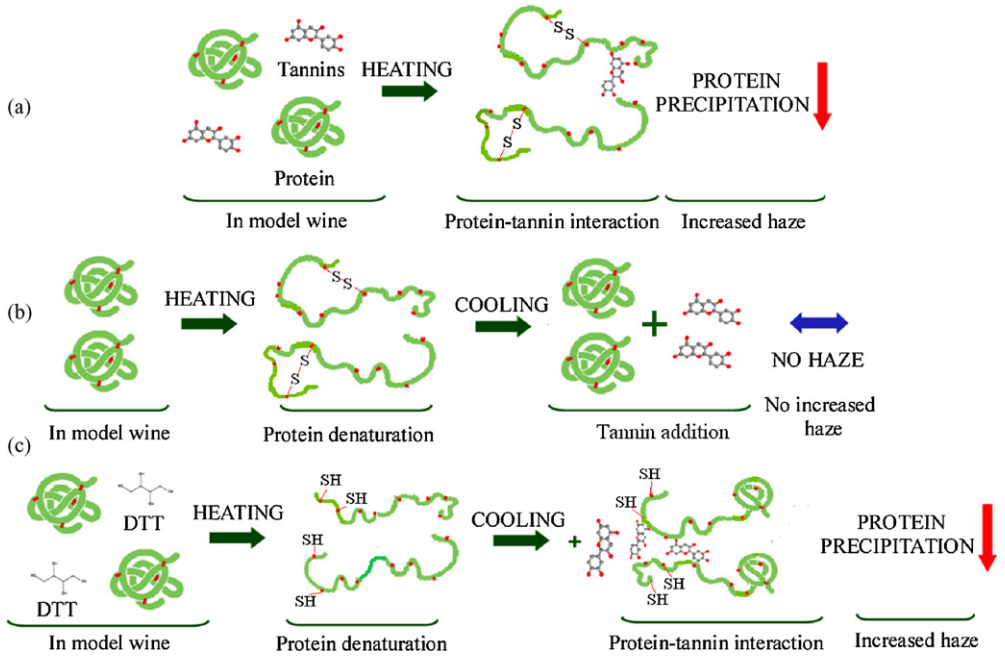

Fig. 9. Hypothetical effects of heating and reduction on protein–tannin interactions: (a) when proteins and tannins are heated together a strong haze develops due to the interactions with the new binding sites exposed on the heat-denatured (unfolded) protein; (b) when proteins are pre-heated in the absence of tannins and then reacted with tannins at room temperature (25 °C) the effect on haze is minimal, because proteins refolds during cooling; (c) when proteins are pre-heated in the absence of tannins and in the presence of a reducing agent (DTT) protein can expose new binding sites; in this case refolding does not occur due to the DTT effect, this fact allowing the protein–tannins interaction and haze formation.

of new tannin-binding sites on the proteins of fraction 2 depended only on heating and did not involve protein regions stabilised by S–S bonds. In this case the increase of turbidity started in the samples treated at relatively low temperatures to reach the highest values in the sample treated at 50 °C. This indicated that the tannin reactivity of the proteins of fraction 2, characterised by a relatively low surface hydrophobicity and mainly composed of a chitinases (Table 3 and Fig. 4), is particularly sensitive to heating, but completely insensitive to reduction.

Fraction 3, the highest HIC peak and containing the largest number of protein bands, mainly TL-proteins (Figs. 2 and 4), showed an opposite behaviour compared to fraction 2. Specifically, heating alone did not lead to any increased reactivity with tannins (Fig. 6), indicating that the proteins of this fraction had the ability to "hide" the tannin-binding sites after being cooled down. In contrast when fraction 3 was treated with DTT, a large increase in turbidity could be noted starting from the samples pre-heated at temperatures higher than 50 °C, which progressively increased for increasing temperature treatments (Fig. 8). These results suggested the presence in the proteins of two types of tannin-binding sites, both exposed to the reaction by heating: one present in a domain stabilised by non-covalent forces and the other internal to a protein region stabilised by S–S bonds. The first protein region would be able to unfold at high temperature and to refold after cooling, whereas the second would need the breakage of the S–S bonds to irreversibly unfold when heated.

The proteins in HIC fractions 4 and 5 also gave higher turbidity under reducing conditions. However, in both fractions, the samples treated with DTT showed a significant ($P \leq 0.01$) increase of haze formation for treatments at a temperature as low as 50 °C, indicating a certain heat instability of the proteins. However, for fraction 4, turbidity formation did not increase for treatments with temperatures higher then 50 °C, whereas these treatments affected fraction 5, containing only TL-proteins (Table 3 and Fig. 4), which gave a maximum turbidity when treated at 80 °C, but only under reducing conditions.

On the whole, the reducing conditions, determined by the presence of DTT, affected significantly the three TL-proteins containing fractions (3–5), which comprised the main part (≈84%, see Table 1) of the total wine proteins. These conditions, by affecting protein stabilisation, aided the action of heating, and resulted in the irreversible exposure of reactive tannin-binding sites on those proteins. On the contrary, the effect of temperature alone seemed somehow to be reversible, since pre-heated TL-proteins containing fractions cooled at 25 °C behaved in a manner similar to that of native (unheated) ones. However, when the proteins were heated in the presences of tannins (as during the heat test) there was obvious turbidity, indicating that proteins in the state of thermal denaturation can react with tannins.

Hence, two types of mechanisms for exposure of tannin-binding sites on the proteins are hypothesised. The first involves the heating alone (thermal protein unfolding) which is sufficient to expose the tannin-binding sites, which can remain exposed also after cooling, as in the case of the fraction containing chitinases (fraction 2), or became again 'hidden' to the tannins after cooling down (protein refolding), as in the case of the TL-proteins containing fractions (3–5). The second mechanism, causing haze formation only under reducing conditions, presupposes that the tannin-binding sites are buried in a protein region stabilised by S–S bonds. After breaking of these covalent bonds, under reducing conditions, irreversible exposure of these sites is made possible by thermal protein denaturation. This latter mechanism seems to be typical of the TL-proteins, which therefore would comprise two distinct tannin-reactive protein regions: one involving non-covalent forces breakable by heating and able to refold by lowering the temperature and the other stabilised S–S bonds. These hypotheses are depicted in Fig. 9.

4. Conclusion

This work highlighted that the purification of both proteins and tannins from the same wine and the determination of their

reactivity is a valuable approach for the characterisation of the still unclear chemical–physical mechanism of white wine hazing. Waters et al. [3] identified proteins causing haze in wines as PR-proteins deriving from the grape berry. In particular, the protein fractions characterized by the highest instability were those containing grape TL-proteins. Our results confirm these findings, showing that TL-proteins, at least in certain conditions, are the most reactive when mixed with wine tannins.

Tannin–protein interaction and complexation can been ascribed mainly to the hydrophobic forces. Indeed, the relation between the order of elution from a hydrophobic column matrix and the level of tannin reactivity has been found for wine proteins. Moreover, the level of exposure of hydrophobic tannin-binding sites for the main wine protein classes (chitinases and TL-proteins) can be differently affected by the physical–chemical conditions, resulting in a different aptitude to form haze.

In particular, the fractions mainly composed of chitinases and characterised by low surface hydrophobicity were able to make available additional binding sites for interactions with tannins with heating alone, and this exposure was irreversible. In contrast, the more hydrophobic and abundant wine protein fractions, mainly composed of TL-proteins, reacted with tannins at high temperature, but seemed to be able to reverse the exposure of tannin-binding sites when cooled down. However, in the presence of reducing agents this protein fraction showed improved aptitude to form haze in the presence of wine tannins. Therefore it can be hypothesised that variations among the hydrophobicity level of different protein classes, affected by variations in the matrix conditions, are involved in protein hazing of white wines. One of the factors influencing protein hazing could be the decrease, during the time after bottling, of the wine red-ox potential [40]. This condition, together with temperature fluctuations during storage, could cause the exposition of hydrophobic binding sites on wine proteins available for tannin complexation, resulting in haze formation during storage of white wines.

Acknowledgements

This work is part of the research activity of the “Dottorato in Viticoltura, Enologia e Marketing delle Imprese vitivinicole”, supported by Provincia di Treviso. We thank Elena Fontana, University of Padua, for her assistance in conducting part of the experiments, and Elizabeth J. Waters, The Australian Wine Research Institute, for helpful discussions regarding this work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2009.10.038.

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THE ROLE OF GRAPE POLYPHENOLS ON *TRANS*-RESVERATROL ACTIVITY AGAINST *BOTRYTIS CINEREA* AND OF FUNGAL LACCASE ON THE SOLUBILITY OF PUTATIVE GRAPE PR PROTEINS

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SUMMARY

The necrotrophic fungus *Botrytis cinerea* is the causal agent of grey mould disease on grapevine. In contact with mature grape berries, the fungus encounters an environment particularly rich in polyphenols and proteins, where the stilbenic phytoalexin *trans*-resveratrol may accumulate. Some grape proteins are structurally and functionally related to plant pathogenesis-related proteins. To mimic conditions similar to those found in grape berries, *B. cinerea* was grown *in vitro* with proteins and polyphenols extracted from mature grapes, and with *trans*-resveratrol. Results showed that in the presence of highly toxic amounts of *trans*-resveratrol, grape polyphenols allowed total recovery of fungal growth, and proteins allowed partial recovery. These resveratrol-polyphenol or resveratrol-protein combinations also induced a strong release into the medium of laccase activity, which is likely to be involved in *trans*-resveratrol detoxification. The protein pattern changed during fungal growth; most grape proteins quickly disappeared from the culture when polyphenols and *trans*-resveratrol were present together. Similar protein patterns were obtained *in vitro* by incubating grape proteins with grape polyphenols and/or *trans*-resveratrol with a purified *B. cinerea* laccase. Under these conditions, most proteins became insoluble. The grape protein pattern obtained from grape berries infected by *B. cinerea* strongly resembled that obtained *in vitro* by incubating grape proteins and polyphenols with fungal laccase. It seems that *B. cinerea*, through laccase secretion and activity and by exploiting the berry polyphenols, easily neutralizes the toxicity of grape stilbenic phytoalexins and makes the grape pathogenesis-related proteins insoluble.

Key words: stilbenic phytoalexins, grey mould, PR proteins, polyphenols.

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INTRODUCTION

Botrytis cinerea causes grey mould disease on grapevine, resulting in loss of grape production and wine quality. *B. cinerea* kills the host plant tissue to acquire the nutrients necessary for growth and reproduction. This process is assisted by the secretion of cell-wall-degrading enzymes and phytotoxin compounds (Williamson *et al.*, 2007). However, to colonize the plant tissue successfully, *B. cinerea* must also neutralize several plant anti-fungal compounds; in the case of grape berries, these compounds are primarily the stilbenic phytoalexins and pathogenesis-related (PR) proteins.

