Dicarboxymethylcellulose as a bentonite alternative to prevent the protein haze phenomenon

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Resumo

Um dos parâmetros organoléticos mais importantes para um consumidor quando avalia um vinho branco é a sua limpidez. Este parâmetro pode ser afetado por diferentes fatores, entre eles a precipitação de proteínas instáveis. De forma a evitar a precipitação destas proteínas, estas são normalmente removidas utilizando bentonite. Neste trabalho foi proposto pela primeira vez a utilização de um novo agente colante, alternativa à bentonite, para a remoção de proteínas instáveis de vinho branco. Vinhos tratados com dicarboxymethylcellulose (DCMC) foram comparados com vinhos colados com bentonite comercial de forma a avaliar a eficiência de remoção por parte do polímero. O polímero dicarboxymethylcellulose teve um grande impacto em todas as amostras, obtendo-se vinhos estáveis do ponto de vista proteico com baixos teores de polímero (quando comparado com a dose necessária de bentonite). Os resultados deste trabalho mostram que o polímero dicarboxymethylcellulose pode ser uma alternativa viável à bentonite na remoção de proteínas instáveis de vinhos brancos.

Palavras chave: Proteínas PR, fenômeno de neblina, bentonita, DCMC, vinho branco.

Abstract

One of the most important parameters required by consumers for a white wine is translucency. This parameter is influenced by the formation of haze that can occur due to various factors, mainly proteins. To remove the protein material from the wines, the most commonly used product is bentonite.

This work proposes for the first time a new fining agent that can remove haze proteins from white wines as an alternative to bentonite.

Wines treated with (dicarboxymethylcellulose) DCMC, with doses of 0.5, 1, 1.5 and 2 g/L have been compared with clarified wines with a type of commercial bentonite with the same doses, to assess the effectiveness of the polymer. In all wines the dicarboxymethylcellulose has had a significant impact, furthermore the stability or near stability was reached with low levels, when compared to bentonite, by the dicarboxymethylcellulose.

The results of this work indicate this polymer can be a great alternative to bentonite for haze protein removal from white wines.

Keyworks: PR proteins, haze phenomenon, bentonite, DCMC, white wine.

Resumo alargado

Um dos parâmetros mais importantes exigidos pelos consumidores para um vinho branco é a translucidez. Este parâmetro é influenciado pela formação de turvação que pode ocorrer devido a vários fatores, principalmente proteínas. Para remover o material proteico dos vinhos, o produto mais usado é a bentonite. Apesar de seu uso geral e mundial, a bentonite causa perdas na ordem de mil milhões de US $ por ano. Embora, nos últimos anos, muitos estudos tenham sido realizados com outros agentes de colagem que possam eventualmente substituir a bentonite, até hoje nenhum alcançou a mesma eficácia.

Este trabalho propõe, pela primeira vez, a utilização de um novo agente de *fining* capaz de remover proteínas formadoras de turvação dos vinhos brancos como uma alternativa equivalente ou até melhor do que a bentonite. Em particular, os vinhos tratados com dicarboxymethylcellulose (DCMC) foram comparados com os vinhos clarificados com bentonite comercial, em quatro doses diferentes, para avaliar a eficácia na quantidade de proteínas removidas e na estabilidade das proteínas, no aumento do pH, na alteração da quantidade de polifenóis totais e no aumento ou diminuição de certos minerais, como o cálcio e o sódio. Estas avaliações foram realizadas em três vinhos com diferentes graus de instabilidade proteica e com diferentes teores de proteínas, de modo a avaliar a eficácia do polímero em diferentes concentrações proteicas. O dicarboxymethylcellulose teve um impacto significativo nos três vinhos analisados. Além disso, a estabilidade total ou quase total do vinho foi alcançada com baixos níveis de dicarboxymethylcellulose, quando comparado com a bentonite. Os resultados deste trabalho indicam que este novo polímero pode ser uma ótima alternativa à bentonite na nossa luta para evitar a formação turvação proteica em vinhos brancos.

Palavras chave: Proteínas PR, turvação proteica, bentonite, DCMC, vinho branco.

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TLPs: Thaumatin-like proteins

PR: Pathogenesis-related

DSC: differential scanning calorimetry

DCMC: Dicarboxymethylcellulose

HST: Heat stability test

1. Introduction

The first impression that the consumer has on the quality of a wine is the translucency - in fact, regardless of the other inherent characteristics, the consumer will reject this product if it contains cloudy precipitates (Ferreira *et al.,* 2004).

Among the different substances present in wine, there are varying amounts of different nitrogenous substances, amongst which proteins. These molecules usually present in concentrations from 15 to 300 mg/L do not contribute significantly to the nutritive value of wines, but they may cause haze formation which results from the slow denaturation of wine proteins (Ferreira *et al.,* 2002; Waters et *al.,* 2005). Therefore, their presence, mainly the unstable proteins, is of great concern to winemakers (Ferreira *et al.,* 2004).

Wine protein denaturation occurs as a result of unfavourable storage conditions, leading to protein aggregation and flocculation which causes a deposit in the bottled wine or the appearance of a haze (Ferreira *et al.,* 2004).

Although wine protein haze does not present health risks (Marangon *et al.,* 2011b) or influences the organoleptic characteristics of wines (Peng *et al.,* 1997), its presence causes a loss of quality in the perception of consumers. For this reason, these proteins, specially chitinases and thaumatin-like proteins (TLP) must be removed in some circumstances before bottling (Waters *et al.,* 1998).

It is the winemaker's job to forecast if proteins need to be removed from wines using different tests on proteins and key components of the wine (Toledo *et al.,* 2017), because formation of haze in white wines is also affected by non-protein components (Waters *et al.,* 2005; Pocock *et al.,* 2007; Marangon *et al.,* 2011a).

To remove the protein material from wines and to avoid this defect, the most commonly used product employed at the industrial level during the clarification operation is bentonite (Toledo *et al.,* 2017). Bentonite seems to be the only clarifier capable to stabilize and prevent the formation of haze after bottling (Van Sluyter *et al.,* 2015). In fact, comparing six different clarifiers, Chagas and colleagues (2012) have shown that bentonite is the only clarifying agent able to ensure wine protein stability. Nevertheless, Sauvage *et al.,* (2010) noted that even with doses of 1.5 g/L bentonite, 30% of thaumatin remained in the stabilized wine. This is important because previous studies (Waters *et al.,* 1992; Pocock *et al.,* 2007) used model wines and protein-free wines, to demonstrate that TLPs alone cause 50% more haze than chitinases. Moreover, it is argued that bentonite use negatively affects the quality of the treated wine since this clarifier is a non-selective entrainment agent - In fact, under certain conditions it removes compounds, such as those involved in flavor, from the matrix of the product (Ribéreau-Gayon *et al.,* 2006).

For world wine production, it has been estimated that the costs of clarification treatments with bentonite are to about $1000 million/year (Majewski *et al*., 2011), and that from 3% to 10% of the volume of treated wine is lost in the form of lees following clarification treatments (Tattersall *et al.,* 2001).

Although there are numerous works in wine protein, the precise factors involved and the mechanism of protein haze formation remain largely to be elucidated (Mesquita *et al.,* 2001).

1.1 Protein haze phenomenon

The protein haze phenomenon in white wines has been considered a multifactorial process, attributed to various protein and non-protein factors (Chagas *et al.,* 2018).The mechanism underlying this phenomenon is cited as a two-stage process, the first in which the unfolding of proteins occurs due to stimuli such as high storage temperature, and in the second the unfolded proteins aggregate and flocculate forming a visible haze. However, all the mechanisms associated with this process are not yet clear (Dufrechou *et al.,* 2010).

1.1.1 Wine proteins

The protein haze phenomenon is obviously mainly due to the presence of proteins (McRae *et al*., 2018), specifically the pathogenesis-related (PR) proteins, such as TLPs and chitinases (Marangon *et al*., 2011b; Van Sluyter *et al*., 2015). In fact, Marangon *et al.,* (2011b) demonstrated the correlation between chitinases content and haze potential, while Mesquita *et al.* (2001) noted that increasing the concentration of proteins causes, under specific conditions, a greater quantity of produced haze. These are proteins of the grapes that unfold and subsequently aggregate causing the defect (Marangon *et al*., 2011b; Van Sluyter *et al*., 2015). They have different deployment and aggregation behaviors, meaning that different wine protein compositions can influence this defect (Marangon *et al.,* 2011a; Gazzola *et al*., 2012; Van Sluyter *et al.,* 2015).

As their name implies, PR proteins are plant defence proteins. In fact, they can prevent or limit the multiplication or spread of pathogens (Van Loon *et al.,* 1999). Due to their high stability, in part derived from the high number of disulfide bonds they contain, they exhibit high resistance against the enzymatic activity of the invading pathogens (Waters *et al.,* 1996; Pocock *et al.;* 2000; Marangon *et al.,* 2011b). They are constitutively accumulated in grapes during the growing season (Tattersall *et al.,* 2001) in healthy berries, generally after veraison (Ferreira *et al.*, 2002). Indeed, after this phenological phase there is a significant increase in grape resistance to pathogen attack, in part derived from the accumulation of PR proteins which reach high concentrations, independently of the exposure to the pathogen, when compared with only a small amount of PR proteins that are synthesized during maturation (Tattersall *et al.,* 1997). PR proteins are also be produced the pre-veraison phase, this time

in response to biotic and abiotic stresses, such as wounds, chemical elicitors and pathogen attack (Jacobs *et al.,* 1999; Robinson and Davies., 2000; Monteiro *et al.,* 2003a, b).

Consequently, all these processes influence the level and proportion of PR proteins in grapes. Therefore, environmental conditions during vegetative growth determine the pattern of proteins that accumulate in grapes (Monteiro *et al*., 2003a, b). In addition, even the vintage (Monteiro *et al.,* 2003b), cultivar (Hayasaka *et al.,* 2001) and harvesting conditions can influence their overall composition (Pocock *et al.,* 1998).

Based on their different structures, 17 different classes of PR proteins (Ferreira *et al.,*2007) have been found, some of which are also present in *Vitis vinifera* (Ferreira *et al.,* 2004). In these classes, there are TLPs and chitinases (Ferreira *et al.*, 2004), considered as the two main soluble grape protein families (Robinson and Davies., 2000) and those most responsible for the haze defect (Dawes *et al.,* 1994).

1.1.1.1 Characteristics of PR proteins

TLPs and chitinases are two classes of PR proteins that are compact and with a net positive charge at the pH of the wine, pH at which they remain soluble (Waters *et al*., 2005, Ferreira *et al*., 2002). They are characterized by resistance to low pH values and to proteolytic attack, as well as by having a low molecular mass (<35 kDa) (Marangon *et al*., 2014). Fig. 1 shows the polypeptide profile of wine I10 Chardonnay used in the work of Sauvage *et al.* (2010). The molecular mass of the polypeptides ranged from 14 to 66 kDa, although most of them possessed between 20 and 30 kDa.

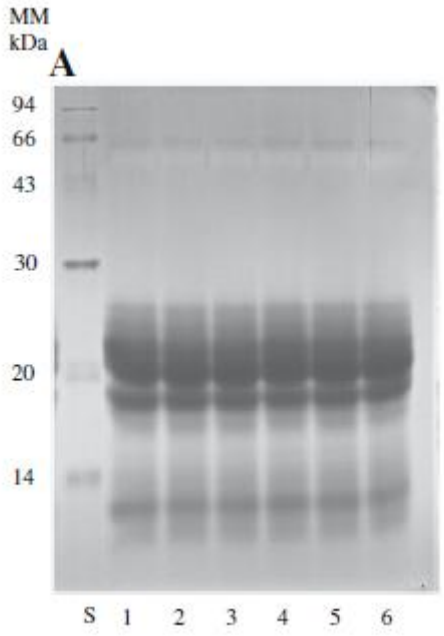


Figure 1. *Polypeptide profiles in the I10 Chardonnay wine obtained by 1D SDS–PAGE electrophoresis (14% w/v acrylamide gel) for a deposited volume equivalent to 400 µL of*

wine. Lane S: Molecular mass markers (kDa); Lanes 1 to 6: Different aliquots of wine I10 Chardonnay (Sauvage *et al.,* 2010).

Sauvage *et al.* (2010) further extracted and separated the polypeptides from the Chardonnaywine by 2D electrophoresis for subsequent identification by mass spectrometry, showing that they were TLPs (spots a1, a2, a3, and a5), chitinases (spots a4 and a6), glucanases (spots a7 and a8) and invertases (spots a9 and a10) (Fig. 2).

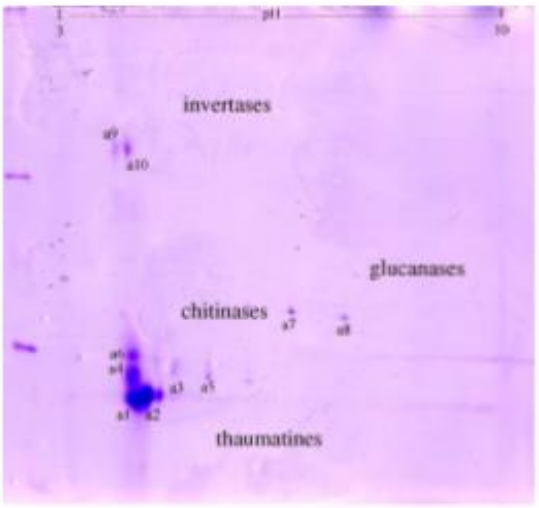


Figure 2. *Polypeptide profile obtained by 2D-electrophoresis separation and staining using CBB-G-250. Proteins in the Chardonnay wine (I10) were identified by mass spectrometry as TLPs (spots a1, a2, a3 and a5), chitinases (spots a4 and a6), glucanases (spots a7 and a8) and invertases (spots a9 and a10)* (Sauvage *et al.,* 2010).

Chitinases are a class of chitin-cleaving enzymes which exhibit a general sensitivity to temperature changes (Falconer *et al.,* 2010), showing less resistance to temperatures than TLPs, and undergoing unfolding at lower temperatures indicating that they are less stable, as shown in Fig. 3.Once the unfolded, due to exposure to their melting temperature (55 °C), chitinases are unable to return to their original structure again after cooling, leading to protein aggregation and subsequent precipitation (Falconer *et al.,* 2010).

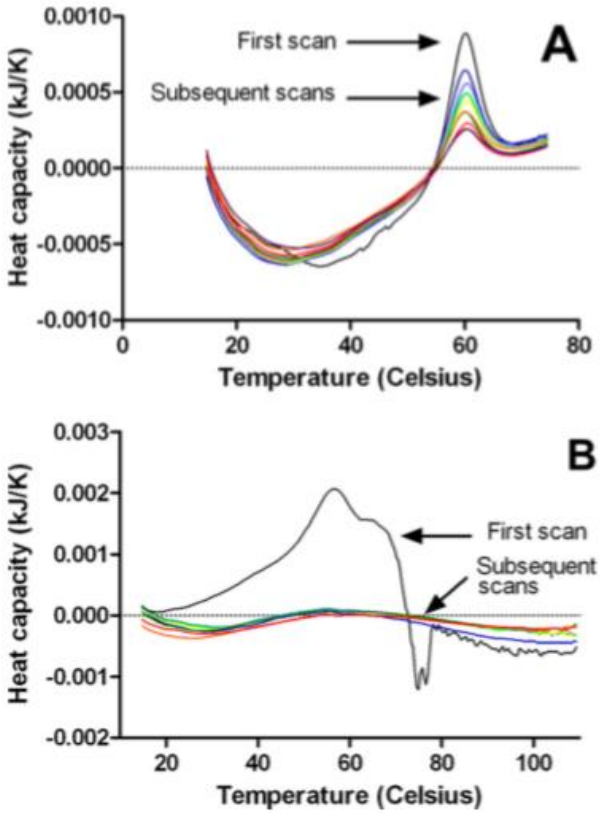


Figure 3. *(A) Repeated differential scanning calorimetry (DSC) scans of TLP C from Semillon grape juice showing a melt temperature of 61 °C and reversibility of thermal unfolding. (B) Repeated DSC scans of chitinase F1 from Sauvignon blanc grape juice showing a melt temperature of 55 °C, no reversibility of thermal unfolding, and aggregation after unfolding.* (Falconer *et al.,* 2010).

Furthermore, based on the type of protein and based on the different temperature (Fig. 4), TLP and chitinases form aggregates with different characteristics, as shown, for example, in table 1: the chitinases can rapidly flocculate and produce large aggregates visible to the naked eye (≥1 µm), whereas TLPs generally produce metastable micro aggregates not visible to the naked eye (<150 nm at normal wine ionic strength) (Dufrechou *et al.,* 2010, Marangon *et al.,*2011a).