Stilbenic phytoalexins accumulate primarily in the skin of grape berries (Jeandet *et al.*, 1991; Montero *et al.*, 2003; Fornara *et al.*, 2008) and *trans*-resveratrol, a 3,5,4'-trihydroxystilbene, is the predominant compound. The amount of *trans*-resveratrol is largely influenced by the grapevine genotype (Li *et al.*, 2006) and is induced by *B. cinerea*, among other fungi (Montero *et al.*, 2003). *Trans*-resveratrol is the precursor of *trans*-dehydrodimers and *trans*-ε-viniferin, both obtained through a coupling reaction catalyzed by plant peroxidase and phenoloxidases, or by fungal laccase (Pezet, 1998; Breuil *et al.*, 1999; Nicotra *et al.*, 2004). In *B. cinerea* cultures, *trans*-resveratrol induces a specific *B. cinerea* laccase gene, and *trans*-ε-viniferin is believed to be the toxic conversion product of *trans*-resveratrol in infected grapevine (Pezet, 1998; Schouten *et al.*, 2002). Thus, by way of laccase activity, *B. cinerea* would, paradoxically, self-intoxicate (Schouten *et al.*, 2002). In contrast, other research has suggested that *B. cinerea*, through laccase activity, degrades *trans*-resveratrol (Sbaghi *et al.*, 1996; Adrian *et al.*, 1998), and the role of *B. cinerea* laccase in grape infection thus remains undecided.

Grapevine proteins may also contribute to protecting berries from fungal infection. Proteome and transcriptome analyses have shown that ripening grape berries constitutively contain large amounts of chitinase, β-1,3-glucanase, osmotin, and thaumatin-like proteins, normally considered PR proteins (Pocock *et al.*, 2000; Sarry *et al.*, 2004; da Silva *et al.*, 2005; Monteiro *et al.*, 2007). These proteins inhibit conidial germination and mycelial growth (Derckel *et al.*, 1998; Monteiro *et al.*,

2003). During host infection, *B. cinerea* expresses several proteases thought to be involved in virulence by degrading plant PR proteins (Schulze Gronover *et al.*, 2004), although *B. cinerea* is reported to degrade grape proteins with difficulty (Marchal *et al.*, 2006); grape proteins are considered to be particularly resistant to enzymatic proteolysis (Waters *et al.*, 1992).

Several studies have investigated the interactions of some plant proteins and polyphenols. Both groups of compounds may interact, forming non-covalent and covalent bonds, a process influenced by the properties of specific proteins and polyphenols, by the level of oxidation of polyphenols, and by the pH (Siebert *et al.*, 1996; Charlton *et al.*, 2002; Kroll *et al.*, 2003; Rawel *et al.*, 2005; Prigent *et al.*, 2007). Grape berries contain large amounts of polyphenolic compounds, such as catechins, flavonols, and hydroxybenzoic and hydroxycinnamic derivatives (Borbalan *et al.*, 2003); in grape juice, some of these may interact with grape proteins to form a colloidal complex (Siebert, 2006).

During berry infection, it is likely that *B. cinerea* develops in an environment in which PR proteins, *trans*-resveratrol, and other polyphenols come into contact with each other. The effects on fungal growth of the interaction among these three factors remain to be investigated.

The aim of this research was to study whether proteins, *trans*-resveratrol, and the other polyphenols extracted from grape berries and used separately or concurrently in fungal cultures affect *B. cinerea* growth and laccase production. Possible alterations in the grape protein pattern in *B. cinerea* cultures were also investigated and compared with those obtained during grape berry infection and *in vitro* with a purified *B. cinerea* laccase.

MATERIALS AND METHODS

***B. cinerea* cultures.** *Botrytis cinerea* strain PM-10 isolated from grape was kindly provided by Prof. Giuseppe Firrao, University of Udine (Italy). The fungus was grown on Petri dishes on potato dextrose agar (PDA, Difco, USA) at 24°C. For spore production, completely colonized plates were incubated under near UV light for 16 h as reported by Schouten *et al.* (2002). After 15 days, conidia were collected into 5 ml of sterile water by gently scraping the plates with a glass rod. Conidia were filtered through sterilized gauze and counted using a haemocytometer. Liquid cultures were produced in a modified Czapek-Dox medium (2 g l^{-1} $NaNO_3$, 0.5 g l^{-1} KCl, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 1 g l^{-1} K_2HPO_4, 0.01 g l^{-1} $FeSO_4$, 1 ml of a solution of 1% $ZnSO_4$ plus 0.5% $CuSO_4$, and 20 g l^{-1} glucose), adjusted to pH 3.5 with tartaric acid (about 10 mM), which was sterilized by autoclaving. The medium was prepared at twice working concentration, and 2 ml aliquots were plated onto Petri dishes (3 cm diameter) and diluted 1:2 with filter-sterilized stock solutions of grape proteins, grape polyphenols, *trans*-resveratrol (Sigma-Aldrich, USA. Purity >99%), and/or water, and with the concentrated spore suspension to obtain a final concentration of 10^4 conidia ml^{-1}.

Absence and presence of each factor (proteins, polyphenols, and *trans*-resveratrol) were compared in a factorial design. Proteins, polyphenols, and *trans*-resveratrol were supplied at 100 μg ml^{-1}, 200 μg ml^{-1}, and 200 μg ml^{-1}, respectively. The *trans*-resveratrol stock solution (25 mg ml^{-1}) was in 95% ethanol, and the same amount of ethanol (0.8%, v/v) was added to the cultures not treated with *trans*-resveratrol. Crystals formed when *trans*-resveratrol was added at the beginning of the experiment, but the compound dissolved during the course of culturing. A higher concentration of ethanol (4%, v/v) has been recommended to increase the solubility of *trans*-resveratrol (Adrian *et al.*, 1998), but was not used because this concentration reduced growth of the fungus. Each treatment was replicated three times.

The cultures were maintained in the dark at 24°C for 4 days. Aliquots of 100 μl were harvested daily from each culture to assess laccase activity and the protein pattern. At the end of the experiment, the content of each culture medium was transferred into 5-ml preweighed tubes and centrifuged at 12000 μg for 30 min. In addition, each plate was rinsed with 3 ml of water, which was then added to each tube, and the mycelium mat was briefly vortexed and centrifuged again. The supernatant was discarded, and the tubes were oven dried at 80°C for 3 days and then weighed.

Grape berry source and inoculation. White ripe grapes (*Vitis vinifera*, cv. IM 6.0.13) were harvested in the first week of September 2008 from a typical vineyard near Conegliano (north east Italy) and stored for 3–6 days at 4°C before protein and polyphenol extraction and *B. cinerea* inoculation.

For inoculation, the berries were detached from the bunch and surface-sterilized with 99% ethanol for 1 min, then rinsed with sterile water. A 3×2 mm piece of PDA, cut from the margin of an actively growing fungal colony, was placed on a small wound created on the berry surface with a razor blade. The berries were placed on a moist filter paper and closed in a plastic bag at about 22°C. After 7 days, the inoculated berries appeared extensively brownish, and were stored at -20°C until extracted. Berries inoculated with non-colonized PDA were incubated in a similar way and used as the control. Healthy berries and naturally infected ones with grey mould symptoms were harvested from the vineyard at the beginning of November 2008.

Grape protein extraction and analysis. Proteins were

extracted from about 1 l of grape juice obtained by hand crushing about 1.6 kg of berries at 4°C in a beaker. The grape juice was immediately filtered through a nylon gauze, and cysteine was added to give a final concentration of 4 mM to prevent polyphenol oxidation. All successive operations were also performed at 4°C. The juice was centrifuged at 30000 × g for 30 min, and the supernatant was filtered in succession through glass-microfibre discs (GMD) and cellulose acetate filters (0.8 µm) (Sartorius, Germany) and dialyzed overnight (membrane cut off =10 kDa) against 10 mM potassium tartrate buffer, pH 3.5. The dialyzed material was adjusted to 20% saturation (106 g l^{-1}) with $(NH_4)_2SO_4$ and stirred for 1 h. After centrifugation at 30000 × g for 30 min, the supernatant was filtered in succession through 0.8, 0.45, and 0.2 µm membranes, concentrated 8 times with a VivaFlow 5000 apparatus (Sartorius, Germany), and loaded on a Sephadex G-25 column (PD-10, GE Healthcare, UK). The protein was eluted with water and further concentrated with a VivaFlow 5000 apparatus and then assayed for protein (Bradford, 1976) and phenol (Folin-Ciocalteau assay) amounts using BSA and gallic acid as the standards, respectively. The protein recovered, about 20 µg g^{-1} of berry fresh weight, was approximately one-third that measured initially in the grape juice. The estimated weight ratio between protein and phenols in the final protein preparation was about 5:1. This protein was used for *B. cinerea* cultures and *in vitro* assays.

The protein profile was analyzed after precipitation of the protein with four volumes of cold ethanol at -20°C for 2 h. The pellet was washed once with cold 70% ethanol. After centrifugation at 12000 × g for 15 min, the precipitated protein was air dried and resuspended in the sample buffer (Laemmli, 1970), and separated by sodium dodecyl sulphate gel electrophoresis on 16% (w/v) polyacrylamide gels (SDS-PAGE). The gel was stained with the colloidal Coomassie G-250 blue silver method (Candiano *et al.*, 2004).

To compare the protein patterns of healthy and infected berries, 10 healthy and 10 infected berries were crushed, and the juice was centrifuged, filtered, and passed through a PD-10 column as described above. A volume of extract of healthy berries containing 5 µg of protein and an identical volume from the infected berries were precipitated and analyzed by SDS-PAGE, as described above.

Grape phenol extraction. Once crushed and divested of seeds, the solid grape residue was mixed with potassium metabisulphite (0.5 g kg^{-1} of fresh grape) and stored at -20°C. Polyphenols were extracted by the method of Kammerer *et al.* (2004) with some modifications. A total of 50 g of the stored material was stirred with four volumes of methanol/0.1% HCl (v/v) for 2 h under nitrogen at room temperature. The extract was filtered through filter paper and vacuum dried using a Rotavapor at 30°C. The residue was dissolved in 100 ml of acidified methanol, centrifuged at 8,600 *g* for 20 min, and dried again. The residue was dissolved in 40 ml of deionized water brought to pH 3.5 with HCl. The aqueous suspension was centrifuged at 8600 × g for 15 min. The supernatant was filtered through 0.45 µm membranes, and 10 ml aliquots were loaded onto SPE DSC-18/6 ml tubes (Supelco, USA) equilibrated with deionized water. After washing with 5% methanol in water, the phenols were eluted with absolute methanol and concentrated using the Rotavapor, as described above. The residue was dissolved in 2 ml of deionized water, and the phenol concentration was determined by the Folin-Ciocalteau assay, using gallic acid as a standard. About 500 µg of phenols per gram of grape fresh weight were obtained, and stored at -20°C until required.

***B. cinerea* laccase production, purification, and assay**. *B. cinerea* laccase was extracted as described by Slomczynski *et al.* (1995). After culturing for 7 days, the 50-ml contents of several 250 ml Erlenmeyer flasks were pooled and filtered through GMD and then 0.8 and 0.45 µm cellulose acetate membranes (Sartorius, Germany). The filtrates were dialysed against deionized water, concentrated to 40 ml using a VivaFlow 5000 apparatus, adjusted to pH 6.0 with 10 mM potassium tartrate, and loaded onto a Q-sepharose column (16×120 mm, GE Healthcare, UK). Bound protein was eluted with a 60 min linear gradient of 0–0.5 M NaCl dissolved in 10 mM K tartrate buffer.

Laccase activity was assayed in the eluted fractions (2 ml), and the fraction with the highest activity was used in the following assays. This fraction showed a 96 kDa band when analyzed by SDS-PAGE (Fig. 4A, lane 1), similar in size to the protein purified by Slomczynski *et al.* (1995).

Laccase activity was determined spectrophotometrically as described by Wolfenden and Willson (1982) in a volume containing 0.7 ml of 0.1 M acetate buffer, 0.1 ml of 2 mM 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) substrate, and variable volumes of samples (1–30 µl). The assay was performed at 30°C by monitoring the A_{420}. One laccase unit (U) was defined as the amount of enzyme that oxidizes 1 µmol ABTS per min.

Polyphenols, protein and *trans*-resveratrol interactions. Grape proteins, grape polyphenols, and *trans*-resveratrol were mixed in the same weight ratio of 1:2:2 as used for the *B. cinerea* cultures. Grape proteins (50 µg ml^{-1}), *trans*-resveratrol (100 µg ml^{-1}), and polyphenols (100 µg ml^{-1}) were dissolved in 0.1 M potassium-tartrate buffer pH 3.5 in the presence or absence of purified laccase (0.02 U ml^{-1}). After 24 h of incubation at 24°C, 100 µl aliquots of the mixtures were centrifuged

at 16000 × g for 20 min, and the pellet containing the precipitated protein was washed once with cold 70% ethanol. The protein in the supernatant (soluble protein) was precipitated with 4 volumes of cold ethanol at -20°C for 2 h and then washed once with cold 70% ethanol. Both soluble and insoluble proteins were recovered by centrifugation and air dried. Proteins were run on SDS-PAGE gels and stained as described above.

Statistical analysis. Data for laccase activity and mycelium dry matter were subjected to analysis of variance according to a multifactorial design with three replicates.

RESULTS

Effects of *trans*-resveratrol, grape proteins, and polyphenols on *B. cinerea* laccase activity and growth. *B. cinerea* was grown on a Czapek-Dox mineral medium supplemented with two concentrations each of *trans*-resveratrol (0 and 200 µg ml^{-1}), grape polyphenols (0 and 200 µg ml^{-1}), and grape proteins (0 and 100 µg ml^{-1}) in a factorial design. Two experiments were performed successively using different preparations of polyphenols, proteins, and spores. Laccase activity was monitored daily from 1 to 4 days of culture. Some results for laccase activity were similar between the two experiments: levels were negligible in all treatments after one day of culture with the highest activity detected on subsequent days when *trans*-resveratrol was combined with polyphenols or with both polyphenols and proteins (Fig. 1). Finally, activity was lower when polyphenols and proteins were supplied without *trans*-resveratrol, and activity was very low in cultures supplied with resveratrol only (Fig. 1). Comparing the two experiments, the major difference was that activity in the first experiment increased earlier and was much higher at day 4 in the three-factor treatment (polyphenols, protein, and resveratrol together). In addition, in the second experiment, higher laccase activity was detected at day 4 in the cultures treated with proteins or polyphenols and in untreated controls (Fig. 1). The main effects and the first- and second-order interactions of the three factors were significant at $P<0.01$ for all data points except in the first experiment; in that experiment, the interaction of proteins × polyphenols and the second-order interaction were not significant at day 3 of culture. During the course of the experiments, the cultures containing polyphenols alone or in combination with proteins and resveratrol became brownish.

Mycelia were collected and dried at day 4 of culture.

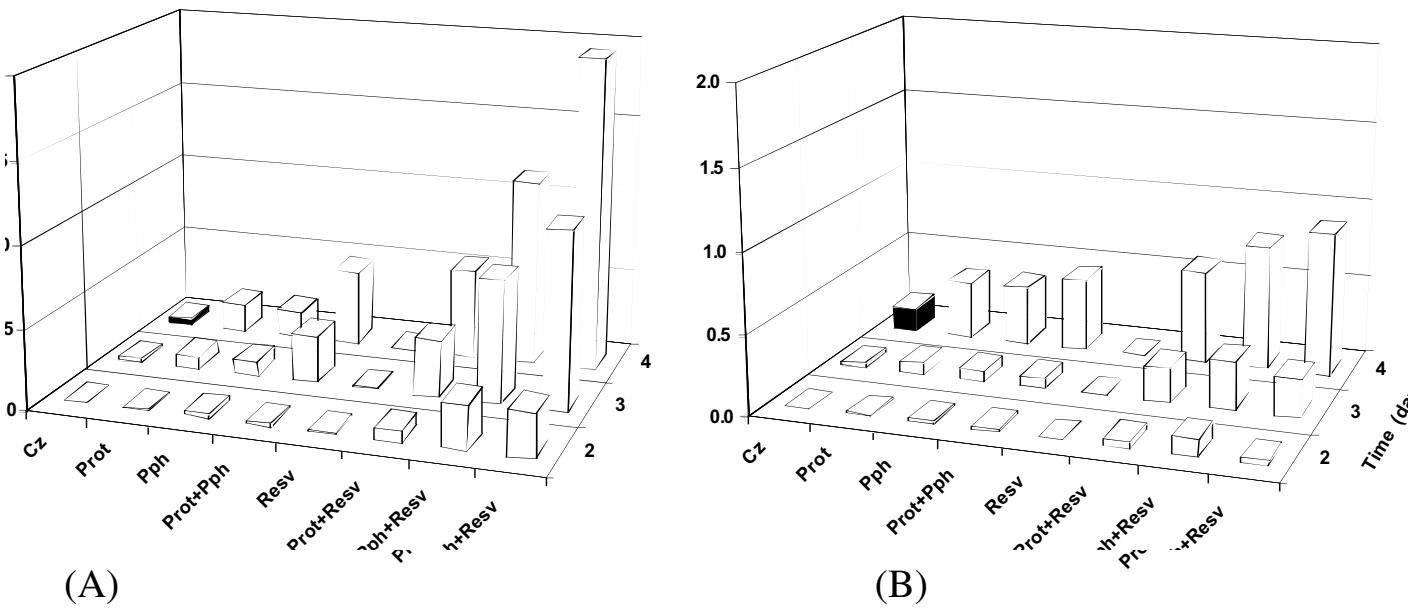

Fig. 1. Laccase activity detected in *B. cinerea* cultures at days 2, 3, and 4 after spore inoculation (10^4 spores ml^{-1}). In a factorial design, the 4-ml cultures were incubated in Czapek-Dox medium at pH 3.5 and supplied without (Cz) or with different combinations of grape proteins (Prot), grape polyphenols (Pph), and *trans*-resveratrol (Resv) at 100 µg ml^{-1}, 200 µg ml^{-1}, and 200 µg ml^{-1}, respectively. At each data point, 1–30 µl aliquots of each culture were assayed for laccase activity. Data from two experiments performed with different batches of proteins, polyphenols, and spore preparations are presented (A and B). Each data point was obtained from three replicated cultures. The main effects and the first- and second-order interactions of the three factors were significant at $P<0.01$ at all data points except in experiment (A), in which the interaction of proteins × polyphenols and the second-order interaction were not significant at day 3 of the culture.

In both experiments, the dry weights were higher than in untreated controls (zero level of each factor) when proteins were supplied alone, in combination with polyphenols, or with both polyphenols and *trans*-resveratrol (Fig. 2). A few sparse mycelium flakes were observed in the culture treated with *trans*-resveratrol alone, and the mycelium dry weight was almost negligible. Mycelium growth was low when *trans*-resveratrol was supplied with proteins, and the detrimental effect of *trans*-resveratrol on mycelium growth was completely abolished when polyphenols were present (Fig. 2). The main effects of the three factors and first-order interactions of protein × *trans*-resveratrol and polyphenols × *trans*-resveratrol were significant at $P<0.01$. Overall, the growth data indicated that grape polyphenols abolish the toxicity of *trans*-resveratrol and that grape proteins promote fungal growth but mostly in the absence of *trans*-resveratrol.

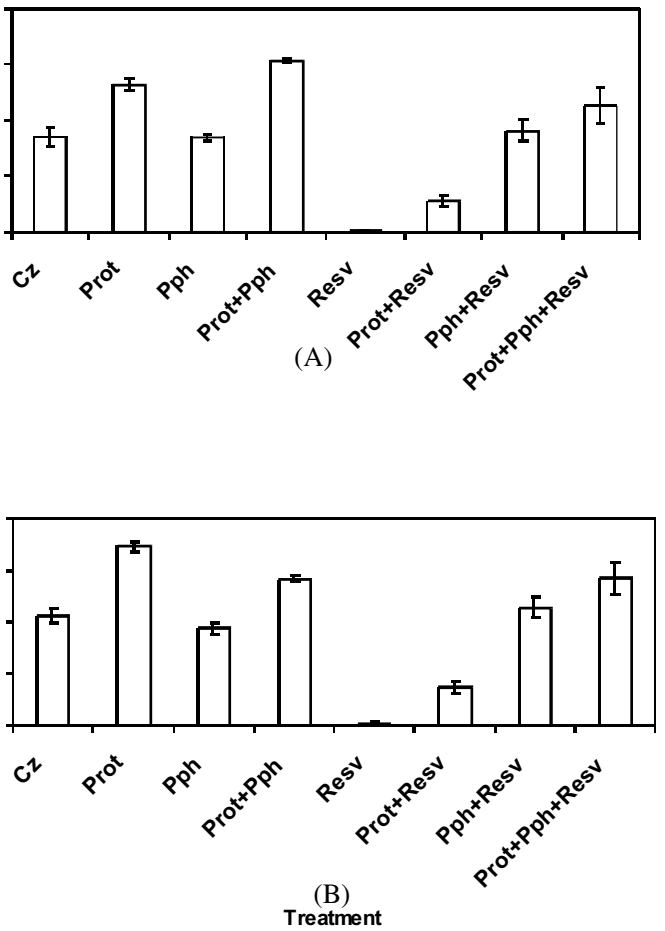

Fig. 2. *B. cinerea* mycelium dry weight harvested at day 4 after spore inoculation (10^4 spores ml^{-1}) from the two experiments (A and B) described in Fig. 1. In a factorial design, the cultures were incubated in 4 ml Czapek-Dox medium at pH 3.5 supplied without (Cz) or with different combinations of grape proteins (Prot), grape polyphenols (Pph), and *trans*-resveratrol (Resv) at 100 µg ml^{-1}, 200 µg ml^{-1}, and 200 µg ml^{-1}, respectively. Each data point is the mean ±SD of three replicated cultures.