Table 1*. Summary of general properties of chitinases and TLPs. Adapted from* Van Sluyter *et al.* (2015).

property	chitinases	stable TLPs
unfolding temperature	55 °C	61–62 °C
aggregate characteristics	visible aggregates (≥1 μm)	microaggregates (<150 nm)
aggregation tendency	self-aggregate	cross-linked with other wine components

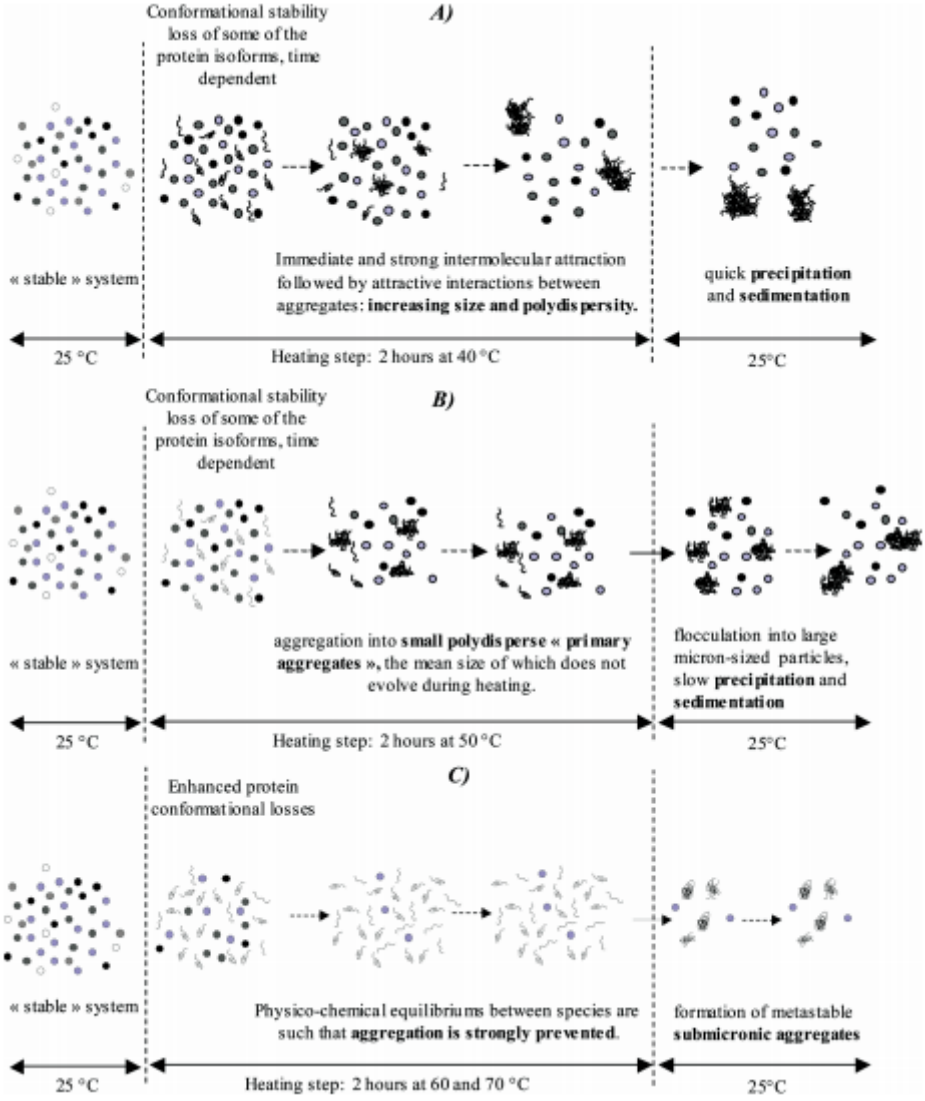


Figure 4. *Schematic representation of protein aggregation in the Sauvignon Blanc wine must during heating at increasing temperatures and cooling, illustrating the temperature impact on physicochemical equilibria and aggregation kinetics. (A) 40 ºC, (B) 50 ºC, (C) 60 and 70 ºC (*Dufrechou *et al.,* 2010).

The differences between these two protein families seem to derive from their different overall structure, that has been described as globular for TLP and elliptic for chitinase (Tattersall *et al.,* 2001; Dufrechou *et al.,* 2013).

Other proteins have also been found which have been shown to contribute to the haze phenomenon, such as β-glucanases, invertases, and osmotins, although they are usually present in much lower amounts than chitinases and TLPs (Esteruelas *et al.,* 2009, Sauvage *et al.,* 2010).

In addition, the different aggregation behaviors of proteins and other wine components can facilitate or prevent the formation of haze. Thus, for example, TLPs are forced to interact with salts and polyphenols which contribute to the haze phenomenon, in contrast to chitinases which can aggregate among them after unfolding and cooling (Marangon *et al.,* 2011a, Dufrechou *et al.,* 2012).

Given the physiological role of TLPs and chitinases, it was thought to modify genetically vine plants in order to increase resistance to pathogens, but due to their characteristics (resistance to proteolytic attack and to low pH values) it is not easy to eliminate them from juices or wines, and this makes their presence a technological discomfort for the influence they have on the clarity and stability of wines (Ferreira *et al.,* 2002).

1.1.2 Non-protein factors

Several components of the wine must be the possible drivers during the two-stage model of haze formation, thus influencing the phenomenon. Furthermore, the temperature can be also influencing the mechanism of protein unfolding, since higher temperatures lead to more rapid protein unfolding. However, temperature alone is not enough to explain the formation of haze that can occur during wine storage, meaning that other non-protein factors should play an important role together with the temperature.

The main known components of the wine matrix that can influence haze formation are pH, ionic strength, phenolic substances, sulphates and sulphur dioxide (Dufrechou *et al.,* 2010, Marangon *et al.,* 2011b; Gazzola *et al.,* 2012; Marangon *et al.,* 2012; Dufrechou *et al.,* 2013).

1.1.2.1 Ionic strength

In white wine, the presence of the haze-forming proteins in solution, bearing a net positive charge at the pH of the wine, increases the ionic strength thus decreasing the electrostatic repulsion among protein molecules (Findenegg *et al.,* 1991). This happens because when the proteins unfold, they expose hydrophobic binding sites, which are normally buried in the core of the proteins, and thanks to the high ionic strength, they can aggregate more easily in a process triggered by hydrophobic interactions (Marangon *et al.,* 2010).

In fact, thanks to the use of the heat stability test and the increase in ionic strength, Marangon *et al.* (2011a) showed that aggregation/precipitation of chitinases, specifically of two isoforms called M1 and O, was strongly influenced by ionic strength (Fig. 5).

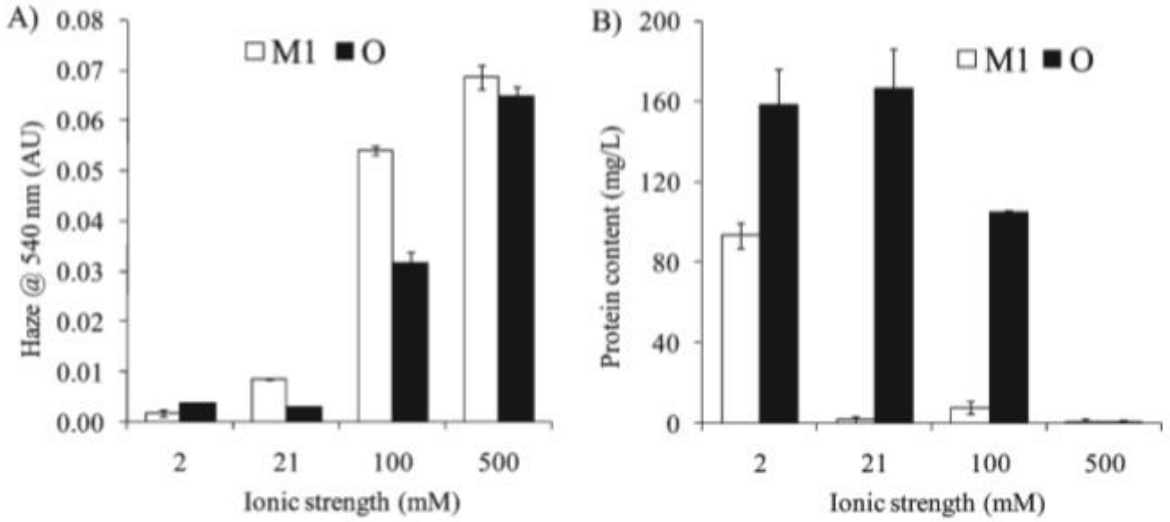


Figure 5. *Chitinases were dissolved in model wine solution containing increasing dosages of NaCl to obtain ionic strength levels of 2 mM (no salt), 21 mM (1.23 g/L NaCl), 100 mM (5.85 g/L NaCl), and 500 mM (29.25 g/L NaCl): (A) haze (at 540 nm) of samples after heat test (analyses were performed after samples were cooled for 20 h at 25 °C); (B) protein content (measured by EZQ protein quantitation kit) in the supernatant obtained after centrifugation of samples (21000* g*, 15 min, 15 °C) following heat test. Protein contents in the untread samples were 93.2 mg/L ± 12.4 mg/L and 158.2 mg/L ± 35.8 mg/L for M1 and O, respectively (*Marangon *et al.,* 2011a).

Regarding the TLPs, in particular two isoforms called M2 and N are less susceptible to a varying ionic strength than chitinases, formation of haze was observed exclusively for the highest values of ionic strength (Marangon *et al.,* 2011a) (Fig 6).

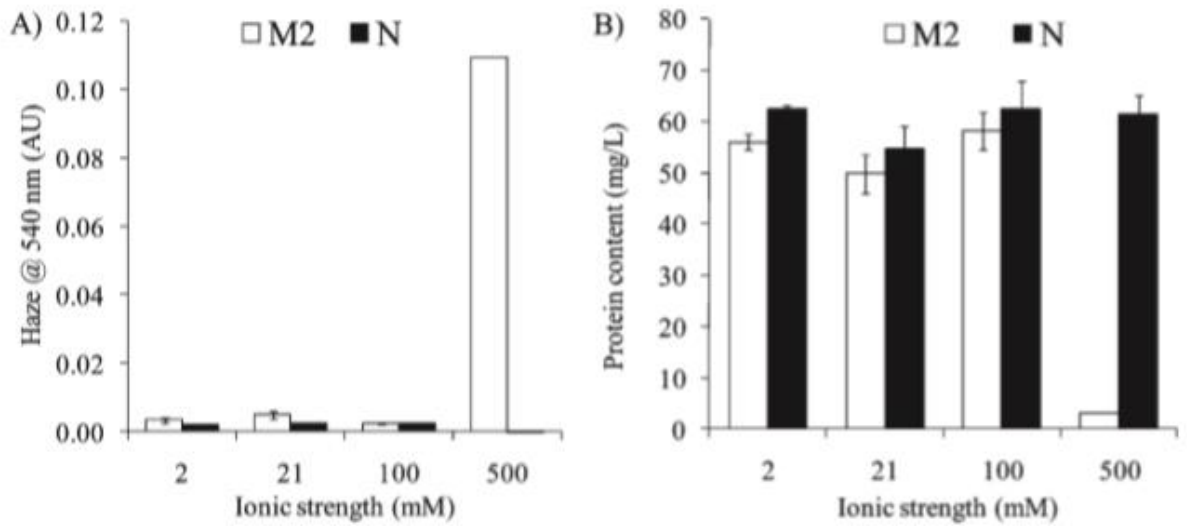


Figure 6. *TLPs were dissolved in model wine solution containing increasing dosages of NaCl to obtain ionic strength levels of 2 mM (no salt), 21 mM (1.23 g/L NaCl), 100 mM (5.85 g/L NaCl), and 500 mM (29.25 g/L NaCl): (A) haze (at 540 nm) of samples after heat test (analyses were performed after samples were cooled for 20 h at 25°C); (B) protein content (measured by EZQ protein quantitation kit) on the supernatant obtained from centrifugation of samples (21000g, 15 min, 15°C) after heat test. Protein contents in the untreated samples were 56.1 mg/L ± 2.9 mg/L and 62.5 mg/L ± 1.4 mg/L for M2 and N, respectively (*Marangon *et al.,* 2011a).

In addition, from this work, Marangon *et al.* (2011) observed that unfolding did not induce aggregation. In fact, no aggregation was observed at high temperatures, but the subsequent cooling induced the onset of aggregation, likely associated with a decrease of thermal agitation.

1.1.2.1 Sulphates and sulphur dioxide

The presence of sulphates or the sulphur dioxide (SO_2) can influence protein aggregation.

The sulphates anions can interact strongly with water, weakening the hydrogen bonds established between proteins and water, thus leading to protein salting-out and aggregation. In this way they allow aggregation to suppress the electrostatic repulsion and even promoting hydrophobic interaction-driven aggregation through kosmotropic effects (Marangon *et al.,* 2011a).

Sulphates do not interact in the process of unfolding PR proteins during heating, but their role is thus to favour protein aggregation when unfolded (Falconer *et al.,* 2010).

The sulphur dioxide instead, interferes with the formation of new disulphide bonds based both on its reducing action and also on its ability to promote sulphonation of SH groups (Bailey & Cole, 1959) involving the formation of inter and intra molecular disulphide bonds

thus inducing the aggregation (Chagas *et al*., 2018). This phenomenon leads to a new conformational change by exposing internal hydrophobic sites that could react with other non-polar sites, thus inducing aggregation and precipitation (Chagas *et al*., 2016). This reaction occurs mostly with the TLPs which at high temperature, in the presence of sulphur dioxide, involves the formation of new configuration states of the protein that precipitates partially during cooling giving rise to quite large protein aggregates (Chagas *et al*., 2018).

1.1.2.2 pH

Another factor that affects the stability of proteins in wines is pH. At room temperature, the change in pH from 2.5 to 4.0 is enough to expose hydrophobic sites of the chitinases that facilitate protein aggregation. In contrast, invertases and TLPs remain stable under the same conditions (Marangon *et al.,* 2011a).

From the work of Batista *et al.* (2009) it has observed that the pattern of turbidity of the Arinto wine used during the experiment, with a protein concentration of 280 mg/L, does not change significantly with pH within the range 2.8–3.8, as shown in Fig. 7.

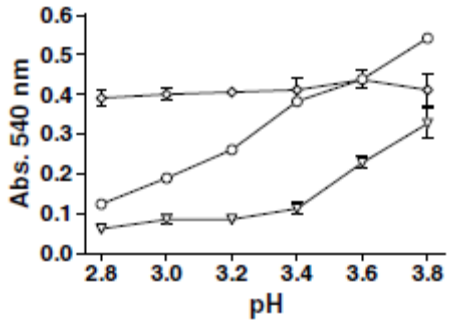


Figure 7. *Heat stability tests of the Arinto wine, first line, (naturally containing 280 mg/L) or of an aqueous solution containing 280 mg/L of the isolated Arinto wine protein, second line, or of a model wine solution composed of isolated protein (thirst line) (280 mg/l), ethanol (12% v/v) and tartaric acid (4 g/l) (Waters et al., 2007)were performed at different pH values (2.8, 3.0, 3.2, 3.4, 3.6 and 3.8). All experiments were performed in triplicate. Vertical bars represent plus or minus the standard deviation, shown when bar is bigger than symbol (*Batista *et al.,* 2009).

The turbidity observed in Fig. 7 formed at pH 2.8 and at pH 3.8 are different. The haze is smooth and homogeneous at pH 2.8 and coarse and flocculated at pH 3.8 of Arinto wine (Fig. 8). This is because there is an increment in the average particle size when the pH increases from 2.8 to 3.8. These observations were confirmed by particle size analysis, as shown in Table 2.

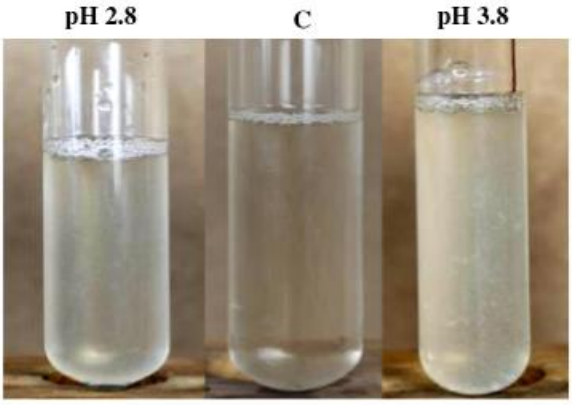


Figure 8. *Samples of Arinto wine were adjusted to pH 2.8 or to pH 3.8 and subjected to the heat stability test. Control (C): Arinto wine that was not submitted to the heat treatment (*Batista *et al.,* 2009).

Table 2. *Samples of Arinto wine were adjusted to pH 2.8 or to pH 3.8 and subjected to the heat stability test. Control (C): Arinto wine that was not submitted to the heat treatment (*Batista *et al.,* 2009).

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

1.1.2.3 Polyphenols

Polyphenols may also contribute to aggregation and precipitation of the proteins, probably as a result of hydrophobic interactions or hydrogen bonds, and subsequently to the formation of larger protein aggregates visible to the naked eye (Waters *et al.,* 1995; Siebert *et al.,* 1996; Batista *et al.,* 2010).

Among the polyphenol compounds responsible for this defect in white wines we find condensed tannins (flavan-3-ol oligomers and polymers), with molecular masses of 500–3000 Da (Serafini *et al.,* 1997), that play a relevant role in this process. The tannins can bind proteins, in fact they are responsible for the astringency in red wines (Gawel *et al.,* 1998).