Alteration of grape protein in *B. cinerea* cultures. Estimation of the total grape protein present in the cultures during fungal growth was impaired because polyphenols and *trans*-resveratrol interfere with standard protein assays. Therefore, aliquots of the *B. cinerea* cultures containing the grape proteins were analyzed by SDS-PAGE. Control samples incubated without the fungus showed the typical grape protein pattern of three main broad bands at about 25, 31, and 66 kDa and a few bands of minor intensity (Fig. 3, controls). The pattern changed with the type of treatment in the *B. cinerea* cultures. In the culture treated with protein only, the intensity of the band at 31 kDa gradually attenuated and a new band of lower size was formed. This

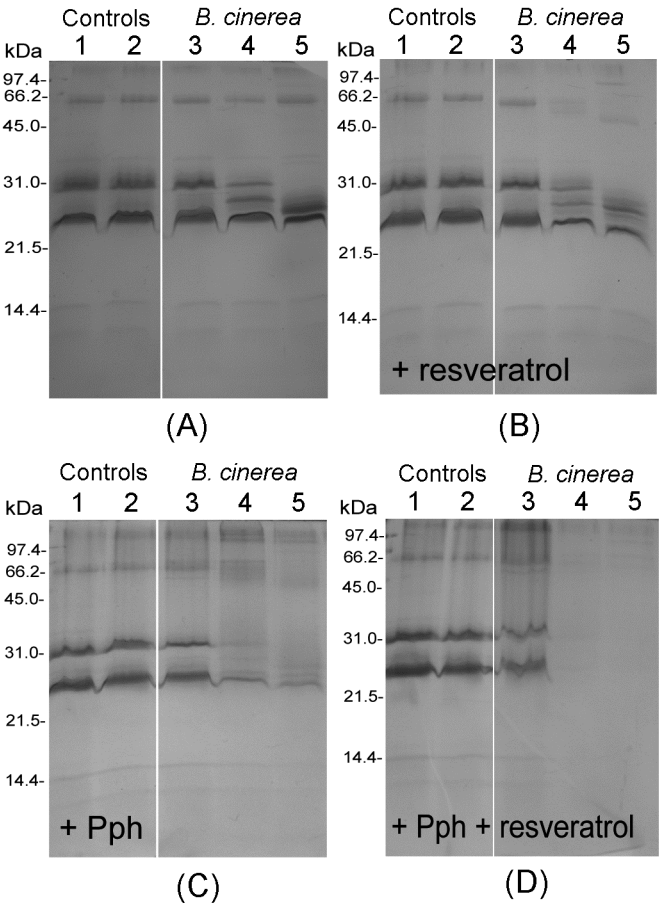

Fig. 3. SDS-PAGE patterns of proteins collected from the *B. cinerea* cultures described in Fig. 2A. Only the cultures supplied with the grape proteins (100 µg ml^{-1}) alone (A) or in combination with resveratrol (B) or grape polyphenols (Pph) (C), or both resveratrol and Pph (D) were analyzed. Samples of 40 µl of inoculated cultures collected at 1, 2, and 4 days (lanes 3, 4 and 5, respectively), and of culture media, incubated without the fungus, collected at 1 and 4 days (lanes 1 and 2, respectively), were centrifuged, precipitated and analyzed as reported in Materials and Methods. The gels were stained with the Coomassie G-250. The low resolution of several bands was probably the result of salts in the medium contaminating the proteins during ethanol precipitation. Similar results (not shown) were obtained with the culture indicated in Fig. 2B.

band is probably the degradation product of the 31 kDa protein, which finally, after 4 days, localized at about 27 kDa (Fig. 3A). A similar pattern was obtained in the culture with *trans*-resveratrol, except that the 25 kDa band was also less intense at the end of the culture, and the 66 kDa band disappeared (Fig. 3B). In the culture supplied with grape polyphenols, the 31 kDa band gradually disappeared and the 25 kDa band faded, becoming poorly visible by the end of the culture period (Fig. 3C). Protein bands almost completely disappeared in the culture containing both polyphenols and *trans*-resveratrol (Fig. 3D).

Alteration of grape protein pattern by *B. cinerea* laccase. From the above results, it seems that polyphenols and *trans*-resveratrol alter the grape protein pattern, mostly when both compounds are added concurrently to the culture. Strong laccase activity was also induced in these cultures. To establish whether *B. cinerea* laccase can mimic these protein alterations, incubations were performed by mixing together proteins with polyphenols and/or *trans*-resveratrol and the fungal laccase. A low laccase concentration (0.02 U ml^{-1}) was used that was less than or comparable to the values measured at the second day of the *B. cinerea* cultures when proteins were mixed with polyphenols and/or *trans*-resveratrol. After a 24 h incubation, these mixtures were centrifuged to separate soluble and insoluble components which were analyzed by SDS-PAGE (Fig. 4). In the supernatants of samples treated with laccase, the major proteins bands became much less intense in the mixture with polyphenols, became weakly visible in the mixtures containing *trans*-resveratrol, and were undetectable in the sample with *trans*-resveratrol and polyphenols (Fig. 4A). These proteins were at least partially recovered from the precipitated materials (Fig. 4B). However, it is worth noting that in the mixture containing polyphenols, the 31 kDa band, which was weak in the soluble fraction (Fig. 4A), was almost undetectable in the insoluble fraction (Fig. 4B). In addition, in the mixture with polyphenols and resveratrol, the 31 kDa band, which had disappeared from the soluble fraction (Fig. 4A), was strongly reduced in the insoluble fraction (Fig. 4B); a smear of protein was clearly visible above 66.2 kDa, probably indicating the formation of high molecular weight aggregates (Fig. 4B). Controls without laccase or with protein plus laccase showed that most protein remained in solution (Fig. 4A). In the control with protein and laccase, a band at 28 kDa was more intense, possibly as a consequence of proteolytic activity in the laccase preparation (Fig. 4A).

Protein pattern in grapes infected by *B. cinerea*. The protein pattern in grape berries infected with *B. cinerea* was analyzed 7 days after inoculation. At this time, the berries appeared completely brown. Grape berries showing grey mould disease symptoms in the vineyard were also analyzed.

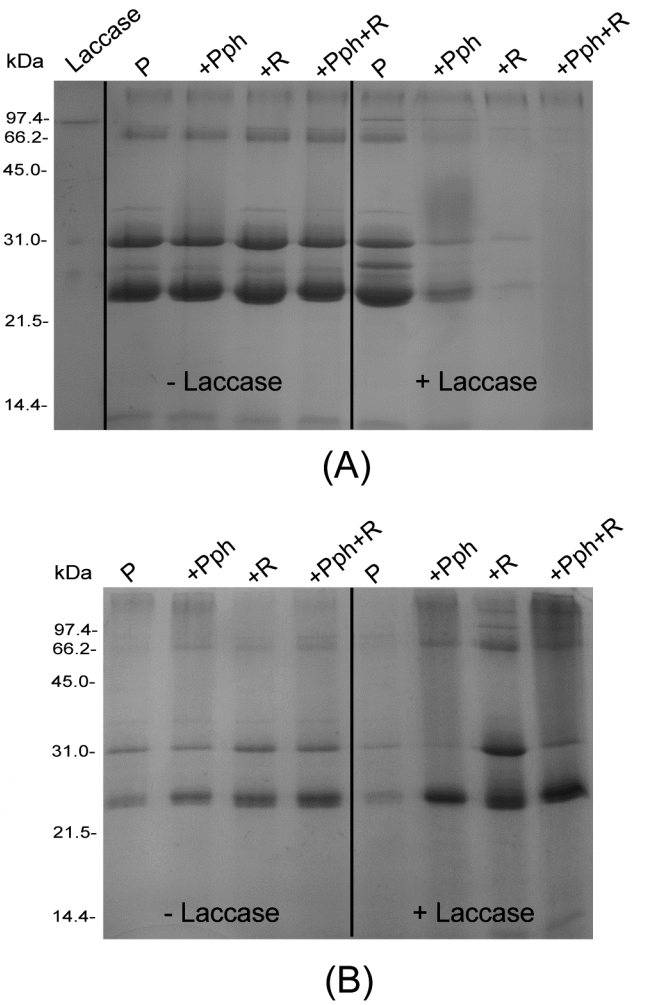

Fig. 4. SDS-PAGE of grape proteins (P) incubated with 0.02 U ml^{-1} of *B. cinerea* laccase activity and with grape polyphenols (Pph), *trans*-resveratrol (R), or polyphenols plus *trans*-resveratrol (Pph+R). Grape proteins, grape polyphenols, and *trans*-resveratrol were supplied at 50, 100, and 100 µg ml^{-1}, respectively (1:2:2 ratio). After a 24-h incubation, 100 µl of each mixture was centrifuged to separate the soluble proteins in the supernatant (A) from the insoluble ones in the precipitate (B). The soluble protein was recovered from the supernatant after precipitation with 4 volumes of cold ethanol. The laccase preparation used in the experiment was also loaded onto the gel (A, first lane from the left). The gels were stained with the Coomassie G-250 as reported in "Materials and Methods".

Compared with healthy grapes (Fig. 5A, lane 1 and Fig. 5B, lane 2), both artificially or naturally infected berries did not show the 31 kDa band and the 25 kDa band showed strongly reduced intensity (Fig. 5A, lane 2 and Fig. 5B, lane 3). These protein patterns resemble those obtained in *B. cinerea* cultures treated with proteins and polyphenols or with incubation of these compounds with *B. cinerea* laccase. A new band at about 45 kDa was observed in the healthy control berries harvest-

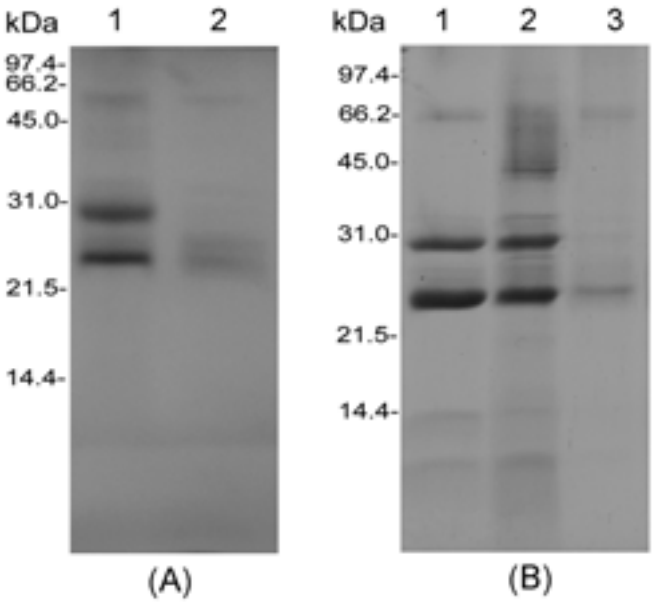

Fig. 5. SDS-PAGE of protein extracted from grape berries either healthy or infected with *B. cinerea*. A volume of extract of healthy berries containing 5 µg of protein and an identical volume from the infected berries were precipitated with four volumes of cold ethanol and analyzed. (A) Protein extracted from healthy (lane 1) and artificially infected berries (lane 2) at 7 days from inoculation. (B) Protein extracted from healthy (lane 2) and infected berries (lane 3) harvested from a vineyard; a sample (approximately 5 µg) of the partially purified grape protein used in the experiment reported in Fig. 4 was also loaded (lane 1).

ed from the field (Fig. 5B, lane 2). This band was not visible in the control grapes used for artificial inoculation, and is possibly linked to the different harvesting dates of the two control samples.

DISCUSSION

During grape infection, the necrotrophic fungus *B. cinerea* encounters an environment rich in phenols and proteins. Among the phenols, the stilbenic *trans*-resveratrol is a phytoalexin precursor toxic to several fungal pathogens, including *B. cinerea* (Adrian *et al.*, 1997). Other grape phenols, which here we generically designated as polyphenols, have no reported effect against pathogenic fungi. In addition, some grape berry proteins have been reported to be toxic against *B. cinerea* and belong structurally and functionally to the PR proteins (Derckel *et al.*, 1998; Monteiro *et al.*, 2003).

To mimic conditions similar to the natural infection of grape berries, the combined effect of *trans*-resveratrol, grape proteins, and grape polyphenols on the growth of *B. cinerea* was studied. It is possible that the grape polyphenol preparations used (about 500 µg g^{-1} of grape fresh weight) could have contained minimal amounts of *trans*-resveratrol. However, the concentration is estimated to be less than 2 µg g^{-1} of skin fresh weight in healthy *Vitis vinifera* grapes (Li *et al.*, 2006). In our experiments, *trans*-resveratrol (200 µg ml^{-1}) was added to cultures at concentration considered lethal for *B. cinerea* (Adrian *et al.*, 1997). An equal amount of polyphenols was also applied. In the literature, there is no clear determination of the *trans*-resveratrol content in grape berries infected by *B. cinerea*, but it is likely to be exceeded by that of polyphenols.