Maragon *et al.* (2010) have observed that when the tannins are heated with the proteins, a strong haze developed, but when the proteins were pre-heated alone and only then exposed to tannins at room temperature (25 °C), the effect on haze was minimal. The authors reasoned that during heating the proteins expose new binding sites, to which polyphenols

may bind if present. In their absence, the proteins refold during the subsequent cooling (Fig. 9).

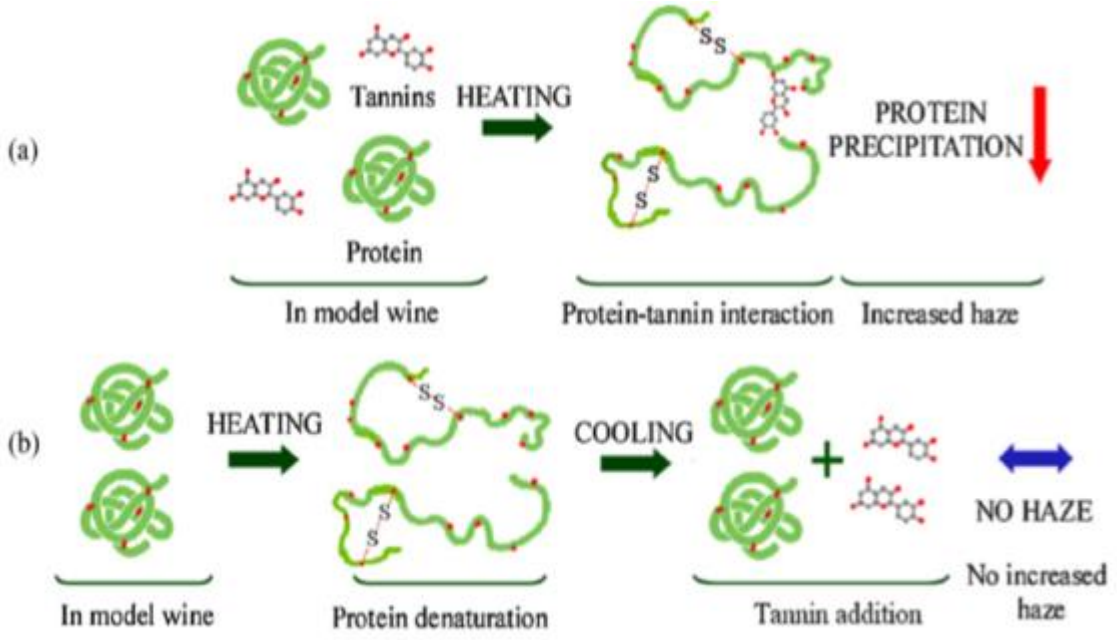


Figure 9. *Hypothetical effects of heating on protein–tannin interactions: (a) when proteins and tannins are heated together a strong haze develops due to the interactions of the phenols with the new binding sites exposed on the heat-denatured (unfolded) protein; (b) when proteins are pre-heated in the absence of tannins and then exposed to tannins at room temperature (25 ºC) the effect on haze is minimal, because proteins refold during cooling, burying the tannin-binding sites (*Marangon *et al.* 2010).

White wines are generally produced under non-oxidative conditions, so they should not contain oxidized phenolic compounds that are highly reactive (Marangon *et al.,* 2010).

1.2 Bentonite

The clarity of a wine, especially of a white wine, is a very important parameter for the consumer. For this reason, it is the winemaker's job to ensure stability from haze phenomenon during storage and transport (Van Sluyter *et al.,* 2015). A common practice in wine making countries to prevent the formation of protein haze is the addition of bentonite (Lambri *et al.,* 2010).

1.2.1 Characteristics of bentonite

Bentonite is the most widely used clarifier in wine industries to remove proteins. It is a montmorillonite clay which bears net negative charge at the pH of the wine capable of

interacting electrostatically with positively charged wine components, amongst which are the positively charged proteins, thus adsorbing them. This is possible due to the cation exchange that takes place - In fact bentonite has some ions such as Al^{3+} in its structure which are displaced by other cations such as Fe^{2+}, Mg^{2+} and Fe^{3+}, thus leading to charge imbalances and therefore a negative charge. This charge is partially balanced by other exchange cations such as Ca^{2+}, Na^{+} and Mg^{2+} (Lambri *et al.,* 2010). Depending on the type of cation exchange functions, bentonites can be subdivided into Na bentonite and Ca bentonite (Catarino *et al.,* 2008).

1.2.2 Factors that influence protein adsorption and bentonite doses

1.2.2.1 pH

The pH is the factor that most influences the adsorption of proteins by bentonite, since the degree of ionization and the surface charge of bentonite, as well as the proteins global charge depend on it (Xifang *et al*., 2007). Furthermore, the adsorption of proteins by bentonite occurs when the proteins change their structure (partial or total unfolding), allowing the establishment of physical-chemical bonds between the interaction sites of bentonite and the amino acids of the proteins (Haynes and Norde, 1995). This change of structure, and therefore the adsorption, occurs when pH is lower than the isoelectric point (Servageant-Noinville *et al.,* 2000; Gougeon *et al.,* 2003).

The isoelectric point of a protein is the pH value at which a protein has a net global charge of zero. At the pH of the wine the proteins are positively charged, allowing bentonite to remove them (Waters *et al.,* 2005).

1.2.2.2 Concentration of ethanol

Different concentrations of ethanol can change the effectiveness of bentonite adsorption towards proteins. Bentonite is generally swollen in water to obtain a separation of its layers so that the wine molecules can enter its structure. Since ethanol molecules are larger than water molecules, wines with a higher ethanol content induce greater separation of the layers, allowing proteins to enter the bentonite structure more easily (Blade and Boulton, 1988).

1.2.2.3 Type of harvest

One of the factors that can influence the doses of bentonite to be used is the type of harvest. Pocock *et al*. (1998) showed that grapes deriving from the same vineyard but harvested by machine rather than by hand, required almost doubling the amount of bentonite to prevent protein haze. This was not due to an increase in protein synthesis, but to the plant's response to stress such as mechanical harvesting.

1.2.3 Disadvantages of bentonite treatment

1.2.3.1 The loss of important aroma and flavour components

As a cation exchanger, bentonite is not a specific clarifier for proteins, therefore it also removes other positively charged aggregates or charged species. Large quantities of bentonite can remove important aroma and flavour components (Voilley *et al.,* 1990; Ribéreau-Gayon *et al.,* 2006). This occurs because the aroma compounds can interact with macromolecules, for example polysaccharides or protein, which are subsequently adsorbed by bentonite. Therefore, the removal of aroma and flavour components takes place as an indirect effect of de-protonization. Instead, some can be directly adsorbed by bentonite (Guichard *et al.,* 2006; Langourieux and Crouzet, 1997).

Furthermore, odor-active compounds can bind in two ways to hydrophilic proteins normally positively charged at the wine pH. If the aromatic compound is hydrophilic, it can bind the protein through weak hydrogen bonds; if instead the aromatic molecule is hydrophobic it can bind to the internal sites of proteins with a greater affinity. For this reason, most of the aromatic compounds inside the wine are removed (Armada *et al.,* 2007)

The interactions among aromatic compounds, proteins and bentonite depend on various factors such as the initial concentration of aromatic compounds, of the proteins and on the chemical nature of these molecules (Lambri *et al.,* 2010).

Among the aromatic compounds or their precursors, mostly eliminated following the treatment with bentonite are the ethyl esters and fatty acids (precursors of aromatic esters). Lambri *et al.* (2010) compared the removal of different aromatic compounds and their derivatives by three different types of bentonite at three different doses. As shown in table 3, the treatment always involves a reduction of aromatic compounds or their derivatives, in some cases significant as for the hexanoic acid and β-phenylethanol.

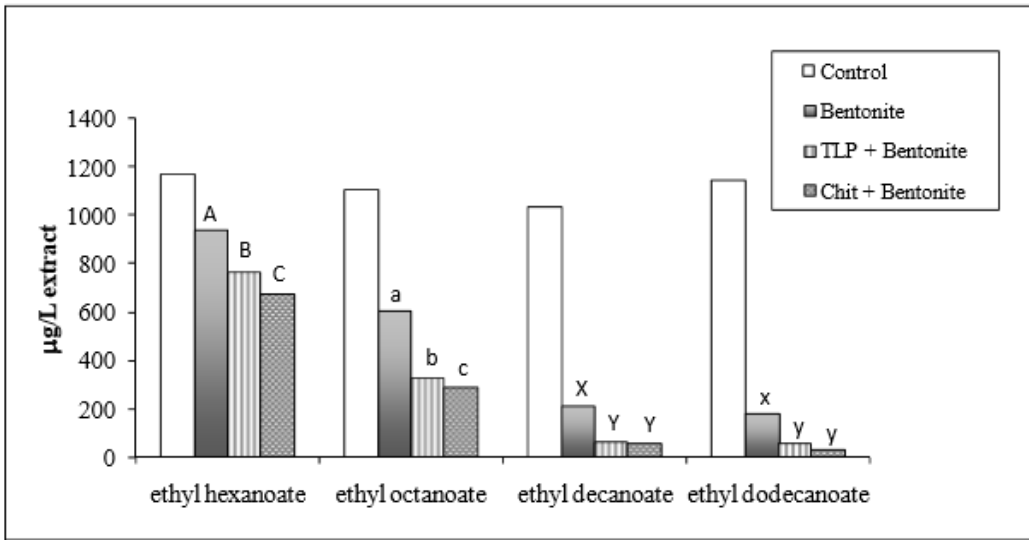
Table 3*. Odor-active compounds (µg/L) in Chardonnay A wine samples treated with bentonite (20, 50, and 100 g/hL) and in untreated control wine A* (Lambri *et al*., 2010).

		Experimental clay dose		
Compound	**Control A**	**20 g/hL**	**50 g/hL**	**100 g/hL**
Ethyl butyrate (µg/L)	3798 ± 228 a[a]	553 ± 21 cd	522 ± 16 d	411 ± 33 e
Ethyl hexanoate (µg/L)	777 ± 62 a	726 ± 28 a	552 ± 34 b	406 ± 28 c
Ethyl octanoate (µg/L)	114 ± 6 a	100 ± 11 ab	97 ± 11 ab	87 ± 10 ab
Isoamyl acetate (µg/L)	2975 ± 208 a	2910 ± 224 a	2168 ± 239 b	1285 ± 141 c
Phenylethyl acetate (µg/L)	812 ± 41 a	699 ± 25 b	629 ± 31 bc	571 ± 17 c
β-Phenylethanol (µg/L)	9595 ± 576 a	9417 ± 485 ab	8032 ± 802 b	7288 ± 802 bc
1-Hexanol (µg/L)	3362 ± 168 a	3040 ± 303 ab	2885 ± 375 ab	2513 ± 219 b
Hexanoic acid (µg/L)	92 ± 8 a	70 ± 6 b	53 ± 4 c	53 ± 5 c
Octanoic acid (µg/L)	1547 ± 155 a	1413 ± 285 a	222 ± 47 cd	163 ± 29 d

		Superbenton dose		
Compound	**Control A**	**20 g/hL**	**50 g/hL**	**100 g/hL**
Ethyl butyrate (µg/L)	3798 ± 228 a[a]	728 ± 44 b	677 ± 13 bc	591 ± 24 c
Ethyl hexanoate (µg/L)	777 ± 62 a	229 ± 16 d	209 ± 12 de	180 ± 14 e
Ethyl octanoate (µg/L)	114 ± 6 a	111 ± 12 a	97 ± 11 ab	96 ± 21 ab
Isoamyl acetate (µg/L)	2975 ± 208 a	1999 ± 100 b	1421 ± 185 bc	1176 ± 164 c
Phenylethyl acetate (µg/L)	812 ± 41 a	793 ± 42 a	672 ± 34 bc	545 ± 65 c
β-Phenylethanol (µg/L)	9595 ± 576 a	7218 ± 519 bc	7051 ± 704 bc	6923 ± 692 cd
1-Hexanol (µg/L)	3362 ± 168 a	3299 ± 160 a	3089 ± 133 a	1664 ± 98 c
Hexanoic acid (µg/L)	92 ± 8 a	86 ± 14 a	42 ± 8 cd	35 ± 4 d
Octanoic acid (µg/L)	1547 ± 155 a	339 ± 34 bc	291 ± 26 c	286 ± 29 c

		Top Gran dose		
Compound	**Control A**	**20 g/hL**	**50 g/hL**	**100 g/hL**
Ethyl butyrate (µg/L)	3798 ± 228 a[a]	784 ± 63 b	614 ± 55 c	222 ± 18 f
Ethyl hexanoate (µg/L)	777 ± 62 a	189 ± 26 e	138 ± 14 f	133 ± 12 f
Ethyl octanoate (µg/L)	114 ± 6 a	94 ± 23 ab	81 ± 15 b	81 ± 12 b
Isoamyl acetate (µg/L)	2975 ± 208 a	2027 ± 203 b	1796 ± 214 b	1699 ± 203 bc
Phenylethyl acetate (µg/L)	812 ± 41 a	621 ± 49 bc	589 ± 53 c	588 ± 58 c
β-Phenylethanol (µg/L)	9595 ± 576 a	6987 ± 699 cd	6479 ± 778 d	6353 ± 670 d
1-Hexanol (µg/L)	3362 ± 168 a	1163 ± 58 d	1101 ± 76 d	1019 ± 79 d
Hexanoic acid (µg/L)	92 ± 8 a	55 ± 5 c	31 ± 5 d	29 ± 5 d
Octanoic acid (µg/L)	1547 ± 155 a	478 ± 24 b	452 ± 28 b	419 ± 33 b

The interaction between protein and aroma or between aroma and bentonite affects only the fermentative esters, in fact the compounds deriving from grapes, such as terpenes, do not seem to be influenced by the bonds with TLPs and chitinases. Many authors have hypothesized that there may also be a synergistic effect between proteins and bentonite in the removal of aromatic compounds (Fig. 10) (Vincenzi *et al.,* 2015).



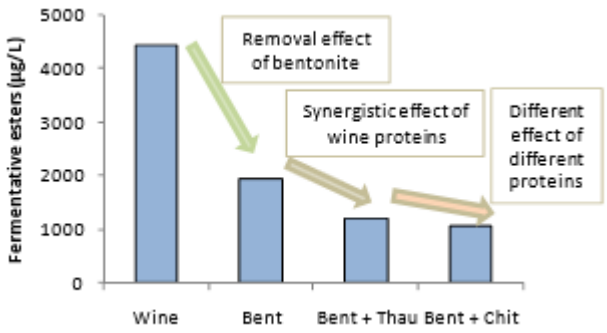


Figure 10. *Mean content of ethyl esters found in a model wine treated with bentonite alone or in presence of 100 mg/L of TLP or chitinase. Samples with different letters are significantly different (p<0.01)* (Vincenzi *et al.,* 2015).

1.2.3.2 The loss of quality of the wine recovered from the lees

The treatment with bentonite can involve a series of disadvantages such as the loss of quality of the wine especially of the wine recovered from the lees (Muhlack *et al.,* 2006). This because the wine could undergo an oxidation process due to the recovery of the wine from the lees through the filtration of the rotating drum vacuum (Waters *et al.,* 2005).

1.2.3.3 Problems arising from its application

Bentonite also presents a health risks of workers for the inhalation of powder, can create interference with increasingly common membrane-based winemaking technologies, treatment has a downtime (Salazar *et al.,* 2007) and it also involves a loss of the wine (Tattersall *et al.,* 2001).

1.2.3.4 Problems with PR proteins

Although bentonite is the most widely used clarifier to prevent haze in white wines, and although chitinase and TLPs show intermediate behaviours to treatment, a specific part of TLPs (about 30%) are generally not affected by bentonite doses less than 150 g/hL. In fact, total adsorption of TLPs is obtained with doses of 200 g/hL, high doses (Sauvage *et al.,* 2010), as illustrated in Fig. 11.

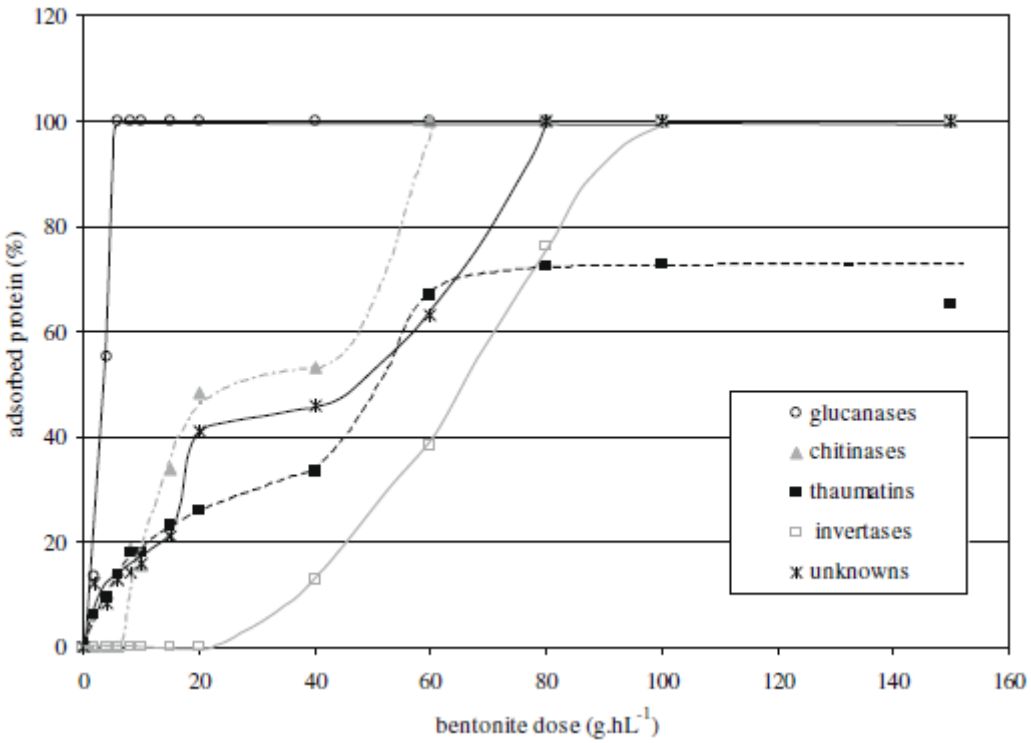


Figure 11. *Percentage of adsorbed proteins as a function of the bentonite dose, represented for each different protein family. Results showed selectivity in protein removal by the clay particles* (Sauvage *et al.,* 2010).