The selected *trans*-resveratrol concentration almost completely prevented fungal growth and laccase activity was negligible in these cultures. In contrast to these findings, Schouten *et al.* (2002) observed only a reduction in fungal growth and a strong induction of the fungal laccase that converts *trans*-resveratrol into the more toxic dimer *trans*-ε-viniferin. The discrepancy between these results may be explained by the lower *trans*-resveratrol concentration (50 µg ml^{-1}) and the high titre of spore suspension (5×10^5 ml^{-1}) used by Schouten *et al.* (2002). Induction of laccase activity and partial fungal growth recovery was also observed after lowering the *trans*-resveratrol concentration to 100 or 50 µg ml^{-1} and increasing the spore concentrations to 10^5 ml^{-1} (data not shown). Hoos and Blaich (1990) previously showed that the effect of *trans*-resveratrol largely depends on the ratio between this molecule and the spore titre. Membrane-bound ATP-binding cassette (ABC) transporters are likely involved in protecting *B. cinerea* germlings from the effects of sub-lethal concentrations of *trans*-resveratrol (Schoonbeek *et al.*, 2001).

Trans-resveratrol became completely ineffective in reducing fungal growth when grape polyphenols where added to the culture, and laccase activity released into the culture was greatly stimulated by this combination of compounds. Thus it can be inferred that under these conditions laccase activity is involved in neutralizing *trans*-resveratrol toxicity. In the presence of grape polyphenols, fungal laccase, which catalyzes a number of phenol coupling reactions (Baldrian, 2006; Riva, 2006), could lead to the formation of more complex compounds, hampering the formation of the toxic *trans*-ε-viniferin. Further work should clarify the role of laccase in the *trans*-resveratrol detoxification process as well as the phenol species involved. Recently, Schouten *et al.* (2008) invoked a similar mechanism for the tannic acid-mediated degradation of the phenolic antibiotic 2,4-diacetylphloroglucinol and also suggested that, at the developing stage, the ABC efflux pump could give *B. cinerea* spores sufficient time to initiate the degradation process.

Grape proteins may also contribute towards making the grape environment inhospitable to fungal attack because they have been described mostly as PR or PR-like proteins. The most highly-expressed proteins in the skin of mature berries are chitinases and β-1,3-glucanase

(Deytieux *et al.*, 2007), which together with thaumatin-like proteins are also largely represented in the mesocarp of mature berries (Sarry *et al.*, 2004), and particularly enriched in grape juice (Tattersall *et al.*, 1997). The protein pattern obtained from grape juice in this study is similar to that obtained in previous studies and is characterized by three major bands at about 25, 31, and 66 kDa, tentatively identified as thaumatin-like proteins, chitinase, and invertase, respectively (Tattersall *et al.*, 1997; Davies and Robinson, 2000).

Grape thaumatin-like proteins and chitinases have significant antifungal activity against *B. cinerea* (Derckel *et al.*, 1998; Monteiro *et al.*, 2003). In contrast, this current study showed an increase in mycelium growth upon supplying *B. cinerea* cultures with the grape proteins at a concentration comparable to that measured in grape juice. The discrepancy with the results of other studies may depend on differences in the bioassay used, including protein concentrations and purity, and the sensitivity of the fungal strain. Indeed, protein preparations used in this work were not pure, and phenol contamination may have played a role in diminishing the effect of grape proteins against the fungus.

The grape protein preparations used also allowed partial recovery of the fungal growth inhibited by the *trans*-resveratrol, suggesting reduction of phytoalexin concentration in the culture, possibly because of interaction with the grape proteins. Indeed, the protein band intensities obtained in the presence of *trans*-resveratrol seem quantitatively less than those obtained without *trans*-resveratrol. However, the major change in the grape protein pattern in the *B. cinerea* culture was observed in the presence of polyphenols or when polyphenols and *trans*-resveratrol were mixed together. In the latter case, almost all proteins were removed from the culture.

We suggest that this disappearance of proteins from liquid culture in the presence of polyphenols and *trans*-resveratrol is the consequence of reduced solubility of grape proteins mediated by the fungal laccase. Covalent and non-covalent interactions between model and food proteins and selected phenolic compounds have been described (Kroll *et al.*, 2003; Rawel *et al.*, 2005; Prigent *et al.*, 2007). Covalent interactions are mediated by the formation of phenol radicals and quinones, which may occur spontaneously at alkaline pH and are produced by laccase activity at acidic pH (Kroll *et al.*, 2003; Baldrian, 2006). Quinones, which include the laccase-oxidized products of *o*-diphenols and *p*-diphenols, may undergo attack by nucleophilic amino acids located on the protein surface (Kroll *et al.*, 2003). This interaction may lead to change in the physicochemical properties, solubility, degradability, and availability of proteins, as well as to alteration in the antioxidant properties of the plant polyphenols (Rawel *et al.*, 2007).

The *in vitro* experiments performed by mixing *B. cinerea* laccase, polyphenols, and/or *trans*-resveratrol with grape proteins confirmed that laccase favours grape protein precipitation, probably through production of oxidized phenol intermediates. However, in the mixture containing polyphenols, the fate of the 31 kDa protein was unclear; its concentration diminished in the soluble fraction and was almost zero in the insoluble fraction.

The *in vitro* experiments strongly supported the conclusion that similar reactions catalyzed by laccase may occur in *B. cinerea* cultures, which would explain the protein alterations observed. The only difference is that in the *B. cinerea* cultures, supplied with resveratrol and proteins, some proteins remained soluble, while they were totally insoluble in the resveratrol-protein mixture used *in vitro* with laccase. A possible explanation is that in the presence of the fungus, a certain amount of *trans*-resveratrol or oxidized derivatives is degraded by the fungus and is less available for interaction with grape proteins.

In general, grape proteins are considered resistant to fungal digestion (Waters *et al.*, 1992; Ferreira *et al.*, 2002). However, this contrasts with the observation that *B. cinerea*–infected grape berries showed a simplified protein pattern with bands of reduced intensity. The pattern observed in infected grape berries (Marchal *et al.*, 1998; this paper) resembles that obtained with protein-polyphenol mixtures after addition of laccase. Laccase activity is usually produced by *B. cinerea* in infected berries (Dewey *et al.*, 2008), and we propose here that this interaction is responsible for the protein pattern observed in the grey mould-rotted grape.

B. cinerea laccase has been suggested as a possible virulence factor during grape infection, causing direct *trans*-resveratrol degradation (Sbaghi *et al.*, 1996). On the other hand, Schouten *et al.* (2002) suggested that laccase increases the toxicity of *trans*-resveratrol by producing *trans*-ε-viniferin, and also showed that knockout mutants in the *Bclcc2* gene, a laccase gene induced *in vitro* by tannic acid and *trans*-resveratrol, were as virulent as wild type on peanut and grapevine leaves.

The data presented here support the conclusion that in a grape environment characterized by an abundance of polyphenols, *B. cinerea* laccase not only detoxifies the *trans*-resveratrol phytoalexin but also modifies the solubility of grape proteins. Future research will address the kind of interaction taking place between *trans*-resveratrol and polyphenol components and between grape polyphenols and proteins. The effect of this interaction on the degradability of the proteins by fungal proteases will also be considered. Because most of the grape proteins are PR-like proteins, it will be interesting to establish whether other laccase-producing fungi use a similar avoidance mechanism to escape the effect of such proteins.

ACKNOWLEDGEMENTS

Research funded by the Veneto Region in the project Azione Biotech 2 of ISIB-CNR, entitled "Biotechnology to improve crop quality, safety and sustainability," and by the Italian Ministry of Research in the project PRIN 2007 entitled "Mechanisms of plant pathogens to overcome the host defence system".

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Received March 6, 2009
Accepted May 7, 2009

Effetto della posizione della bottiglia durante la rifermentazione e la maturazione del Prosecco Sur Lies

Effect of the bottle position during second fermentation and ageing in Prosecco Sur Lies wine

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Riassunto

Il Prosecco "Sur Lies", a differenza del Prosecco Frizzante prodotto con il metodo in autoclave, viene rifermentato in bottiglia e conservato sui lieviti. Poiché la tecnologia di produzione del "Sur Lies" si basa tuttora su scelte di lavorazione che derivano da osservazioni empiriche, lo scopo di questo lavoro è stato quello di analizzare l'effetto della posizione della bottiglia durante la rifermentazione, operazione critica della produzione.

Campioni di Prosecco vinificati in due aziende diverse, subito dopo l'inoculo dei lieviti, sono stati conservati per un periodo di sei mesi tenendo le bottiglie in posizione orizzontale (O) o verticale (V) per tutto il periodo di maturazione o orizzontale solo per le prime due settimane (O/V). Le analisi condotte durante i sei mesi hanno dimostrato che nella tesi O avviene una fermentazione più veloce, probabilmente grazie alla maggiore superficie di scambio tra i lieviti ed il liquido circostante. Infatti in questo campione è stata osservata una più rapida evoluzione della pressione. E' stata inoltre seguita, durante la maturazione, anche l'evoluzione dei parametri di schiumabilità che sono risultati migliori per le tesi O e O/V. Infine, dalle analisi sensoriali è emerso che il vino delle tesi V e O/V risulta più equilibrato rispetto a quello della tesi O.

Si può quindi concludere che la tesi O/V è quella preferibile, in quanto permette di raggiungere un maggior equilibrio fra la componente sensoriale e l'aspetto visivo della schiuma.

Summary

The Prosecco "Sur Lies", unlike the Prosecco Frizzante produced with the "bulk method", is fermented a second time in the bottle and preserved on yeasts. Since the production technology of "Sur Lies" wines is based on processing techniques deriving from empirical observations, the aim of this work is to analyze the effect of the bottle position during the second fermentation, the most critical operation of the production.

Samples of Prosecco vinified in two different wineries, after yeast inoculation for second fermentation, were maintained for six months, holding the bottles in horizontal (O) or vertical (V) position for the whole period of maturation or horizontal only for the first two weeks (O/V). Analyses made during the six months showed that in the thesis O a faster fermentation happens, probably thanks to the greater contact surface between the yeasts and the wine. In fact in this sample a more rapid evolution of pressure was observed. Besides, during the maturation, also the evolution of the foamability parameters was followed and the best results was noticed for the theses O and O/V. Finally, from the sensory analyses it emerged that the wine of the theses V and O/V is more balanced in comparison to that of the thesis O.

In conclusion the thesis O/V is preferable because it allows to reach a greater balance between the sensory component and the visual aspect of the foam.

Parole chiave: vini spumanti, schiumabilità, autolisi dei lieviti
Key words: sparkling wines, foaming properties, yeast autolysis

Introduzione

Il Prosecco "Sur Lies", a differenza del Prosecco Frizzante prodotto con il metodo consueto in autoclave (metodo Charmat), viene rifermentato in bottiglia, conservato sui lieviti per alcuni mesi senza subire l'operazione di sboccatura e quindi consumato in presenza delle fecce formatesi durante la fermentazione. Questo vino, ancora prodotto artigianalmente, pur avendo caratteristiche interessanti e tipiche sotto il profilo sensoriale, viene apprezzato quasi solo nella zona di produzione a causa di due fattori principali: la presenza di residui dei lieviti sul fondo, che può essere percepita dal consumatore come un difetto, e l'elevato livello di eterogeneità tra bottiglie, dovuto alla difficoltà di controllo del processo di produzione. Proprio a causa della sua scarsa diffusione, la tecnologia di produzione del "Sur Lies" non è stata molto studiata e quasi sempre le scelte di lavorazione derivano dalle osservazioni empiriche dei produttori.