1.3 Bentonite alternatives

To prevent wine protein haze, is possible to use also other strategies in addition to bentonite fining, such as reduction of polyphenol concentration, decrease in the ionic strength of the wine, enzymatic degradation of proteins, ultrafiltration to remove proteins, interruption of hydrophobic interactions among proteins, and the use of other fining agents.

Reducing the amount of polyphenol from wine or decreasing the ionic strength are processes that inevitably modify wine sensory attributes. The interruption of the interactions among proteins is instead an applicable process, for example thanks to the addition of mannoproteins deriving from yeasts. In this way, it is possible both to hinder the interactions among proteins to even to stabilize them. However, it is not clear if these proteins remain protected from denaturation or if they protect proteins when they are already denatured. Another alternative to reduce the interactions among proteins could be the addition of surfactants to wine (molecules able to stabilize emulsions), such as polysorbates, but currently their use is not allowed. Moreover, they could have a negative influence on the foam of sparkling wines.

The most promising alternative to the use of bentonite is the use of enzymes capable of degrading proteins under wine conditions, as it minimizes the loss of wine and aromas. In

addition, yeasts could also use these degradation products as a source of nitrogen, thus improving the wine aroma quality and reducing the need for nitrogen addition. However, given the resistance of chitinases and TLPs to the enzymatic action of proteases, and due to their rigid structure, it seems a rather difficult but challenging task to eliminate them by proteolysis under wine conditions (Van Sluyter *et al.,* 2015).

A strategy could be to use high temperature resistant proteases before fermentation. At higher temperatures, the wine proteins unfold becoming more susceptible to proteolytic attack and thus making the treatment effective (Marangon *et al.,* 2012). This treatment requires high energy to heating and could have detrimental influences on the final characteristics of the wine (Lloyd e*t al.,* 2005). Nevertheless Marangon *et al.* (2012) have shown that with a careful heating process, those disadvantages can be minimized.

Marchal *et al.* (2006) noted that musts deriving from grapes infected with *Botrytis cinerea* showed less quantity of PR proteins than musts obtained from healthy grapes, in contrast to other pathogens. This effect has been attributed to the action of a specific *B. cinerea* protease which has an effect against PR proteins, without the need for heating because it is apparently capable to work at winemaking temperatures (Van Sluyter *et al.,* 2012).

From the tests done, the wines to which the enzymes from *B. cinerea* were added showed less haze after stability test than wines where the enzyme had not been added (Van Sluyter *et al.,* 2015).

The use of other fining agents would also be another solution. Chagas *et al.* (2012) compared six different fining agents (casein, isinglass, chitin, PVPP, egg albumin and chitosan) using as control the treatment with bentonite (Table 4).

Table 4. *Commercial fining agents used for fining. Moscatel of Alexandria white wine (*Chagas *et al.,* 2012).

Agent	Concn	Manufacturer
Casein	40 g/hL	Sigma-Aldrich
Egg albumin	10 g/hL	Sigma-Aldrich
Isinglass	4 g/hL	Cristalline
Chitosan[a]	100 g/hL	Sigma-Aldrich
Chitin[b]	100 g/hL	Sigma-Aldrich
PVPP	80 g/hL	Fluka
Bentonite	100 g/hL	Enartis

[a]Minimum 85% deacetylated.
[b]From crab shells.

Except egg albumin and chitosan, none of the other four fining agents produced significant differences in the decrease in haze formation after the heat stability test (Fig. 12). Nevertheless, although egg albumin and chitosan showed a decrease in the haze phenomenon, the wines treated were still considered susceptible to forming protein haze. Otherwise, the control treated with bentonite was stable (Chagas *et al.,* 2012), confirming

that bentonite is the most effective fining agent to prevent the haze phenomenon (Pocock and Waters, 2006).

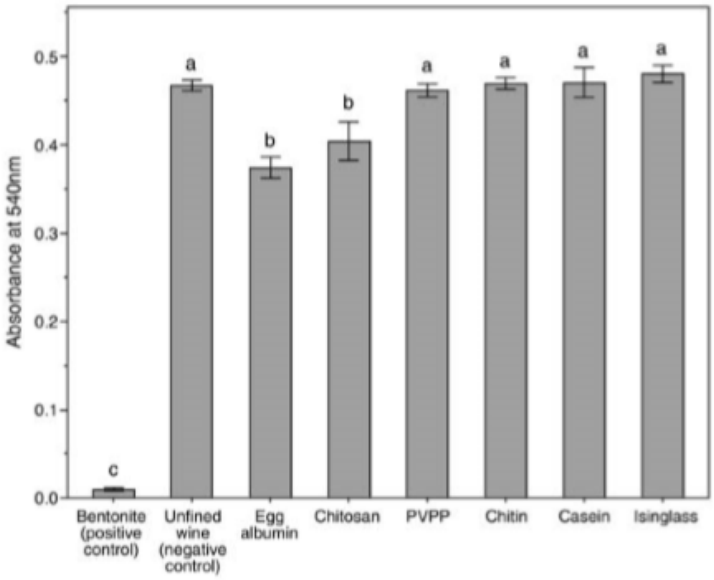


Figure 12. *Changes in turbidity detected by the difference in the absorbance at 540 nm in samples of fined and unfined (negative control) wines measured after heat stability test. Bars indicate mean ± SD (n = 3). Different letters represent different homogeneous subsets for p = 0.05 (*Chagas *et al*., 2012).

1.4 Aim of the work

The purpose of this work was to assess the potential of a novel cellulose-based polymer for wine protein removal, capable of replacing bentonite. In this work fourteen wines, seven from 2017 year and seven from 2018, were studied. On these, the routine analyses and quantification of proteins was performed to characterize the samples.

After this phase, dicarboxymethylcellulose (DCMC) was used at four different doses on three different wines, evaluating how many proteins have been retained by the polymer by the Bradford method. Specifically, the wines were Moscatel of Setúbal 2018, Viosinho 2018 and Encruzado 2018.

These wines were chosen based on their different protein content and based on their different stability to the heat stability test. Both the untreated and the treated wines were subjected to the heat stability test to correlate the amount of proteins and the stability of the wine after treatment.

Besides, to have a comparison, the same trials were performed with bentonite on the same wines to see the differences with the use of DCDM and to compare the effect of DCMC to that of bentonite.

2. Materials and methods

2.1 Fining agents and experiments

The fining agent analysed in this work was the DCMC that was compared with bentonite, here used as the positive control. The DCMC is able to remove positively charged molecules from wines, thanks the cation exchange that take places.

The fining experiments involved the addition of standard concentrations of the DCMC, 0.5, 1, 1.5 and 2 g/L., and then compared with the wines treated with the same dosages of bentonite. The trials were performed at a laboratory scale using 20 mL aliquots of wine. The unfined wines were used as the negative control. The DCMC and the bentonite were added to wine, previously clarified by centrifugation at 10,000 *g* for 15 min and incubated for 48 h at 25 °C under agitation. The samples were then centrifuged at 10,000 *g* for 15 min and filtered. All trials were performed in triplicate.

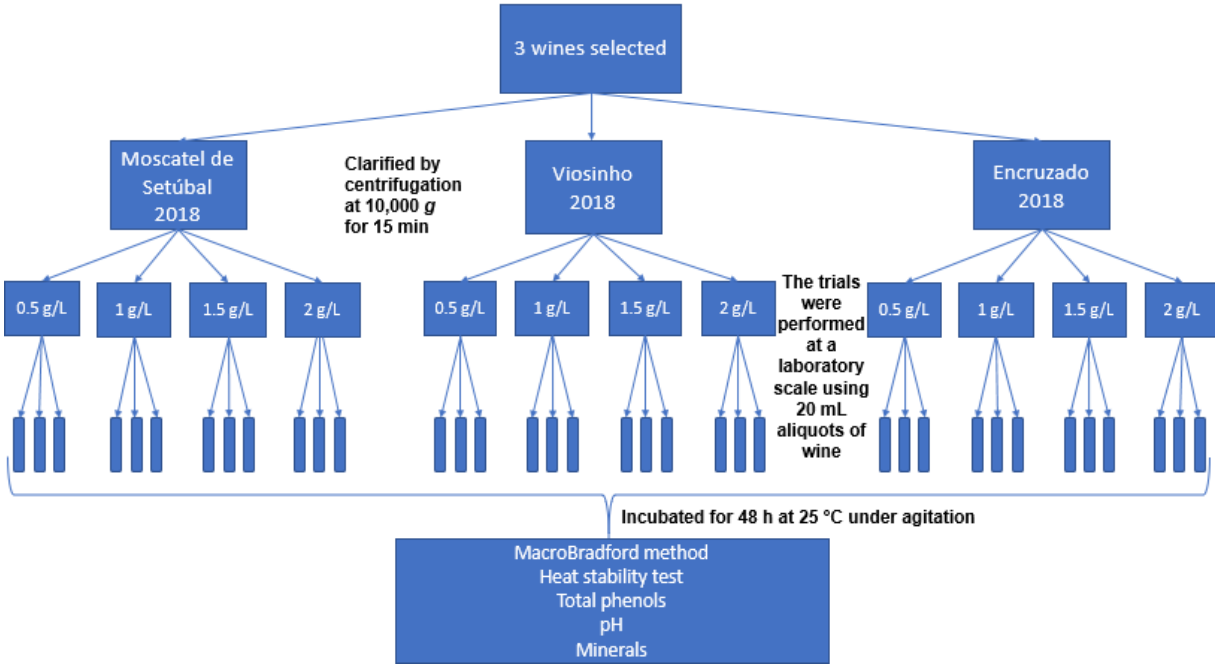


Figure 13. *Experimental design.*

2.2 Wine sample

Wine samples prepared from seven different white grape varieties were taken from the winery of Instituto Superior de Agronomia, University of Lisbon, Lisbon, Portugal. Specifically, for each variety two wines of two different years were taken, one from 2017, the other from 2018. The varieties were: Arinto, Moscatel of Setúbal, Moscatel Galego, Encruzado, Alvarinho, Viosinho and Macabeu. These samples were always stored in a dark room, and in bottles filled up with nitrogen.

2.3 Protein instability test

Wine samples were heated at 80 °C for 2 h in a thermomixer and subsequently cooled in ice for 2 h. After equilibration at ambient temperature, the increase in turbidity was detected spectrophotometrically at 540 nm. Differences in wine turbidity (before and after the heat treatment) have been shown to correlate directly to wine protein instability. The value of 0.02 is the pass-fail point in protein stability tests suggested by Pocock and Waters (2006).

All measurements were performed in triplicate (Marangon et al., 2014; Chagas et al., 2015).

2.4 Protein instability test with tannins

To compare two different protein instability tests, the protein instability was also tested with the tannins test. In two test tubes of 20 mm × 200 mm, 20 mL of clear (centrifuged) wine and about 200 mg of ascorbic acid were added; to one test tube, 1 mL of tannin solution was added.

The temperature was maintained at 80 °C for 10 min and then cooled down. The absorbance at 650 nm was measured in a cuvette of 1 cm path length. The difference in absorbance between the tube with tannin and the tube without tannin represents the protein haze.

The value of 0.1 is the pass-fail point in protein stability with tannins. All measurements were performed in triplicate.

2.5 Sulfur dioxide quantification

Total sulfur dioxide was determined by potentiometric titration with an iodide/iodate solution after alkaline hydrolysis.

The semiautomatic apparatus Sulfilyser was used to measure free and total SO2 following the Ripper method (Method OIV-MA-AS323-04B), making easier the measurement. With a double platinum electrode and a LED indicator, it detects the electric current as soon as the oxidizing solution of iodide/iodate is in excess. The user controls the flow of this solution, leading to a change of LED signs that indicates the end of the measurement. All measurements were performed in duplicate.

2.5.1 Free SO_2

First, a burette was filled up with the solution of iodide/iodate N/64. A beaker was placed under the injection point to collect the solution and the titration button was pressed to fill in the hose and the injection point with the solution of iodide/iodate. The level of iodide/iodate was readjusted in the burette.

A solution of 25 mL of wine or sample to analyse and 5 mL of sulfuric acid in 1/3 (v/v) was then prepared in a beaker of 50 mL, and to this solution a stirring bar was added. The solution was stirred.

When everything was ready, the titration button was pressed regularly (at a rate of one pressure every second) till the red led remained on. The volume of solution of iodide/iodate used from the burette was obtained and called VF. Free SO_2 concentration was then obtained with the following formula:

Concentration (Free SO_2) = VF × 20 (expressed in mg/L).

2.5.2 Total SO_2

To quantify total SO_2, 10 mL of wine was added to 2 mL of NaOH 2N in a beaker of 50 mL. After the addition, we waited 5 min to free the combined SO_2.

Twenty mL of sulfuric acid 1/10 (v/v) and a stirring bar were added.

Once everything was prepared in this way, the titration button was pressed regularly (at a rate of one pressure every second) till the red led remained on. The volume of solution of iodide/iodate used from the burette was obtained and called VT. The Total SO_2 concentration was then given with the following formula:

Concentration (Total SO_2) = VT × 50 (expressed in mg/L).

2.6 Volatile acidity

The volatile acidity of our wine samples was analysed according with the Method OIV-MA-AS313-02. Each sample must be prepared before the distillation process. First, carbon dioxide had to be eliminated by placing about 50 mL of wine in a vacuum flask and applying vacuum to the flask with the water pump for one to two min while shaking continuously.

Around 20 mL of wine, freed from carbon dioxide, were taken into the flask. Before starting the distillation procedure about 0.5 g of tartaric acid were added. At least 250 mL of the distillate was collected.

Titration was done with a sodium hydroxide solution 0.1 M, using two drops of phenolphthalein as indicator. “n” mL was the volume of sodium hydroxide used. The pink coloration must be stable for at least 10 s.

After the addition of four drops of diluted HCl 1/4 with distilled water, 2 mL of starch solution to 5 g/L and a few crystals of potassium iodide, free sulfur dioxide was titrated with the iodine solution 5 mM. “n'” mL was the volume used.

The saturated sodium tetraborate solution was added until the pink coloration reappeared. The combined sulfur dioxide was titrated with the iodine solution 5 mM. “n'” mL was the volume used.

The volatile acidity, expressed in g of acetic acid per L to two decimal places, was given by: 0.300 (n - 0.1 n' - 0.05 n'').

All measurements were performed in duplicate.

2.7 Total acidity

According with the method OIV-MA-AS313-01, the total acidity of our wine samples was analysed. The samples must be prepared by avoiding CO_2, by placing about 50 mL of wine in a vacuum flask and applying vacuum to the flask with the water pump for one to two min while shaking continuously. This is because the carbon dioxide should not be included in the total acidity determination.

Follow the titration with indicator (bromothymol blue), 25 mL of boiled distilled water, 1 mL of bromothymol blue solution and a volume prepared equal to 10 mL of wine were added to a beaker. Sodium hydroxide solution (100 mM) had to be added until the colour changed to blue-green. Then 5 mL of the pH 7 buffer solution (107.3 g of potassium dihydrogen phosphate, 500 mL sodium hydroxide solution 1 M and water to 1000 mL) was added.

Into a beaker, 30 mL of boiled distilled water was added with 1 mL of bromothymol blue solution and 10 mL of wine. Sodium hydroxide solution 0.1 M was added until the same colour was obtained as in the preliminary test described above. “n” mL was the volume of sodium hydroxide solution (0.1 M) added.

Total acidity expressed in milliequivalents per L was given by A = 10 n and was recorded to one decimal place. Total acidity expressed in g of tartaric acid per L was given by A' = 0.075 x A. The result was quoted to two decimal places.

All measurements were performed in duplicate.

2.8 Alcoholic strength

The alcoholic strength was calculated by ebulliometry. First, the boiling point of water was determined. After this the instrument was cooled down. The wine to be analysed was used to rinse the instrument. Approximately 50 mL of wine was placed in the boiling chamber and the condenser was filled up with cold water. The thermometer was placed inside the instrument and the latter was placed on the source. When the thermometer reached a stable value, the temperature was taken. The boiling point of the wine was located on the inner “Degrees du thermometer” scale and the corresponding alcohol point was recorded (% v/v) on the outer scale. Only one measurement was taken.

2.9 pH

According with the method OIV-MA-AS313-15, the pH of each wine sample was measured. Zeroing of the apparatus was carried out before any measurements were made. The pH meter must be calibrated at 20 °C using standard buffer solutions. The pH values selected

must encompass the range of values that may be encountered in wines. The electrode was dipped into the sample to be analysed, the temperature of which should be between 20 and 25 °C and as close as possible to 20 °C. The pH value was read directly from the scale. At least two determinations of the same sample were carried out.

2.10 Reducing Sugars

According with Method OIV-MA-AS311-01A, sugars were detected in the samples of wines as reducing substances. The density of the wine was measured and if it was less than 0.997 the sample should not be diluted. Fifty mL of wine were placed in a 100 mL volumetric flask; 5 mL of solution I: potassium ferrocyanide and water (150 g and 1000 mL, respectively) and 5 mL of solution II: zinc sulfate and water (300 g and 1000 mL, respectively) were also added. The solution was stirred and made up to the mark with water. It was then left for 10 min before the filtration (1 mL of filtrate contains 0.50 mL of dry wine). Once enough filtrate is obtained, 25 mL of the alkaline copper salt solution, 15 mL water and 10 mL of the clarified solution are mixed in a 300 mL conical flask. Few small pieces of pumice stone were added. The mixture was brought to boiling within 2 min. The mixture was boiled for 10 min. The flask was cooled immediately under cold running water. When completely cooled, 10 mL of potassium iodide solution 30% (m/v), 25 mL sulfuric acid 25% (m/v), and 2 mL starch solution were added. Sodium thiosulfate solution 0.1 M was used to titrate. “n” is the number of mL used. A blank titration in which the 25 mL of sugar solution was replaced by 25 mL of distilled water was also made, where “n'” is the number of mL of sodium thiosulfate used.

The quantity of sugar, expressed as inverted sugar, contained in the test sample is given as a function of the number (n' - n) mL of sodium thiosulfate used. The sugar content of the wine is to be expressed in g of inverted sugar per L to one decimal place, account being taken of the dilution made during clarification and of the volume of the test sample.

All measurements were performed in duplicate.

2.11 Density

The density of the wine samples was measured be the method OIV-MA-AS2-01B. Density is the mass per unit volume of wine or must at 20 °C, and is expressed in g/mL.

Two hundred and fifty mL of the wine sample was placed in the measuring cylinder; and then the hydrometer and thermometer were inserted. The sample was allowed to reach room temperature, stirred for for 1 min and the temperature recorded. The thermometer was removed and after a further 1 min the apparent density was taken.

These measurements were taken only one once.

2.12 Total dry matter

Total dry extract was calculated indirectly from the specific gravity of the alcohol-free wine, following the method OIV-MA-AS2-03B: R2012. The specific gravity of the "alcohol free wine" was calculated using the following formula:

dr = dv - da + 1.000

where:

dv = specific gravity of the wine at 20 °C (corrected for volatile acidity (1))

da = specific gravity at 20 °C of a water-alcohol mixture of the same alcoholic strength as the wine, obtained using the formula:

dr = 1.00180** (rv - ra) + 1.000

where:

rv = density of the wine at 20 °C (corrected for volatile acidity)

ra = density at 20 °C of the water-alcohol mixture of the same alcoholic strength as the wine.

** The coefficient 1.0018 approximates to 1 when rv is below 1.05 which is often the case.

The value for specific gravity of the alcohol-free wine is used to obtain the total dry extract (g/L).

These calculations were done only once, taking the average value of the factors considered.

2.13 Colour intensity

According to the method OIV-MA-AS2-07B, the colour intensity was measured. If the wine was cloudy, it was clarified by centrifugation. The optical path b of the glass cell used must be chosen so that the measured absorbance values (A) fell between 0.3 and 0.7. The spectrophotometric measurements were taken using distilled water as the reference liquid, in a cell of the same optical path b, thus, to set the zero on the absorbance scale of the apparatus at the wavelength of 420. Using the appropriate optical path b, the absorbencies were read off at each of these three wavelengths for the wine.

The color intensity “I” is conventionally given by: $I = A_{420} + A_{520} + A_{620}$.

and is expressed to three decimal places.

However, since we worked only with white wines, was not necessary read the values at 520 nm and 620nm. At 520 nm and 620 nm is used only for red wines to evaluate the evolution for color.

The values were taken in triplicate.

2.14 Total phenols

The determination of total phenols firstly implies a dilution of wine with distilled water (1:20), followed by the absorbance reading at 280 nm at the spectrophotometer. Specifically, 2.5 mL

of wine and 47.5 mL of water were used (Somers and Evans, 1977). Therefore, the final values were multiplied by 20 because of the previous dilution factor. The values were taken in triplicate and transformed, using the calibration curve in mg/L of gallic acid equivalents.

2.15 Non-flavonoids and flavonoids

The quantification of non-flavonoids (mainly phenolic acids) in a wine is based on the determination of the phenolic content after the precipitation of the flavonoids through the reaction with the formaldehyde, under certain conditions (low pH, room temperature, darkness) at which non-flavonoids do not precipitate. Five mL of wine, 5 mL of HCl and 2.5 mL of formaldehyde were mixed, and we put the nitrogen to avoid the oxidation.

After 24 h a dilution with distilled water (1:10) was carried out and the absorbance was measured at 280 nm at the spectrophotometer (Kramling and Singleton, 1969). The final value is multiplied by 10 because of the previous dilution. On the other hand, flavonoids came out from the difference between total phenols and non-flavonoids. The values were taken in triplicate and transformed using the calibration curve in mg/L of gallic acid equivalents.

2.16 Sulphates

According with the method OIV-MA-AS321-05A, sulphates were quantified. Forty mL of the wine sample were placed into a 50 mL centrifuge tube with 2 mL hydrochloric acid 2 M, and 2 mL of barium chloride solution 200 g/L. The sample was centrifuged for 5 min, and then the supernatant carefully decanted. The barium sulphate precipitate was washed as follows: ten mL hydrochloric acid 2 M were added. The precipitate was suspended and centrifuged for 5 min, then the supernatant was carefully decanted. The washing procedure was repeated twice as before using 15 mL distilled water each time. The precipitate was transferred, with distilled water, into a tared platinum capsule and placed over a water bath at 100 °C until fully evaporated. The dried precipitate was calcined several times briefly over a flame until a white residue was obtained. The dried residue was cooled down and then weighed (m = mass in mg of barium sulphate obtained). The sulphate content, expressed in mg of potassium sulphate per L is given by:

18.67 x m

The sulphate content of the wines is expressed in mg of potassium sulphate per L of wine, to the nearest whole number. All analyses were done in triplicate.

2.17 Tartaric stability

For tartaric stability determination, 100 mL of wine was placed in a beaker with a magnetic bar, all inside an ice bath. The temperature was ca. 0 ºC. The conductivity value was measured in a conductivimeter. One g of potassium hydrogen tartrate powder was added to the wine, and the conductivity was taken at each 1 min. The conductivity value was measured until the readings remained stable for at least three successive 1-min-intervals. The final value of conductivity corresponds to that at which the wine is stable. If, in the 5 to 10 min after seeding, the drop in the conductivity reading was lower than 5% of the wine's initial conductivity (measured before adding potassium bitartrate), the wine was considered to be properly treated and stabilized. If the drop of conductivity was over 5%, the wine was considered unstable. All analyses were done in duplicate (Angele, 1992; Ribérau-Gayon *et al.,* 2006).

2.18 Chloride

According with method OIV-MA-AS321-02, the chloride analysed was performed as follows: five mL of standard chloride solution, approximately 100 mL of distilled water and 1.0 mL of nitric acid were poured into a 150 mL cylindrical vessel placed on a magnetic stirrer.

After immersing the electrode, silver nitrate solution was added with the micro-burette, with moderate stirring using the following procedure: at the begin 4 mL in 1 mL fractions was added and the corresponding mV values read. The next 2 mL in fractions of 0.20 mL were added. Finally, the addition in fractions of 1 mL was continued until a total of 10 mL had been added. After each addition, a wait of approximately 30 s was done before reading the corresponding mV value. The values obtained were plotted on a graph against the corresponding mL of titrant and the potential corresponding to the equivalence point determined.

If n represents the number of mL of silver nitrate titrant, the chloride content in the tested liquid, is given by 32.9 x n, expressed as mg of NaCl per L. All measurements were performed in triplicate.

2.19 Minerals

2.19.1 Sodium

Sodium was analysed according to the method OIV-MA-AS322-03A. A volume of wine (2.5 mL) was pipetted into a 50 mL volumetric flask, to which 1 mL of the cesium chloride solution to 5% was added, and the flask was made up until the mark with distilled water. For the calibration, the matrix solution was prepared as follows: 3.5 g of citric acid monohydrate, 1.5 g of sucrose, 5 g of glycerol, 50 mg of anhydrous calcium chloride, 50 mg of anhydrous magnesium chloride, 50 mL of absolute alcohol and de-ionized water to 500 mL.

5.0 mL of the matrix solution was inserted in each one of five 100 mL volumetric flasks and 0, 2.5, 5.0, 7.5 and 10 mL were added respectively of a 1:100 dilution of the 1 g/L sodium solution. Two mL of the caesium chloride solution was added to each flask, which was filled up to 100 mL with distilled water. The standard solutions prepared in this way contained 0.25, 0.50, 0.75 and 1.00 mg of sodium per L respectively, and each contained 1 g of caesium per L. These solutions were taken in polyethylene bottles. The absorbance wavelength was set to 589.0 nm. The absorbance scale was zeroed by using the zero-standard solution. The diluted wine was aspired directly into the spectrophotometer, followed in succession by the standard solutions. Each absorbance value was recorded. The calculation of the results was made by plotting a graph of measured absorbance versus the sodium concentration in the standard solutions. The absorbance obtained was recorded with the diluted wine on this graph and its sodium concentration was determined in mg/L. The sodium concentration in mg/L of the wine was then calculated as F x C expressed to the nearest whole number, where F is the dilution factor and C the sodium concentration.

2.19.2 Potassium

Potassium was measured according to the method OIV-MA-AS322-02B. Different volumes of the reference solution (25, 50, 75 and 100 mL) were placed into a set of four 100 mL volumetric flasks, which was filled up to 100 mL with the dilution solution to give solutions containing 25, 50, 75 and 100 mg of potassium per L respectively. The measurements were made at 766 nm. The standard solutions were successively aspirated directly into the burner of the photometer, followed by wine diluted 1/10 with distilled water and the readings were noted. Whenever necessary, the wine already diluted 1/10 was further diluted with the dilution solution. A graph of the variation in transmittance is plotted as a function of the potassium concentration in the standard solutions. The transmittance values obtained for the samples of diluted wine on this graph were recorded and the corresponding potassium concentration C was determined. The potassium concentration in mg potassium per L to the nearest whole number was calculated as F x C, where F is the dilution factor.

2.19.3 Calcium

Calcium was determined according to the method OIV-MA-AS322-04. One mL of wine and 2 mL of the lanthanum chloride solution to 50 g/L were placed in a 20 mL volumetric flask, which was filled up to the mark with distilled water. The diluted wine contained 5 g lanthanum per litre. Zero, 5, 10, 15 and 20 mL of dilute standard calcium solution to 50 mg/L were placed respectively into each of five 100 mL volumetric flasks, followed by 10 mL of the lanthanum chloride solution and filled up to 100 mL with distilled water. The solutions prepared in this way contained 0, 2.5, 5.0, 7.5 and 10 mg of calcium per L respectively, and

each contained 5 g of lanthanum per L. These solutions were stored in polyethylene bottles. The absorbance wavelength was set to 422.7 nm. The absorbance scale was zeroed using the zero standard. The diluted wine was aspired directly into the spectrophotometer, followed in succession by the five standard solutions and the absorbance values were recorded. A graph was plotted showing the variation in absorbance as a function of the calcium concentration in the standard solutions. The mean values of the absorbance obtained with the sample of diluted wine on this graph were recorded and their calcium concentration C was read. The calcium concentration in mg/L of the wine to the nearest whole number was given by 20 x C.

2.19.4 Magnesium

Magnesium was determined according to the method OIV-MA-AS322-07. The wine was diluted 1/100 with distilled water. Five, 10, 15 and 20 mL of the diluted standard magnesium solution to 1 g/L were placed into each one of a set of four 100 mL volumetric flasks and each made up to 100 mL with distilled water. The standard solutions prepared in this way contained 0.25, 0.50, 0.75 and 1.0 mg of magnesium per L, respectively. These solutions were kept in polyethylene bottles. The absorption wavelength was ser at 285 nm. The absorbance scale was zeroed using distilled water. The diluted wine was directly sucked into the spectrophotometer, followed in succession by the standard solutions. The absorbance of each solution was recorded, and each measurement repeated. A graph showing the variation in absorbance was plotted as a function of the magnesium concentration in the standard solutions. The mean value of absorbance with the diluted samples of wine was recorded on this graph and the magnesium concentration C in mg/L was read. The magnesium concentration in mg/L of the wine to the nearest whole number was given by 100 x C.

2.19.5 Copper

Copper was determined according to the method OIV-MA-AS322-06. Twenty mL of wine sample were placed in a 100 mL volumetric flask and made up to 100 mL with double-distilled water. The dilution had to be modified whenever necessary to obtain a response within the dynamic range of the detector. The absorbance was measured at 324.8 nm. The zero was set with double distilled water. Different volumes of copper solution to 1 g/L (0.5, 1 and 2 mL) were pipetted into each of three 100 mL volumetric flasks and was filled up to 100 mL with double distilled water; the solutions contained 0.5, 1 and 2 mg of copper per L, respectively. The absorbance of the standard solutions was measured. A graph was plotted showing the variation in absorbance as a function of the copper concentration in the standard solutions. Using the measured absorbance of the samples the concentration C in mg/L from

the calibration curve was read off. If F is the dilution factor, the concentration of the copper present is given in mg/L by F x C.

2.19.6 Iron

Iron was measured according to the method OIV-MA-AS322-05A. First of all it's necessary to remove the alcohol from the wine by reducing the volume of the sample to half its original size using a rotary evaporator (50 to 60 °C). The original volume was made up with distilled water. If necessary, wine samples were diluted prior to analysis with distilled water. One, 2, 3, 4, and 5 mL of the solution containing 100 mg iron per L respectively were added into each of five 100 mL volumetric flasks and made up to 100 mL with distilled water. The solutions prepared in this way had 1, 2, 3, 4 and 5 mg of iron per L, respectively. The measurements were made at 248.3 nm.

The diluted samples were aspired directly into the spectrophotometer, followed in succession by the five standards. The absorbance was recorded.

A graph of the variation in absorbance was plotted as a function of the iron concentration in the standard solutions. The absorbance obtained for the sample of diluted wine on this graph was recorded and the corresponding iron concentration C was determined. The iron concentration in mg per L to the nearest whole number will be F x C, where F is the dilution factor.

2.20 Protein quantification: MacroBradford method

The Bradford protein assay (1976) was the method used to quantify wine proteins, here in part modified. This is a colorimetric method based on the change in absorbance of the Brilliant Blue dye Coomassie G (Bradford's reagent) when binding to the amino acid residues and their aromatic side chains. Under acidic conditions, this dye is in its protonated cationic form, presenting a red color with maximum absorption at 470 nm. When the Bradford reagent binds to the aromatic amino acid residues of the proteins it assumes a stable unprotonated form of blue staining and maximum absorption at 595 nm. Thus, the absorption value will be proportional to the protein concentration present in each sample and the greater the intensity of the blue coloration in each well the greater the amount of protein present. A calibration line was prepared with bovine serum albumin (BSA) with a concentration of 0.5 mg/mL (bovine serum albumin) solution at different concentrations: 2.5 µg/mL, 5 µg/mL, 7.5 µg/mL, 10 µg/mL, 15 µg/mL and 20 µg/mL.

Protein samples (400 µL aliquots) dissolved in the various matrices were mixed with an equal volume of deionized water to which 200 µL of Bio-Rad Protein assay reagent were added. A_{595} readings were taken after holding samples at room temperature for 10 min. The protein

concentration was then calculated by the absorbance results obtained (Marchal et al., 1997). All measurements were performed in triplicate.

2.21 Fining agents and experiments

The fining agent analysed in this work was the DCMC that was compared with bentonite, here used as the positive control. The fining experiments involved the addition of standard concentrations of the DCMC, and then compared with the wines treated with the same dosages of bentonite. The trials were performed at a laboratory scale using 20 mL aliquots of wine. The unfined wines were used as the negative control. The DCMC and the bentonite were added to wine, previously clarified by centrifugation at 10,000 *g* for 15 min and incubated for 48 h at 25 °C under agitation. The samples were then centrifuged at 10,000 *g* for 15 min and filtered. All trials were performed in triplicate.