E' ormai ben noto che l'affinamento sui lieviti arricchisce il vino di nuovi componenti come composti azotati, sostanze volatili, polisaccaridi e mannoproteine (Fornairon-Bonnefond *et al.*, 2001), che proteggono il vino dalle precipitazioni tartariche e proteiche (Flanzy, 1998), ne accentuano il "flavor" e ne migliorano la corposità (Leroy *et al.*, 1990). Numerosi studi condotti su vini prodotti con il metodo classico, inoltre, hanno dimostrato che la maturazione sui lieviti migliora le caratteristiche di schiumabilità (formazione delle bolle e loro stabilità) (Pueyo *et al.*, 1995;

Andrès-Lacueva *et al.*, 1996; Moreno-Arribas *et al.*, 2000), le quali sono considerate tra i più importanti parametri qualitativi dei vini spumanti (Senèe *et al.*, 1998).

Lo scopo di questo lavoro è stato quello di analizzare in dettaglio una variabile potenzialmente critica per la produzione, ovvero, la posizione di conservazione della bottiglia, che chiaramente ha un effetto sulle modalità di contatto del vino con i lieviti.

Materiali e metodi

La sperimentazione è stata eseguita su bottiglie derivanti da due masse omogenee di Prosecco prodotte nell'anno 2007 e vinificate in due aziende diverse situate rispettivamente a Valdobbiadene [A] e ad Ogliano [B].

La stabilizzazione e la chiarifica sono state realizzate con bentonite (15 g/hL) per il campione A mentre per il campione B è stata effettuata solo una decantazione naturale. Dopo otto mesi dalla vendemmia gli zuccheri necessari sono stati addizionati mediante aggiunta di mosto di Prosecco.

Si è poi proceduto alla rifermentazione e, come lieviti, sono stati utilizzati S. *bayanus* ceppo T18 [A] e S. *cerevisiae* ceppo CGC62 [B].

E' stato quindi effettuato l'imbottigliamento e per ogni massa (A e B) sono state organizzate 3 tesi: un terzo delle bottiglie tenute in posizione orizzontale per tutto il periodo di maturazione (O); un terzo delle bottiglie tenute in posizione verticale per tutto il periodo di maturazione (V); un terzo delle bottiglie tenute in posizione orizzontale per le prime due settimane (fino alla fine della fermentazione alcolica) e poi messe a maturare per il restante periodo in posizione verticale (O/V).

Il campionamento è stato eseguito dopo due settimane (fine fermentazione alcolica), cinque settimane (fine fermentazione malolattica), tre mesi ed infine sei mesi dall'imbottigliamento.

Da ogni tesi e per ogni campionamento sono state prelevate 3 bottiglie su cui è stata misurata la pressione tramite afrometro. Il vino delle tre bottiglie è stato quindi mescolato e sottoposto alle analisi chimiche in triplice ripetizione.

Per la determinazione dei polisaccaridi è stato utilizzato il metodo del fenol solforico (Dubois *et al*., 1956; Segarra *et al*., 1995).

La qualità della schiuma è stata valutata con un apparato che si basa sul sistema di Rudin. Esso è costituito da una colonna di vetro, destinata a contenere il vino da esaminare, con alla base un setto poroso (porosità 101-160 μm). Un campione di 50 mL di vino, opportunamente filtrato e degasato, è stato introdotto nella colonna e tramite un flussimetro è stata insufflata anidride carbonica regolata a 1 bar di pressione e 260 mL/min di flusso. L'evoluzione della schiuma è stata valutata misurandone l'altezza ad intervalli di 15 secondi per un periodo totale di 15 minuti. Alla

fine della misurazione è stato registrato anche il tempo necessario per la scomparsa totale della schiuma dopo aver cessato l'insufflazione del gas (Brissonnet *et al*., 1993). Per ogni campione sono state eseguite tre repliche.

L'analisi sensoriale dei vini è stata condotta dopo 3 e 6 mesi dall'imbottigliamento da un panel di 12 degustatori esperti sulla base di descrittori sintetici usuali e con l'ausilio di schede non strutturate; i campioni sono stati presentati a ciascun degustatore in ordine randomizzato.

I dati sono stati analizzati statisticamente mediante ANOVA e test di Tukey-Kramer (XLSTAT 2007).

Risultati e discussione

Evoluzione della pressione

Considerando che nel metodo "Sur Lies" la seconda fermentazione non avviene su una massa unica come per il metodo in autoclave è evidente che ogni bottiglia evolve indipendentemente dalle altre comportando una variabilità nel risultato finale. Per questo da ogni tesi (O, V, O/V) e per entrambi i campioni (A e B), sono state prelevate tre bottiglie e per ognuna di queste è stata effettuata la misura della pressione; il Grafico 1 (campione A) riporta le medie dei tre valori di pressione misurati in momenti diversi durante il periodo di maturazione del vino. Lo sviluppo di pressione si è verificato, come atteso, nelle prime due settimane, seguito da un ulteriore aumento nel tempo, anche se molto modesto, fino a 3 mesi. Il campione B ha mostrato la stessa cinetica di evoluzione della pressione.

In entrambi i campioni, già a due settimane, le bottiglie tenute in orizzontale (tesi O e O/V) hanno sviluppato una pressione significativamente maggiore rispetto alla tesi V ($p<0.05$). Ciò può essere un'indicazione di una fermentazione più veloce nelle bottiglie in posizione orizzontale, probabilmente dovuta ad una maggiore superficie di scambio tra i lieviti ed il liquido circostante.

Dopo 5 settimane, momento che potenzialmente corrisponde alla fine della fermentazione malolattica, e dopo 3 e 6 mesi dall'imbottigliamento, non sono state riscontrate differenze di pressione statisticamente rilevabili tra le bottiglie conservate nelle diverse condizioni.

Comunque, le bottiglie derivanti dalla massa A mostrano una maggiore pressione rispetto a quelle della massa B. Tale differenza non è imputabile ad un diverso contenuto di zuccheri di partenza, peraltro molto simile nei due campioni, ma piuttosto ai diversi ceppi di lievito utilizzati per la rifermentazione. E' già stato dimostrato, infatti, che S. *bayanus* (utilizzato per la

fermentazione del campione A) ha una maggiore resistenza all'alcol rispetto a S. *cerevisiae* (utilizzato, invece, nel campione B) (Gaia, 1983).

Analisi dei polisaccaridi

Il Grafico in Figura 2 riporta l'andamento del contenuto in polisaccaridi totali delle bottiglie della massa A. La cinetica di evoluzione di queste molecole è risultata molto simile in entrambe le masse A e B. Per quanto riguarda la massa B è stato riscontrato un maggiore contenuto di polisaccaridi già nel vino base (218 mg/L rispetto ai 178 mg/L del vino A). Oltre alla normale variabilità legata alle differenze che si possono riscontrare tra due campioni che provengono da diverse aree di produzione e che sono stati processati in cantine diverse, questa diversità potrebbe essere riconducibile anche al fatto che il campione B, al contrario di quanto avvenuto per il campione A, non ha subito il trattamento con bentonite. E' noto infatti che la bentonite, oltre ad adsorbire le proteine, ha un effetto, seppur minimo, anche sulla frazione polisaccaridica (Okuda *et al.*, 2003).

In entrambi i campioni, durante il periodo di rifermentazione (fino a 5 settimane), i polisaccaridi solubili sono diminuiti, mentre successivamente essi sono aumentati anche oltre il contenuto inizialmente misurabile all'inizio. Questo indicherebbe una iniziale perdita di polisaccaridi, forse per insolubilizzazione indotta dall'aumento di etanolo e/o per consumo attivo da parte dei lieviti in fermentazione attiva. Seguirebbe poi, nelle fasi successive, un rilascio dei polisaccaridi di parete per autolisi dei lieviti; è ormai infatti ampiamente descritto che il fenomeno di disgregazione delle pareti cellulari dei lieviti può iniziare nelle bottiglie dopo pochi mesi dalla fine della fermentazione alcolica (Charpentier *et al.*, 1993).

Analisi della schiumabilità

Le modalità di conservazione potrebbero avere un effetto sulle caratteristiche della schiuma, un parametro importante per la qualità del Prosecco.

Con il sistema da noi utilizzato è possibile ricavare tre parametri utili alla caratterizzazione della schiuma: "altezza massima" (HM), correlata con il volume massimo di schiuma che si può sviluppare in un determinato vino; "altezza alla stabilità" (HS), cioè l'altezza della schiuma nel momento in cui la velocità di formazione di nuove bolle è pari alla velocità della loro scomparsa, correlata con la stabilità della schiuma nel bicchiere; "tempo di scomparsa" (TS), corrispondente al tempo necessario perché la schiuma scompaia dopo aver bloccato il flusso di CO_2, parametro che

determina la velocità di scomparsa della schiuma dopo il versamento del vino nel bicchiere (Brissonet *et al.*, 1993).

Per entrambe le masse A e B ed indipendentemente dalla posizione della bottiglia è stata osservata una graduale diminuzione di HM nel tempo (Figura 3 e Tabella 1). Questo fenomeno è stato già osservato in precedenti lavori che prendevano in considerazione vini prodotti con il Metodo Classico (Andrès-Lacueva *et al.*, 1996; Moreno-Arribas *et al.*, 2000). L'aumento di etanolo, che ha un noto effetto negativo sulla schiuma, non può essere la causa di questo fenomeno, in quanto l'altezza massima del campione dopo 2 settimane dall'imbottigliamento (quando quasi tutto l'etanolo possibile si è già sviluppato) è quasi sovrapponibile a quella del vino base. E' quindi più probabile che la diminuzione di HM osservata nel tempo sia da collegare a fenomeni conseguenti l'autolisi dei lieviti.

Gli stessi dati mostrano che, aumentando il tempo di contatto con i lieviti, il picco corrispondente ad HM si raggiunge in tempi sempre più brevi, arrivando anche nell'ultimo campionamento ad un anticipo di 30-45 secondi rispetto al vino base. Una spiegazione di questo fenomeno, mai osservato prima, potrebbe essere ricercata in una variazione qualitativa e/o quantitativa di molecole tensioattive (come ad esempio le proteine e le mannoproteine) in grado di rendere più elastica, e quindi meno resistente all'espansione, la parete delle bolle.

L'andamento della stabilità della schiuma (HS) si è dimostrato, invece, altalenante nel tempo (Tabella 1), ma comunque ripetibile sia tra i trattamenti sia tra le due diverse masse di vino. Nelle prime due settimane HS non è variata, mentre dopo 5 settimane essa è diminuita in modo più evidente nel campione non trattato con bentonite. Dopo 3 mesi invece HS è aumentata per poi nuovamente diminuire nell'ultimo campionamento.