2.22 Statistical Analysis

To assess if there were statistically significant differences between bentonite and DCMC treatments, the data obtained by the MacroBradford, heat stability test, pH and total phenols was subjected to analysis of variance (ANOVA) and comparisons among treatments (Tukey's post-hoc test) were performed using R- project 3.4.3.

To study the correlation among the different instabilities of wines and their protein concentrations, the R- project 3.4.3 was used.

Statistical significance (at $p<0.05$) of the differences between mean values was assessed by Tukey's test.

3 Results and discussion

3.1 Routine analyses

3.1.1 Protein instability test

The heat test was done on all wine samples, to group them in stable and unstable and to choose the best wines to do the experiments with fining agents. Particularly, from heat stability test results it was noted that all wines from 2017, except Moscatel de Setúbal, were stable, highlighting the peculiarity of Moscatel of Setúbal to be one of the most unstable varietal wines, as reported also by Chagas *et al.* (2016). Furthermore, the heat stability test performed with added tannins was carried out on all fourteen wines under study. However, all wines were found to be unstable, showing that this test overestimates protein instability (Ribérau-Gayon *et al*., 2007). Table 5 shows the results obtained with the heat stability test (HST).

Regarding the seven wines from 2018, only the wines of the varieties Moscatel of Setúbal, Viosinho, Moscatel Galego and Encruzado were unstable, with different degrees of instability: Moscatel of Setúbal was highly unstable, Viosinho and Moscatel Galego were moderately unstable and Encruzado was almost stable, presenting an absorbance value near 0.02 after the heat test.

To assess the effect of the fining agents on different wines with different levels of instability, Moscatel de Setúbal, Viosinho and Encruzado were selected.

Table 5. *Protein instability test performed with tannins (HST+T) and without tannins (HST). Regarding the HST, a wine is considered unstable if the absorbance at 540 nm is higher than 0.02 AU. According with the HST+T, a wine is considered unstable if the absorbance at 650 nm is higher than 0.01 AU. Values are mean ± SD (n = 3).*

Wine	HST	stability	HST+T	stability
Encruzado 2017	0.015 ± 0.005	stable	0.099 ± 0.007	unstable
Alvarinho 2017	0.014 ± 0.005	stable	0.056 ± 0.002	unstable
Viosinho 2017	0.017 ± 0.007	stable	0.240 ± 0.05	unstable
Arinto 2017	0.011 ± 0.002	stable	0.130 ± 0.006	unstable
Moscatel Galego 2017	0.009 ± 0	stable	0.128 ± 0.007	unstable
Macabeu 2017	0.003 ± 0.001	stable	0.051 ± 0.004	unstable

Moscatel de Setúbal 2017	0.050 ± 0.005	unstable	0.770 ± 0.01	unstable
Encruzado 2018	0.019 ± 0.002	stable	0.720 ± 0.02	unstable
Alvarinho 2018	0.016 ± 0.005	stable	0.259 ± 0.009	unstable
Viosinho 2018	0.073 ± 0.008	unstable	0.454 ± 0.02	unstable
Arinto 2018	0.017 ± 0.001	stable	0.299 ± 0.01	unstable
Moscatel Galego 2018	0.040 ± 0.005	unstable	0.343 ± 0.008	unstable
Macabeu 2018	0.012 ± 0.001	stable	0.177 ± 0.003	unstable
Moscatel de Setúbal 2018	0.19 ± 0.01	unstable	1.347 ± 0.02	unstable

3.1.2 Protein quantification by the MacroBradford method

To quantify the protein in the fourteen wine samples under study the Bradford protein assay (1976) was used. The work of McRae *et al.* (2018) showed that, in general, the protein concentration was more influential on the amount of haze produced than the protein composition. In addition, these authors suggested that the wine components previously shown to have significantly impact on haze formation, such as pH, ionic strength, phenolic substances and polysaccharides, did not play a significant role in the wines of their study. However, the protein concentration taken alone isn't enough to explain the protein instability of a wine, since it is important to correlate it with many other parameters (Chagas *et al*., 2016).

In contrast, from the study of Van Sluyter *et al.* (2015) has been shown that Chitinases and TLPs are the most important proteins involved in wine haze formation demonstrating that protein composition is an important factor for haze formation

The Bradford method was used to estimate the total proteins present in all wines, as shown in table 6. From this analysis, for both years, the wines from the Moscatel de Setúbal variety have typically high protein values than the other wines from the same year, while Viosinho and Encruzado showed intermediate behaviours. Furthermore, all wines were in the range of the concentration of proteins reported by Ferreira *et al.* (2002), that is from 15 to 230 mg/L.

Table 6. *Protein concentration (in mg/L) in all wines quantified by the Bradford method. Values are mean ± SD (n = 3).*

Wine	Protein concentration (mg/L)
Encruzado 2017	*45.5 ± 2*
Alvarinho 2017	*42.5 ± 3*
Viosinho 2017	*61.2 ± 4*
Arinto 2017	*50.8 ± 4*
Moscatel Galego 2017	*54.5 ± 4*
Macabeu 2017	*27.2 ± 1*
Moscatel de Setúbal 2017	*94.6 ± 7*
Encruzado 2018	*80.1 ± 8*
Alvarinho 2018	*61.9 ± 2*
Viosinho 2018	*87.9 ± 6*
Arinto 2018	*73.1 ± 3*
Moscatel Galego 2018	*122.2 ± 6*
Macabeu 2018	*39.4 ± 2*
Moscatel de Setúbal 2018	*218.7 ± 10*

3.1.3 Wines identity card

All fourteen wines under study were subjected to routines analyses in order to have an identity card of each one of them. Some of these analyses are important because they show how the treatment with bentonite and DCMC affect the increase or decrease of different compounds of the wine, such as total polyphenols, pH and minerals. The results of the routine analyses, that are within the ranges expected, are shown in table 7 a, b and c.

Table 7. *Routine analyses of the fourteen wines from 2017 and 2018 under study*; *values are mean ± SD (n = 3).*

(a)

Parameters	Free SO_2 (mg/L)	Total SO_2 (mg/L)	pH	Total acidity (g/L of tartaric acid)	Volatile acidity (g/L of acetic acid)	Alcohol (% v/v)	Reducing substances (g/L)
Encruzado 2017	11 ± 0	50 ± 0	3.54 ± 0	6.6 ± 0.11	0.32 ± 0.01	14.1 ± 0.03	0.8 ± 0
Alvarinho 2017	10 ± 0	50 ± 0	3.30 ± 0	7.5 ± 0.12	0.27 ± 0.01	14 ± 0.03	0.6 ± 0

Viosinho 2017	9 ± 0	57 ± 0	3.44 ± 0	6.3 ± 0.12	0.33 ± 0.03	15.4 ± 0.02	0.7 ± 0.01
Arinto 2017	8 ± 0	60 ± 0	3.32 ± 0	7.4 ± 0.10	0.28 ± 0.03	13.1 ± 0.0	1.0 ± 0.01
Moscatel Galego 2017	16 ± 0	80 ± 0	3.45 ± 0	7.2 ± 0.09	0.44 ± 0.02	15.8 ± 0.01	0.7 ± 0.02
Macabeu 2017	20 ± 0	90 ± 0	3.24 ± 0	7.7 ± 0.08	0.23 ± 0.02	12.3 ± 0.02	0.5 ± 0
Moscatel of Setúbal 2017	12 ± 0	85 ± 0	3.39 ± 0	6.8 ± 0.09	0.29 ± 0.01	13.2 ± 0.02	0.3 ± 0.01
Encruzado 2018	25 ± 0	90 ± 0	3.12 ± 0	8.4 ± 0.11	0.28 ± 0.03	13.5 ± 0.01	0.1 ± 0
Alvarinho 2018	32 ± 0	100 ± 0	3.3 ± 0	8.1 ± 0.11	0.2 ± 0.02	14 ± 0.01	0.7 ± 0.02
Viosinho 2018	28 ± 0	82 ± 0	3.28 ± 0	7.4 ± 0.06	0.4 ± 0.01	16 ± 0.02	1.2 ± 0.01
Arinto 2018	25 ± 0	95 ± 0	3.2 ± 0	7.8 ± 0.07	0.29 ± 0,01	14.5 ± 0.01	1.5 ± 0
Moscatel Galego 2018	20 ± 0	85 ± 0	3.4 ± 0	7.8 ± 0.08	0.55 ± 0.02	16.3 ± 0.02	1.6 ± 0.01
Macabeu 2018	24 ± 0	75 ± 0	3.19 ± 0	6.3 ± 0.12	0.26 ± 0.02	12.7 ± 0.02	0.4 ± 0.03
Moscatel of Setúbal 2018	21 ± 0	80 ± 0	3.47 ± 0	6 ± 0.11	0.32 ± 0.01	14.1 ± 0.0	0.43 ± 0.02

(b)

Parameters	Dry matter (g/L)	Colour (AU)	Total phenols (mg/L of gallic acid)	Non-flavonoids (mg/L of gallic acid)	Flavonoids (mg/L of gallic acid)	Chloride (mg NaCl/L)	Sulphates (g/L of potassium sulphate)
Encruzado 2017	21.4 ± 0.2	0.048 ± 0	189.6 ± 3.2	68.04 ± 2.6	121.58 ± 1,2	11.0 ± 0.11	0.1 ± 0
Alvarinho 2017	20.6 ± 0.5	0.184 ± 0	263.5 ± 3.5	100.32 ± 2	163.17 ± 1.5	7.1 ± 0.16	0.3 ± 0
Viosinho 2017	22.9 ± 0.15	0.119 ± 0	288.2 ± 3	113.7 ± 1.9	174.6 ± 1.8	8.7 ± 0.12	0.2 ± 0.05
Arinto 2017	25.2 ± 0.17	0.177 ± 0	263.5 ± 1.7	99.3 ± 1.5	164.2 ± 2.2	9.4 ± 0.12	0.3 ± 0.02

Moscatel Galego 2017	22.9 ± 0.4	0.091 ± 0	258.4 ± 2.1	92.2 ± 1.4	166.2 ± 0.8	18.1 ± 0.15	0.3 ± 0.01
Macabeu 2017	22.9 ± 0.3	0.072 ± 0	200.2 ± 1.4	67.8 ± 1.9	132.4 ± 1.4	14.70 ± 0.14	0.2 ± 0.02
Moscatel of Setúbal 2017	22.4 ± 0.22	0.115 ± 0	234.7 ± 1.1	84.3 ± 2.2	150.4 ± 2	11.19 ± 0.1	0.3 ± 0.04
Encruzado 2018	22.2 ± 0.18	0.067 ± 0	192.6 ± 1.4	67.8 ± 1.4	124.8 ± 1	8.12 ± 0.1	0.2 ± 0.03
Alvarinho 2018	25.2 ± 0.1	0.098 ± 0	249.4 ± 1.8	86.6 ± 2.3	162.9 ± 0.9	9.32 ± 0.16	0.2 ± 0.02
Viosinho 2018	23.6 ± 0.35	0.066 ± 0	227.5 ± 3.5	77.5 ± 1.1	149.6 ± 1.7	12.72 ± 0.18	0.2 ± 0.01
Arinto 2018	25.4 ± 0.44	0.11 ± 0	212.3 ± 2.2	76.6 ± 1.6	135.6 ± 2.6	9.76 ± 0.15	0.3 ± 0
Moscatel Galego 2018	26.1 ± 0.35	0.071 ± 0	311.0 ± 1,5	109.6 ± 2	201.4 ± 1.7	16.45 ± 0.18	0.2 ± 0
Macabeu 2018	20.9 ± 0.4	0.045 ± 0	192.6 ± 2.7	62.0 ± 2.4	130.6 ± 2	12.83 ± 0.11	0.2 ± 0.03
Moscatel of Setúbal 2018	21.4 ± 0.1	0.048 ± 0	189.6 ± 1.8	68.0 ± 1.7	121.6 ± 2.3	10.97 ± 0.13	0.1 ± 0.01

(c)

Parameters	**Tartaric stability (%)**	**Cu (mg/L)**	**Fe (mg/L)**	**Ca (mg/L)**	**Mg (mg/L)**	**Na (mg/L)**	**K (mg/L)**
Encruzado 2017	7.8 ± 1	0.06 ± 0	0.6 ± 0,02	42.78 ± 0.4	74.5 ± 1.2	7.63 ± 0.3	808.6 ± 4
Alvarinho 2017	11.0 ± 1.1	0.16 ± 0	1.05 ± 0,02	90.32 ± 2.6	73.74 ± 1.5	10.51 ± 0.2	748.23 ± 3.2
Viosinho 2017	6.3 ± 1.4	0.15 ± 0	1.32 ± 0,01	54.52 ± 1.8	72.39 ± 1.3	13.04 ± 0.1	717.62 ± 2.7
Arinto 2017	10.4 ± 1.2	0.14 ± 0	1.61 ± 0.02	66.74 ± 1.6	59.61 ± 1.7	11.61 ± 0.15	885.88 ± 4.3
Moscatel Galego 2017	5.9 ± 1	<0.01 ± 0	1.16 ± 0.02	58.48 ± 1.3	94.14 ± 1.6	14.11 ± 0.2	743.18 ± 2.2

Macabeu 2017	12.1 ± 0.5	<0.01 ± 0	1.20 ± 0.01	76.09 ± 1.9	51.03 ± 1.4	11.81 ± 0.4	796.25 ± 3.2
Moscatel of Setúbal 2017	10.7 ± 1.6	<0.01 ± 0	0.52 ± 0.01	57.00 ± 1.2	51.03 ± 1.3	5.66 ± 0.15	726.74 ± 4.2
Encruzado 2018	10.7 ± 1	<0.01 ± 0	0.75 ± 0.01	55.59 ± 1	68.42 ± 0,9	6.76 ± 0.17	756.57 ± 3
Alvarinho 2018	10.4 ± 0.1	<0.01 ± 0	0.59 ± 0	56.17 ± 0.9	101.74 ± 1.1	6.92 ± 0.1	631.07 ± 1.9
Viosinho 2018	3.6 ± 0.8	<0.01 ± 0	0.81 ± 0.01	44.13 ± 0.5	87.49 ± 0.8	9.85 ± 0.25	525.04 ± 1,5
Arinto 2018	9.5 ± 0.05	<0.01 ± 0	0.64 ± 0	61.71 ± 1.2	72.57 ± 0.9	6.08 ± 0.1	739.9 ± 4.6
Moscatel Galego 2018	2.1 ± 0.07	<0.01 ± 0	0.79 ± 0,02	38.17 ± 0,3	91.89 ± 1.2	8.77 ± 0.2	653.25 ± 3.4
Macabeu 2018	10.3 ± 0.09	<0.01 ± 0	0.52 ± 0	57.00 ± 0.6	51.03 ± 1.1	5.66 ± 0.3	726.74 ± 3.7
Moscatel of Setúbal 2018	7.8 ± 0.07	0.062 ± 0	0.60 ± 0.01	42.78 ± 0.45	74.50 ± 0.5	7.63 ± 0.25	808.60 ±

3.1.4 Effects of the DCMC on protein removal and on wine stability

Three unfined white wines from different varieties were treated with DCMC or with bentonite at four different dosage rates of 0.5, 1, 1.5 and 2 g/L. The wines were treated for 48 h and then on these the Macro-Bradford and the HST were done to assess the possible effects of the fining agent on the removal of proteins and on the haze forming potential.

Regarding the samples of Moscatel de Setúbal treated with the DCMC, which had an initial concentration of proteins of about 200 mg/L, treatment decreased the amount of proteins by about 75% for any DCMC doses tested (Fig. 13). However, all samples treated with the DCMC produced significant differences when compared to the negative control. Furthermore, the samples treated with 0.5 and 1g/L of DCMC produced significant differences when compared to the positive control with 0.5 g/L of bentonite, while when they were compared to the positive control treated with 1 g/L of bentonite the differences were smaller. In any case, DCMC-treated wine samples had always less protein than their respective positive controls.

With doses of 1.5 and 2 g/L of DCMC, the samples treated had more proteins than those treated with equivalent doses of bentonite, and so there were differences when compared to the positive control with 2 g/L of bentonite, but less when compared at control with 1.5 g/L (Fig. 13).

As shown by Dawes et al. (1994), the haze formation and the levels of protein in the wine decreased with the clarification with bentonite. In this work the same trend occurred with clarification with the DCMC.

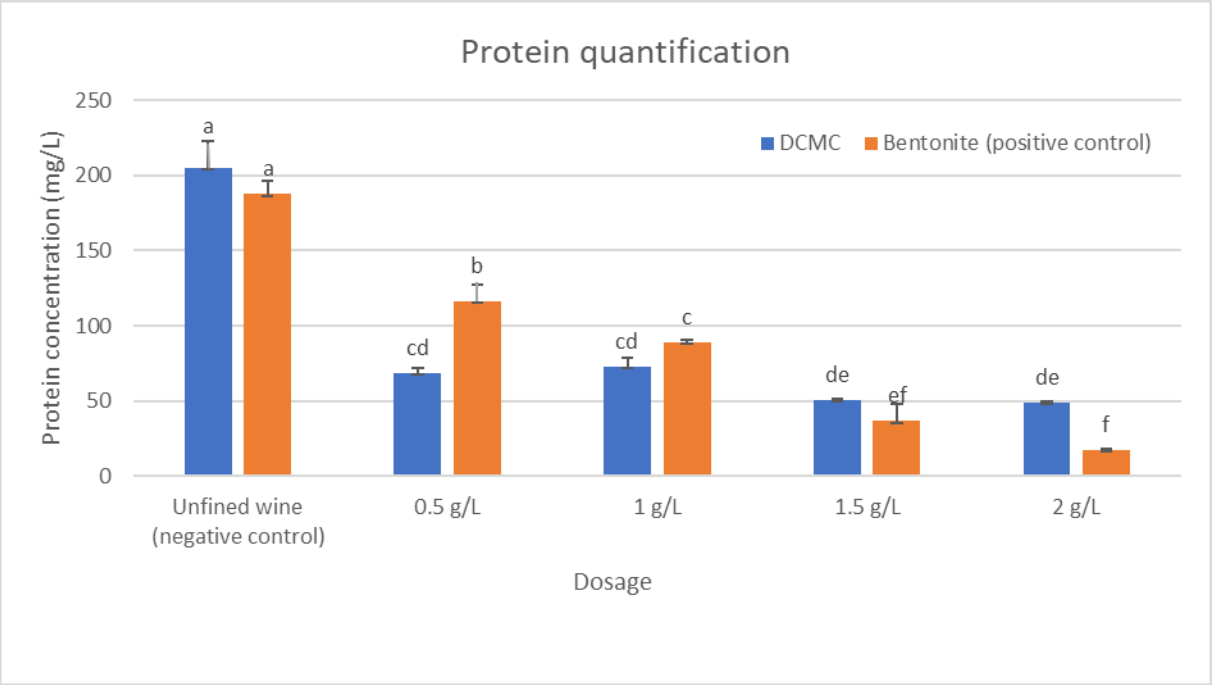


Figure 14. *Protein concentration (in mg/L) in Moscatel of Setúbal, quantified by the Bradford method, with increasing amounts of bentonite and DCMC. Different letters represent different homogeneous subgroups for p = 0.05.*

For the HST the trend was similar to the results obtained for the protein quantification. In fact, all samples treated with DCMC produced significant differences when compared to the negative control, as shown in Fig. 14, but in this case only the samples treated with 0.5 g/L of DCMC had values of absorbance smaller than at its respective control (i.e. 0.5 g/L bentonite). Indeed, only these trials produced significant differences when compared to its respective control. Besides, even with a very low turbidity forming after the HST, as can be seen in Fig.15, the samples didn't become stable because the A_{540} values were greater than 0.02. Only the positive controls with 1.5 and 2 g/L of bentonite became stable.

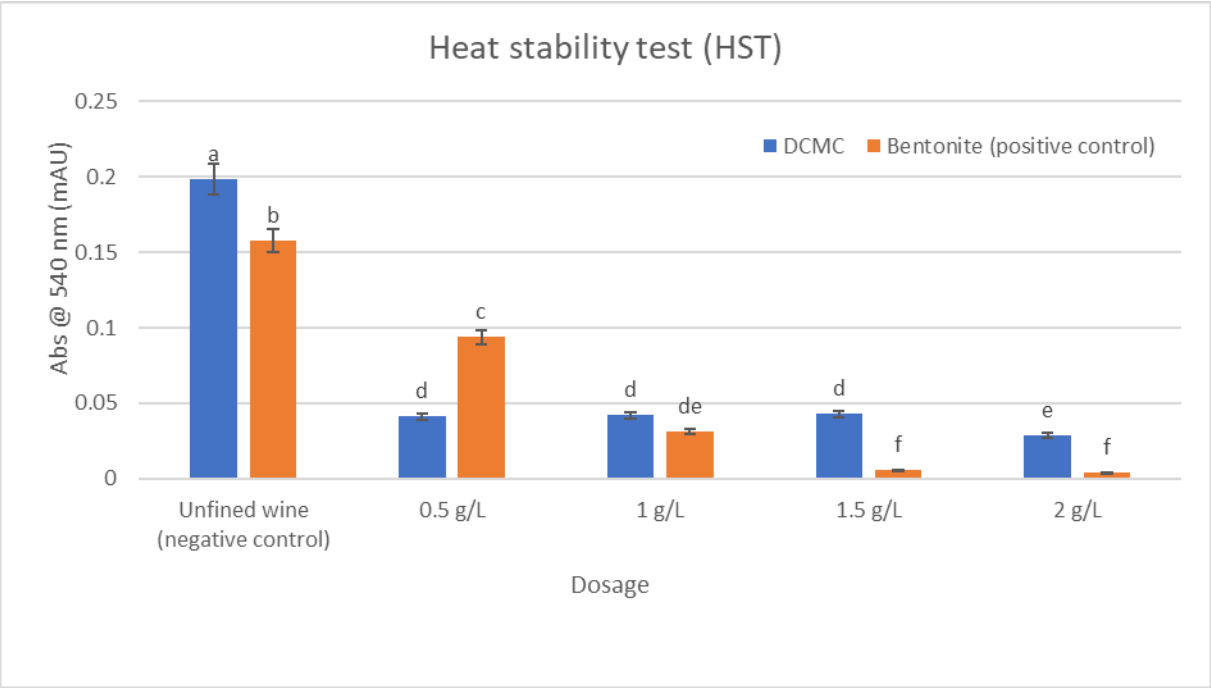


***Figure 15**. Effect of the bentonite and DCMC treatments on turbidity of the Moscatel de Setúbal wine with increasing amounts of bentonite or DCMC. Different letters represent different homogeneous subsets for p = 0.05.*

Figure 16. *Samples of Moscatel de Setúbal wine untreated (left) and treated with 0.5 g/L of DCMC (right).*

The samples from the Viosinho varietal wines, after the treatment with DCMC, produced a significant effect on the protein concentration when compared to the negative control. Also, in this experiment, DCMC showed that it was able to remove more than half of the wine proteins at all doses tested. Besides, all DCMC-treated samples were statistically similar among them and only the sample treated with 0.5 g/L of DCMC had less protein than its positive control (i.e. 0.5 g/L bentonite), and the sample with 1.5 g/L of polymer, that had more proteins than the positive control (i.e. 1.5 g/L bentonite) produced significant differences when compared to their positive controls (Fig. 16).

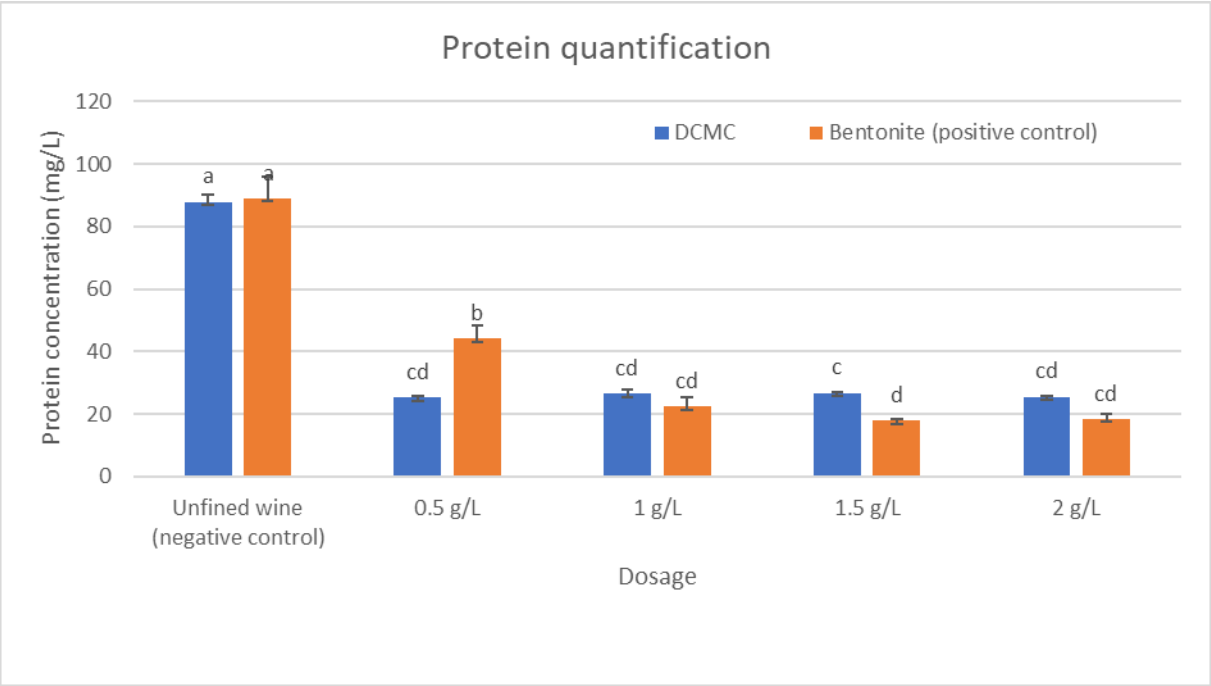


Figure 17. *Protein concentration (in mg/L) in Viosinho wine samples, quantified by the Bradford method, with increasing amounts of bentonite or DCMC. Different letters represent different homogeneous subsets for p = 0.05.*

In what concerns the HST, the samples treated with DCMC produced significant differences compared to the negative control, but in this experiment the impact of bentonite on the prevention of haze formation was greater: all samples treated with bentonite were stable, with low values of absorbance after the treatment, while the wines treated with DCMC reached stability, with lower values of absorbance obtained with doses of 1.5 and 2 g/L, and only the sample treated with 2 g/L of DCMC produced less significant statistical differences when compared to its positive control (Fig. 17). Besides, the sample treated with 0.5 and 1 g/L of DCMC were considered almost stable but with the values of absorbance slightly higher than 0.02.

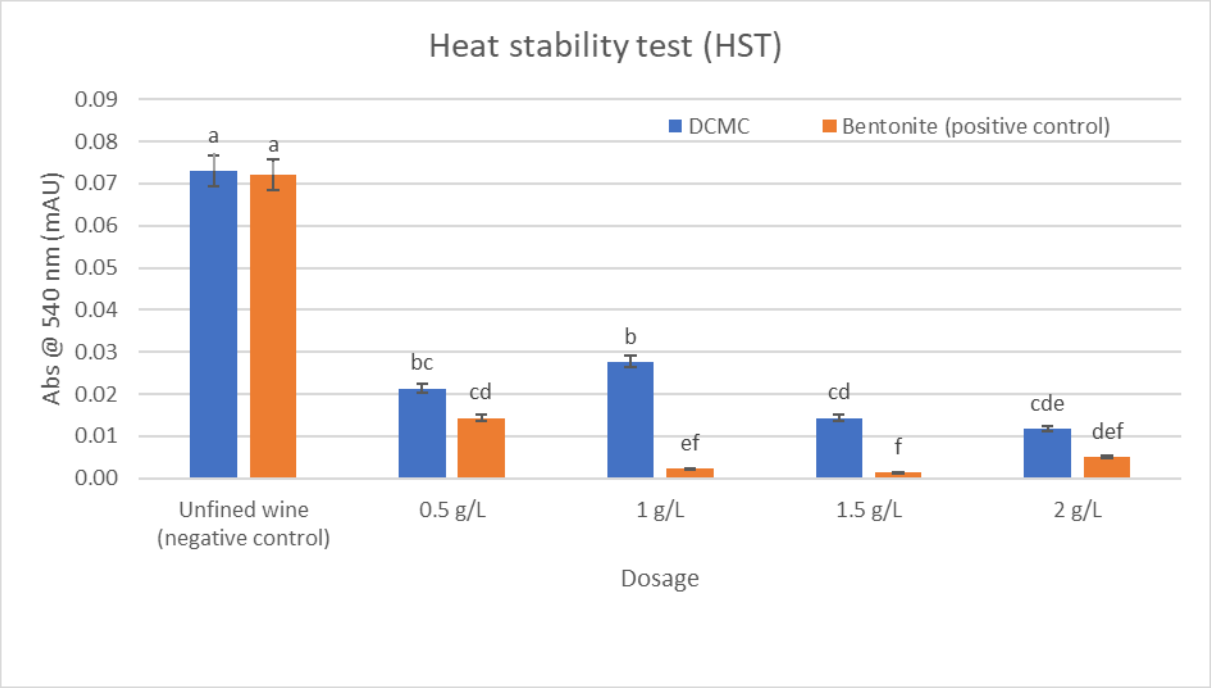


Figure 18. *Effect of the bentonite or DCMC treatments on turbidity of the Viosinho wine with increasing amounts of bentonite or DCMC. Different letters represent different homogeneous subsets for p = 0.05.*

Regarding the samples of Encruzado varietal wines, which had a turbidity value close pass-fail point in protein stability tests suggested by Pocock and Waters (2006)(table 2), lost half of their proteins after the treatment with DCMC, producing a statistically significant effect when compared to the negative control, but only the samples treated with 0.5 g/L of DCMC were statistically similar at their positive controls (Fig. 18). However, all samples treated with DCMC, at any doses, were stable after the HST. In fact, there weren't statistically significant differences between the positive control and the samples, but just between the samples and the negative control (Fig. 19).

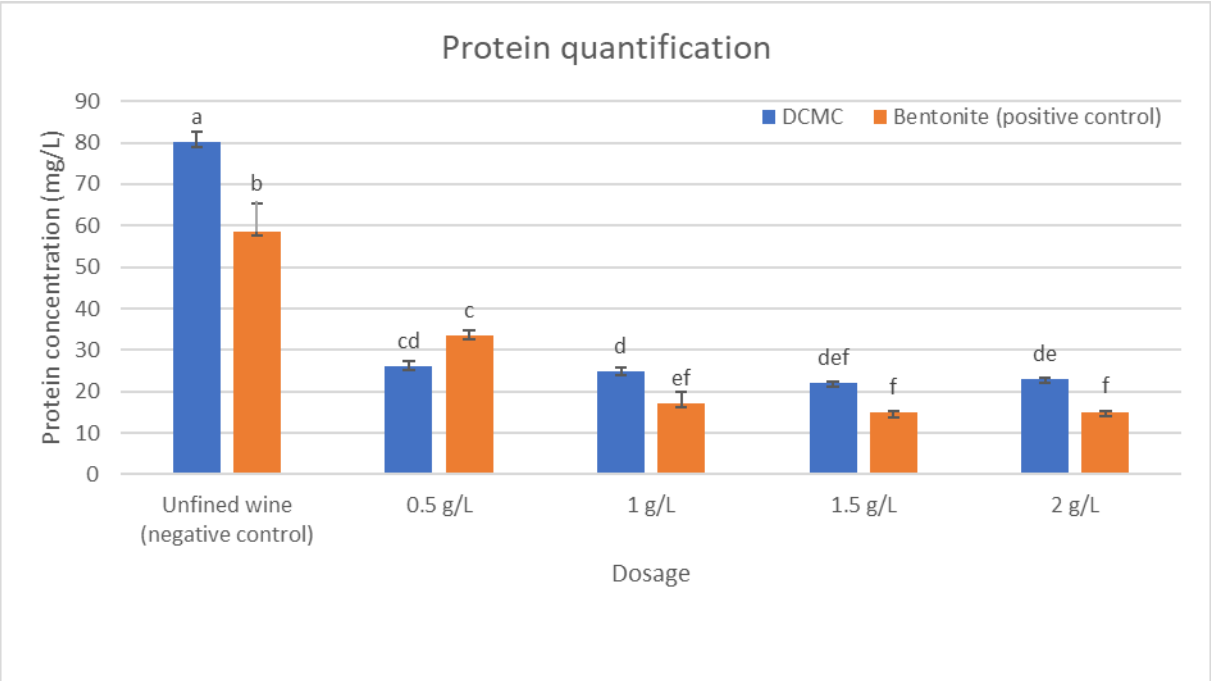


Figure 19. *Protein concentration (in mg/L) of Encruzado wines, quantified by the Bradford method, after treatment with increasing amounts of bentonite or DCMC. Different letters represent different homogeneous subsets for p = 0.05.*