Infine, in tutte e due le masse (A e B) non è stata osservata nessuna variazione significativa del parametro TS. Questo contrasta con i dati di letteratura che indicano come durante la rifermentazione in bottiglia e la maturazione sui lieviti, TS tenda ad aumentare di 10-20% nei primi 2-4 mesi, per arrivare addirittura ad un aumento di oltre il 400% dopo 15 mesi (Maujean *et al.*, 1990). L'andamento di TS nei campioni da noi analizzati potrebbe essere legato alla varietà di uva: infatti già i vini base hanno mostrato un valore di TS molto più basso (in media circa 19 secondi) rispetto alle varietà tradizionalmente utilizzate per la produzione di Champagne (120-230 secondi) (Maujean *et al.*, 1990) o di Cava (70-200 secondi) (Andrès-Lacueva *et al.*, 1996). Questi bassi valori di TS, che sembrerebbero determinati principalmente dal vitigno, sono considerati, per il vino Prosecco, una caratteristica positiva. Infatti, una valutazione di 23 vini Prosecco da parte di 106 soggetti, che prendeva in considerazione solo l'aspetto visivo collegato all'evoluzione della schiuma sul bicchiere ha dimostrato che il prodotto considerato ottimale dal consumatore medio è

quello con una schiuma che raggiunge sì un volume consistente, ma anche che sparisce rapidamente (Franceschi *et al.*, 2005).

I campioni sono stati confrontati tra loro con lo scopo di valutare l'effetto della posizione della bottiglia (Tabella 1 e Grafico 4). Già dopo le prime due settimane di rifermentazione, per entrambe le masse, l'altezza massima nella tesi V è risultata superiore a quella delle altre due tesi, in cui sono apparsi già evidenti la diminuzione dell'altezza massima e l'anticipo del picco HM. Questo dato confermerebbe che nelle bottiglie tenute in posizione orizzontale la fermentazione, e quindi le variazioni qualitative ad essa associate, avviene più velocemente.

Dopo 6 mesi, comunque, le tesi O e O/V hanno dimostrato di avere una HM maggiore rispetto alla tesi V. Questi risultati indicherebbero che il volume della schiuma è influenzato soprattutto dai fenomeni che avvengono durante i primi 15 giorni dopo l'imbottigliamento, in cui avviene la fermentazione alcolica attiva, che sembrerebbe dunque influenzata dalla posizione delle bottiglie. Al contrario non sono state riscontrate differenze significative tra le tesi O e O/V, indicando che la superficie di scambio tra i lieviti ed il liquido, maggiore per le bottiglie conservate in posizione orizzontale, non sembrerebbe avere effetto su HM, almeno per tempi di sosta fino a 6 mesi, che sono relativamente brevi se confrontati con quelli tipici del Metodo Classico.

Analisi sensoriale

Alcune bottiglie per ogni tesi sono state sottoposte ad analisi sensoriale.

Dopo 3 mesi non sono state riscontrate differenze tra bottiglie conservate nei tre diversi modi (dati non mostrati), indicando che a questo tempo la posizione di conservazione è ininfluente dal punto di vista organolettico.

A 6 mesi dall'imbottigliamento (Grafico 5 relativo alla massa B), invece, l'analisi della varianza ha mostrato differenze statisticamente significative per alcuni descrittori tra i campioni conservati nelle diverse posizioni.

Dal punto di vista olfattivo, le note floreali e fruttate sono state percepite in media con un valore superiore nel vino conservato in posizione verticale. Anche se l'analisi statistica ha rilevato che queste differenze, prese singolarmente, non sono significative, i parametri di intensità ed armonia olfattiva (in cui i descrittori sono considerati nel loro insieme) sono invece risultati superiori in maniera significativa ($p<0.05$) nel vino conservato in posizione verticale rispetto a quello tenuto in posizione orizzontale. I vini conservati in posizione orizzontale solo per le prime due settimane sono risultati più simili, dal punto di vista olfattivo, a quelli mantenuti per tutto il tempo in posizione verticale. Questo comportamento può essere ricondotto ad una maggiore

modificazione delle molecole aromatiche tipiche, risultante in un invecchiamento organolettico in quelle bottiglie in cui è maggiore la superficie di scambio tra le cellule di lievito ed il liquido. Uno studio svolto sui vini Cava ha messo in evidenza la correlazione tra il tempo di sosta sui lieviti e la diminuzione di alcune molecole odorose come gli acetati di alcoli superiori, responsabili di aromi fruttati (Riu-Aumatell *et al.*, 2006). Inoltre, anche se non ci sono studi riguardanti l'evoluzione di terpenoli, benzenoidi e norisoprenoidi, che sono i principali composti aromatici del Prosecco (Tomasi *et al.*, 2000), non è da escludere che una maggiore cessione, da parte del lievito, di altri composti aromatici comporti comunque una loro minore percezione. Infatti, nei vini conservati per tutto il periodo in posizione orizzontale, sembrerebbe più intenso l'aroma di pane che deriva tipicamente dai lieviti.

Anche la percezione del descrittore gustativo amaro risultava mediamente maggiore (anche se non in modo significativo) nei campioni tenuti tutto il tempo in orizzontale rispetto a quelli delle altre tesi. Anche in questo caso, la possibile spiegazione potrebbe essere una maggiore velocità di rilascio di molecole da parte dei lieviti, tra cui piccoli peptidi che potrebbero contribuire alla sensazione amara (Alexandre *et al.*, 2001).

Differenze significative tra i vini sono state rilevate anche per il parametro "tipicità" che ha premiato i vini tenuti in posizione orizzontale solo per i primi 15 giorni.

Conclusioni

In questo lavoro è stato notato come la rifermentazione e l'evoluzione del vino in bottiglie di Prosecco Sur Lies mantenute in posizione orizzontale siano risultate più veloci di quelle riscontrabili in bottiglie mantenute in posizione verticale. Questi fenomeni sono chiaramente collegabili all'estensione della superficie di scambio tra i lieviti ed il liquido circostante, maggiore nelle bottiglie mantenute orizzontali. Tali dati sono ricavati da un più rapido sviluppo della pressione durante le prime due settimane e da una diversa evoluzione dei parametri relativi alla schiumabilità e alle caratteristiche sensoriali. In particolare è apparso che, quando la fermentazione alcolica avviene in posizione orizzontale (tesi O e O/V) si osserva un miglioramento del volume della schiuma, probabilmente legato al maggior rilascio di molecole dai lieviti rispetto ai vini mantenuti sempre in posizione verticale.

Dalle analisi sensoriali, al contrario, è emerso che mantenere il vino in posizione orizzontale (tesi O) oltre le 2 settimane, in cui avviene la fermentazione, comporta una riduzione nella percezione degli aromi floreali e fruttati tipici del Prosecco a favore della comparsa di sentori di lievito. Benché una evoluzione di questo tipo sia desiderata nei vini prodotti con il Metodo

Classico, è invece considerata sfavorevole per il Prosecco. In conclusione, condurre la fase di fermentazione alcolica (primi 15 giorni) mantenendo le bottiglie in posizione orizzontale sembrerebbe quella che permette di raggiungere il miglior compromesso tra armonia olfattivo-gustativa e schiumabilità.

Infine, se dal punto di vista organolettico l'effetto della posizione della bottiglia si può notare solo dopo 3 mesi di conservazione, tempo compatibile con la messa in commercio del prodotto, le caratteristiche della schiuma possono essere migliorate mantenendo, per i primi 15 giorni, le bottiglie in posizione orizzontale. Il cambiamento di posizione, da orizzontale a verticale, dopo questi primi 15 giorni, da una parte garantisce la possibilità di deposito dei lieviti sul fondo della bottiglia, dall'altro sembra permettere una migliore conservazione nel tempo degli aromi freschi caratteristici del Prosecco Sur Lies, consentendo così una maggiore flessibilità nei tempi di commercializzazione.

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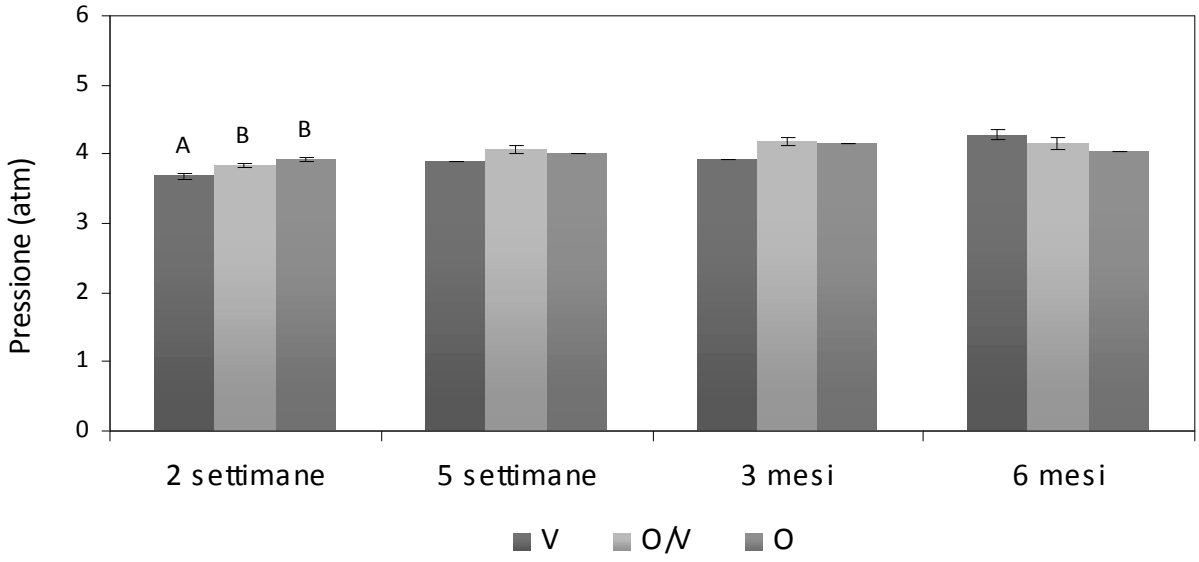

Fig. 1. Evoluzione della pressione nella massa A.

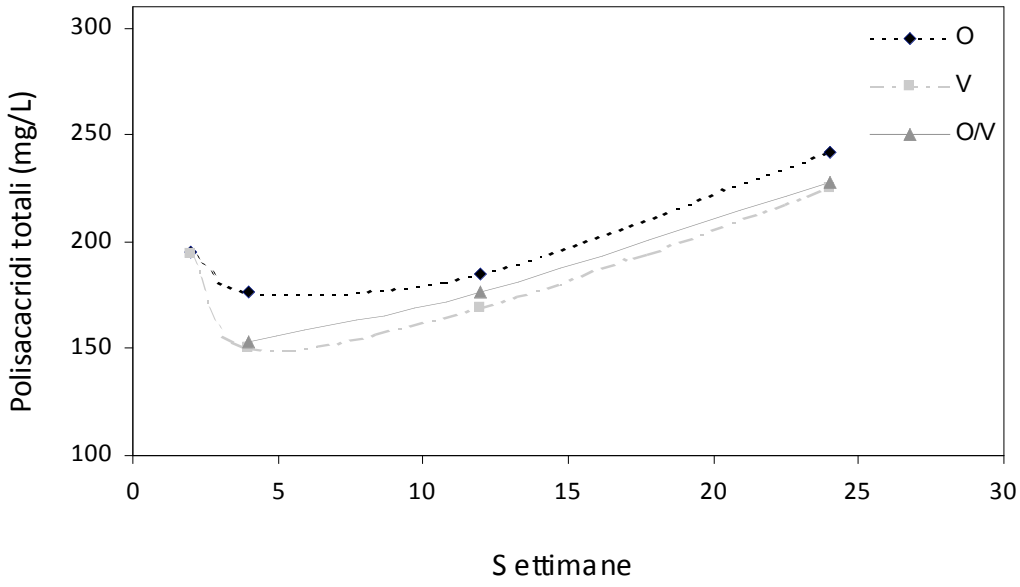

Fig. 2. Contenuto in polisaccaridi totali nella massa A.

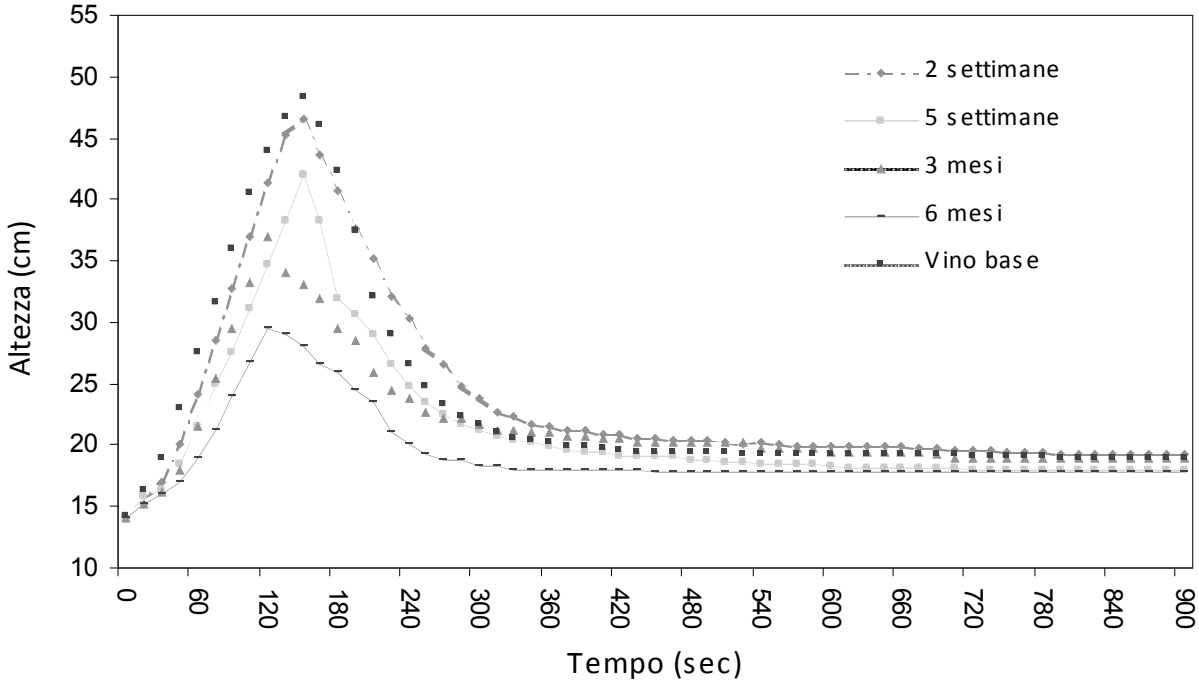

Fig. 3. Andamento della curva di schiumabilità relativa alla posizione O nella massa A

Tab. 1. Parametri di schiumabilità nella massa A.

Tesi	Tempo	HM (cm)	HS (cm)	TS (sec)
V	2 settimane	52.2 ± 1.0	19.1 ± 0.4	17.7 ± 0.6
	5 settimane	46.3 ± 0.6	18.0	17.7 ± 0.6
	3 mesi	35 ± 4.0	18.5	15.8 ± 0.4
	6 mesi	27.5 ± 3.9	17.2 ± 0.3	16.3 ± 0.6
O	2 settimane	47.8 ± 2.3	19.3 ± 0.3	20.7 ± 0.6
	5 settimane	43.2 ± 0.8	18.0	20.3 ± 0.6
	3 mesi	37 ± 4.0	19.0	18.3 ± 0.4
	6 mesi	23.3 ± 0.6	17.7 ± 0.3	17.0
O/V	2 settimane	48.3 ± 3.8	19.0	19 ± 2.6
	5 settimane	46 ± 1.0	18.1 ± 0.1	19 ± 1.0
	3 mesi	30.5 ± 3.0	18.9 ± 0.7	16.9 ± 0.8
	6 mesi	29.0	17.5	16.0

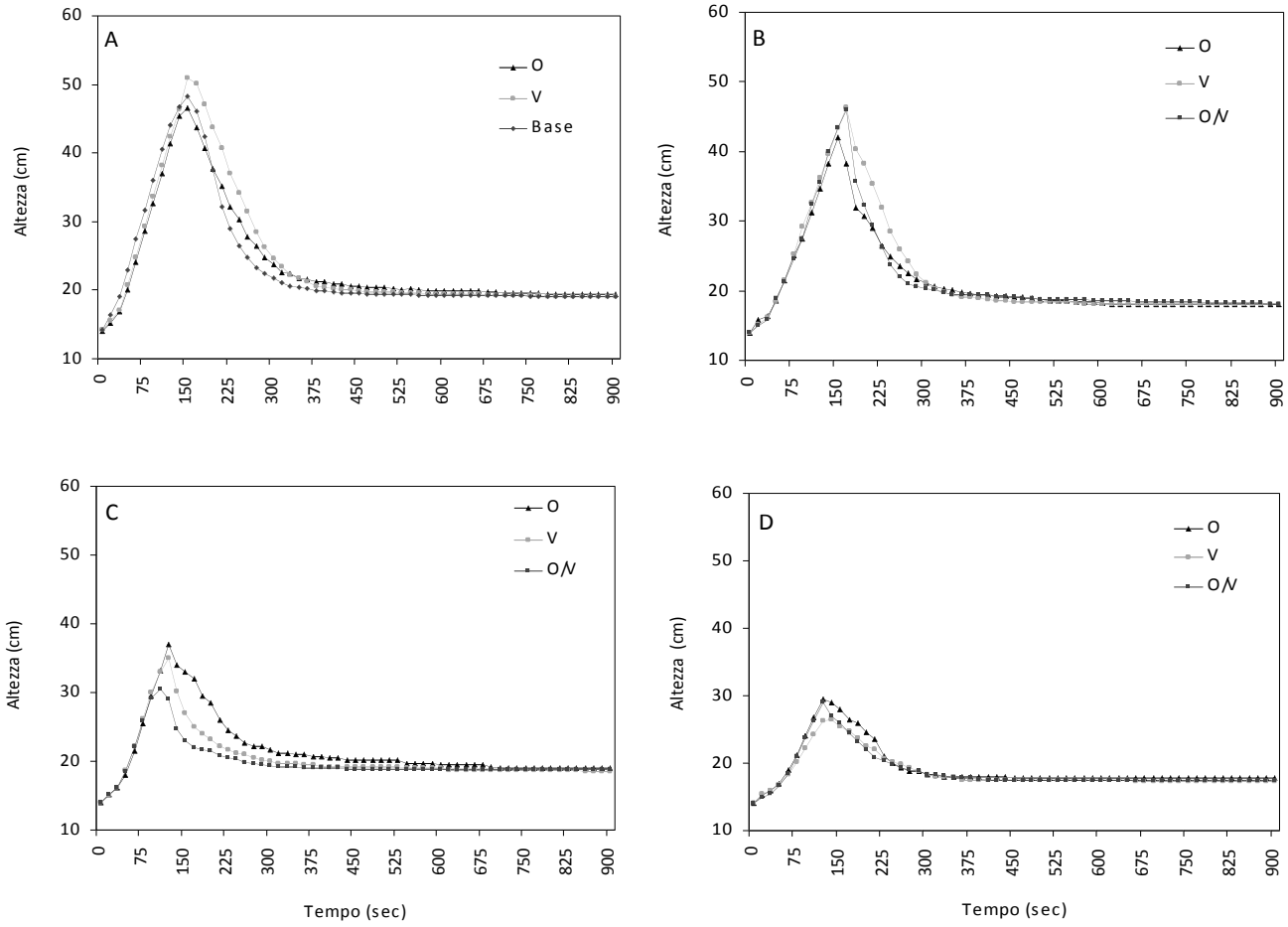

Fig. 4. Andamento della curva di schiumabilità a 2 settimane (A), 5 settimane (B), 3 mesi (C), 6 mesi (D) dall'imbottigliamento nella massa A.

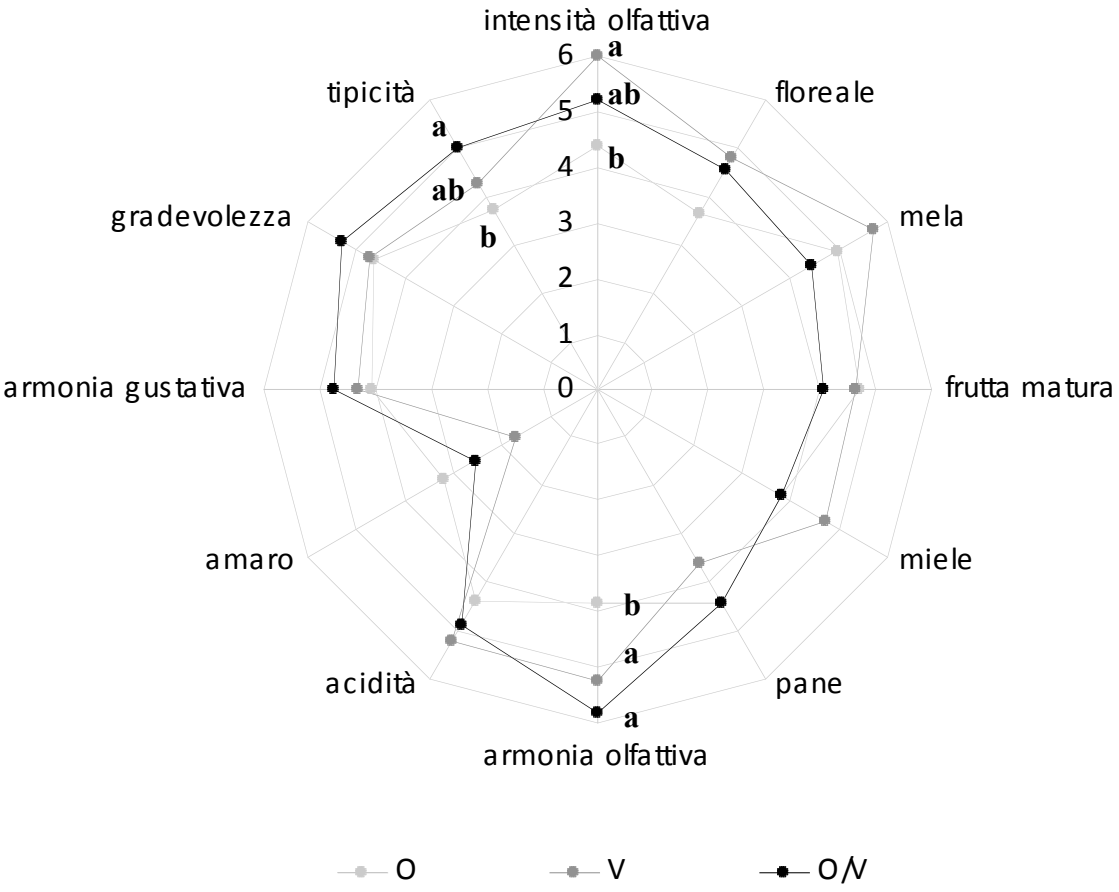

Fig. 5. Descrittori olfattivi e gustativi a 6 mesi dall'imbottigliamento nella massa A.