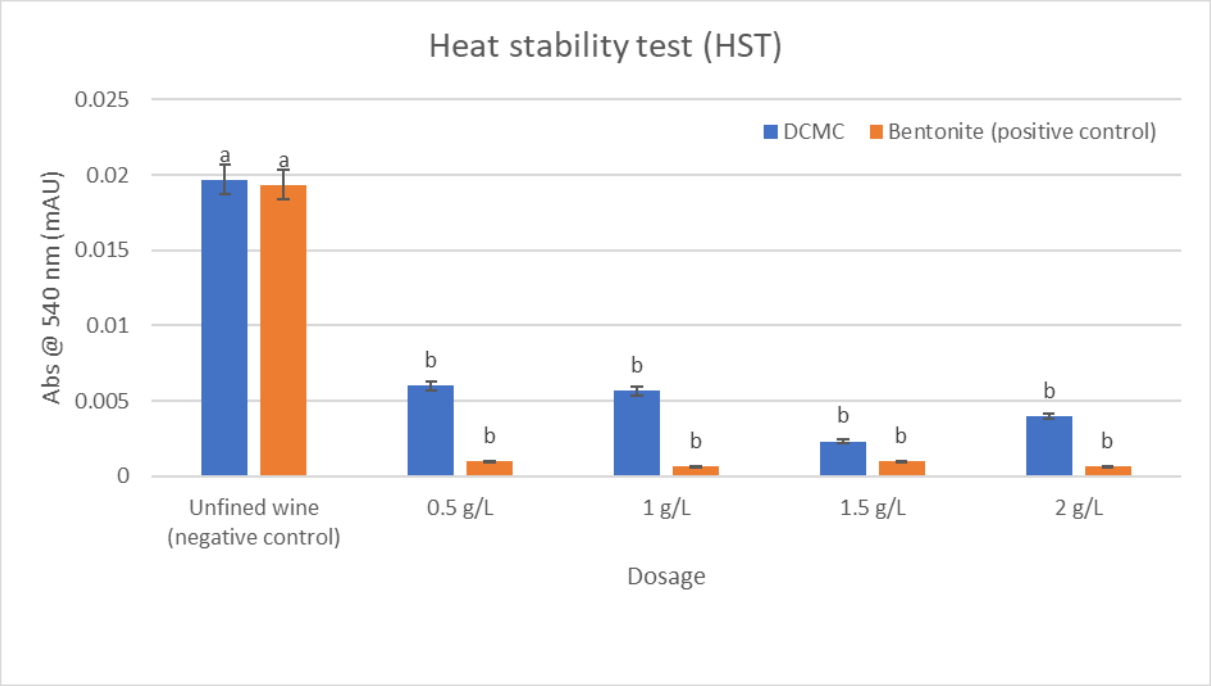


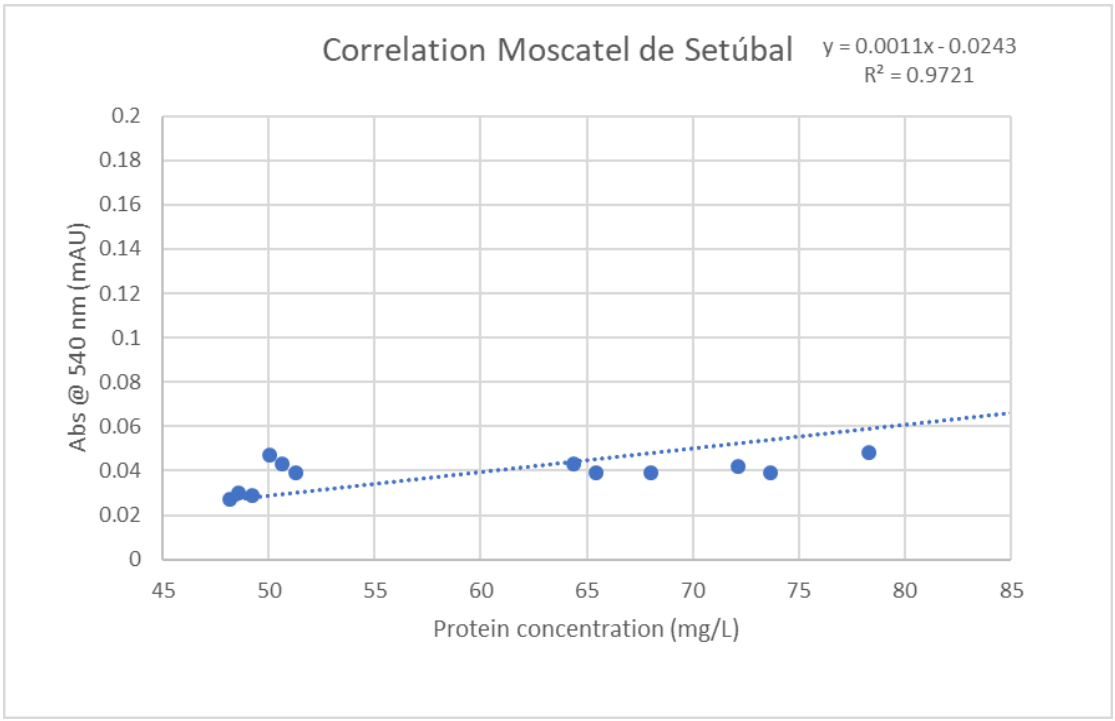
Figure 20. *Effect of bentonite or DCMC on turbidity of the Encruzado wines with increasing amount of bentonite or DCMC. Different letters represent different homogeneous subsets for p = 0.05.*

The work of Lambri *et al.* (2010), has shown that the different initial protein concentration changed the efficacy of bentonite clarification. Indeed, as previously reported by Achaerandio

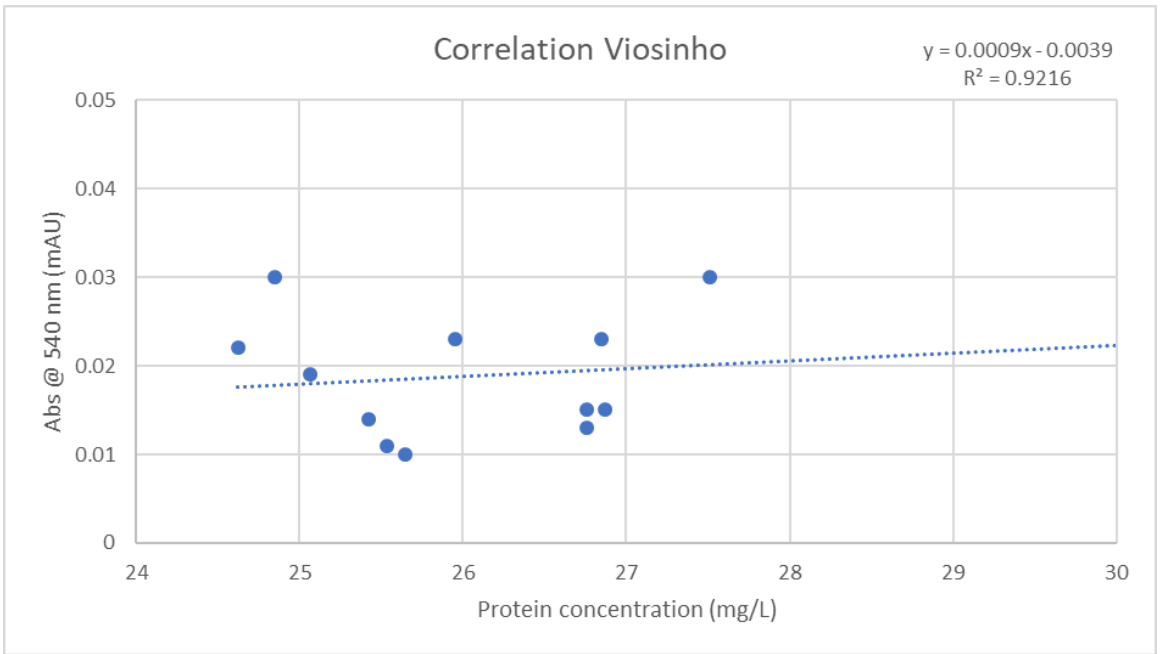
et al. (2001), in general, wines with less initial protein suffer a greater percentage of protein removal by bentonite. Instead, in the present work, the bentonite and even the DCMC exhibited the same performance both for wines with high protein content and for wines with low protein content.

Furthermore, following statistical analysis, the protein concentration was positively correlated with haze formation after the heat stability test for all trials, with R^2 = 0.97 for the Moscatel de Setúbal, 0.92 for the Viosinho and 0.87 for the Encruzado (20 a, b and c).

(a)



(b)



(c)

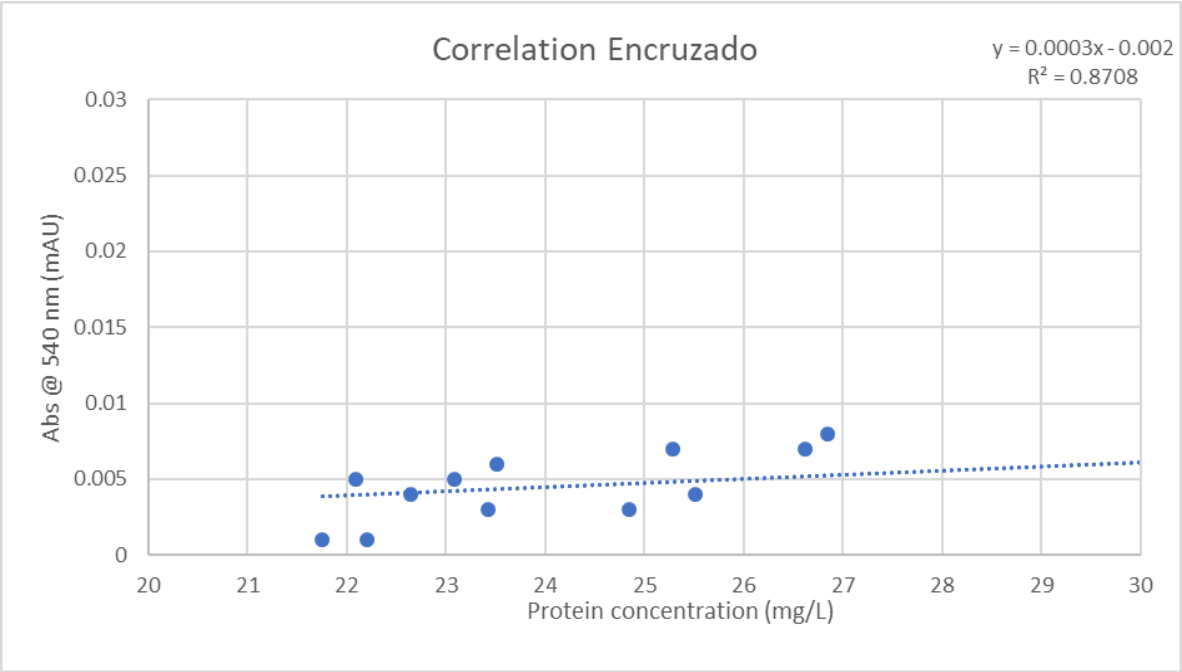


***Figure 21**. Relationship between the wine total protein concentration and haze formation after the HST. (a) . Trendline was for Moscatel de Setúbal, y = 0.0011x - 0.0243, R^2 = 0.9721. (b) Trendline was for Viosinho, y = 0.0009x - 0.0039, R^2 = 0.9216. (c) Trendline was for Encruzado, y = 0.0003x - 0.002, R^2 = 0.8708.*

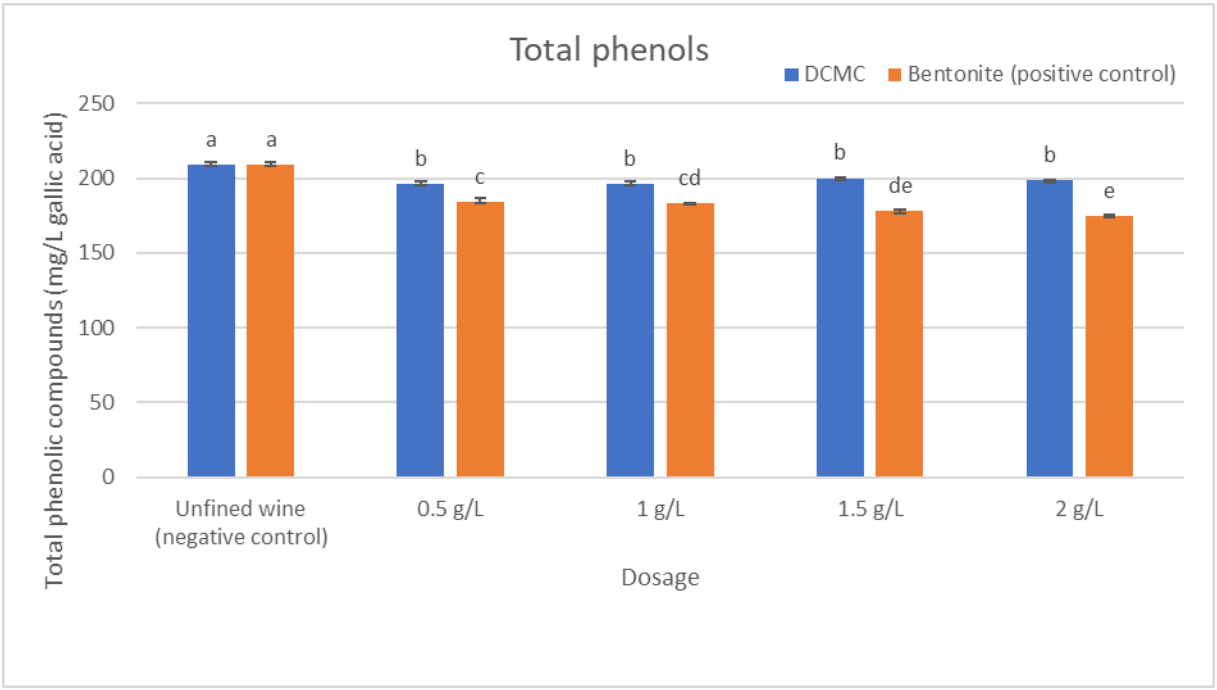
3.1.5 Effect of DCMC on phenolic compounds

DCMC showed an interesting effect on the removal of proteins and a decrease on haze formed, but the same did not happen in what concerns the removal of phenols. Indeed, for all three wines analysed, the decrease of total phenols was low, and just in the test with Moscatel de Setúbal 2018 the samples treated produced a statistically significant effect when compared to the negative control. In the tests with the other two wines, the DCMC-treated samples were statistically similar to the negative control and the DCMC didn't involve a large decrease in phenolics.

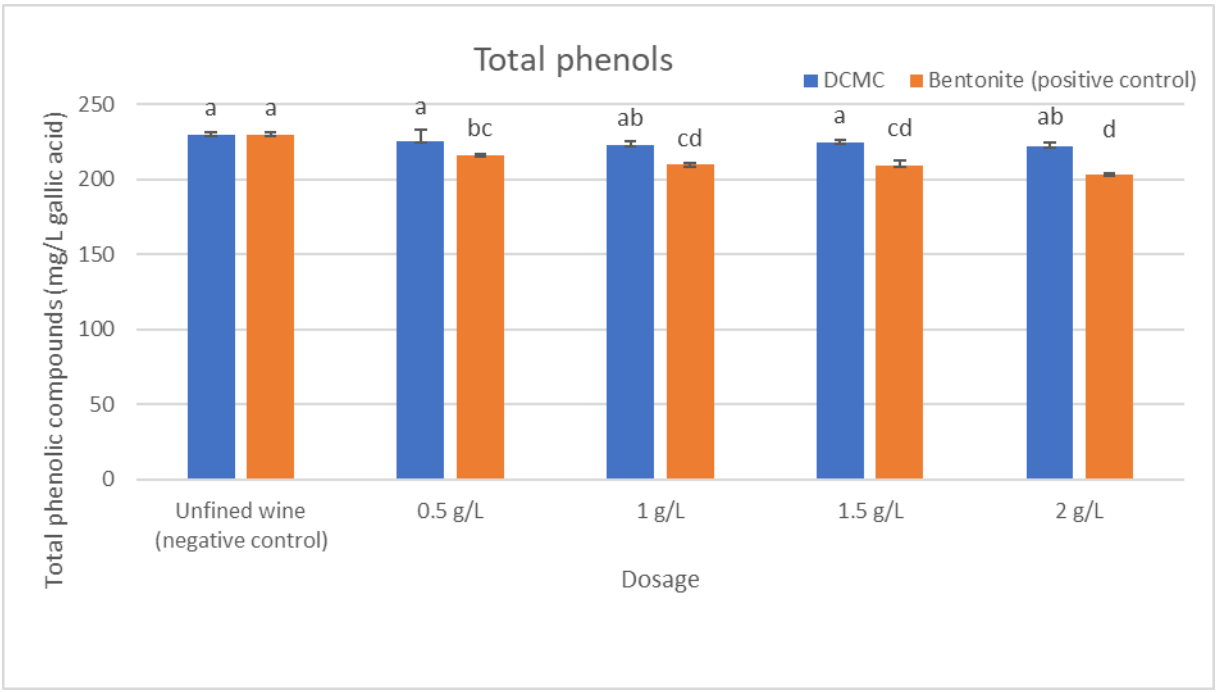
Furthermore, in all three cases, the samples treated with DCMC produced statistically significant differences when compared to the positive controls, since the treatment with bentonite always involves a linear significant in the amount of total wine phenols when the bentonite doses increase, showing that bentonite has a significant effect on the removal in total phenols, as already showed in the work of Main and Morris (1994).

In contrast, the DCMC treatment has little influence on the wine phenolics content and at any dose tested it only removes a small part of the phenolic compounds (Fig. 21 a, b and c).

(a)


Total phenols
DCMC
Bentonite (positive control)
Total phenolic compounds (mg/L gallic acid)
250
200
150
100
50
0
a a
b c
b cd
b de
b e
Unfined wine (negative control)
0.5 g/L
1 g/L
1.5 g/L
2 g/L
Dosage

(b)


Total phenols
DCMC
Bentonite (positive control)
Total phenolic compounds (mg/L gallic acid)
250
200
150
100
50
0
a a
a bc
ab cd
a cd
ab d
Unfined wine (negative control)
0.5 g/L
1 g/L
1.5 g/L
2 g/L
Dosage

(c)

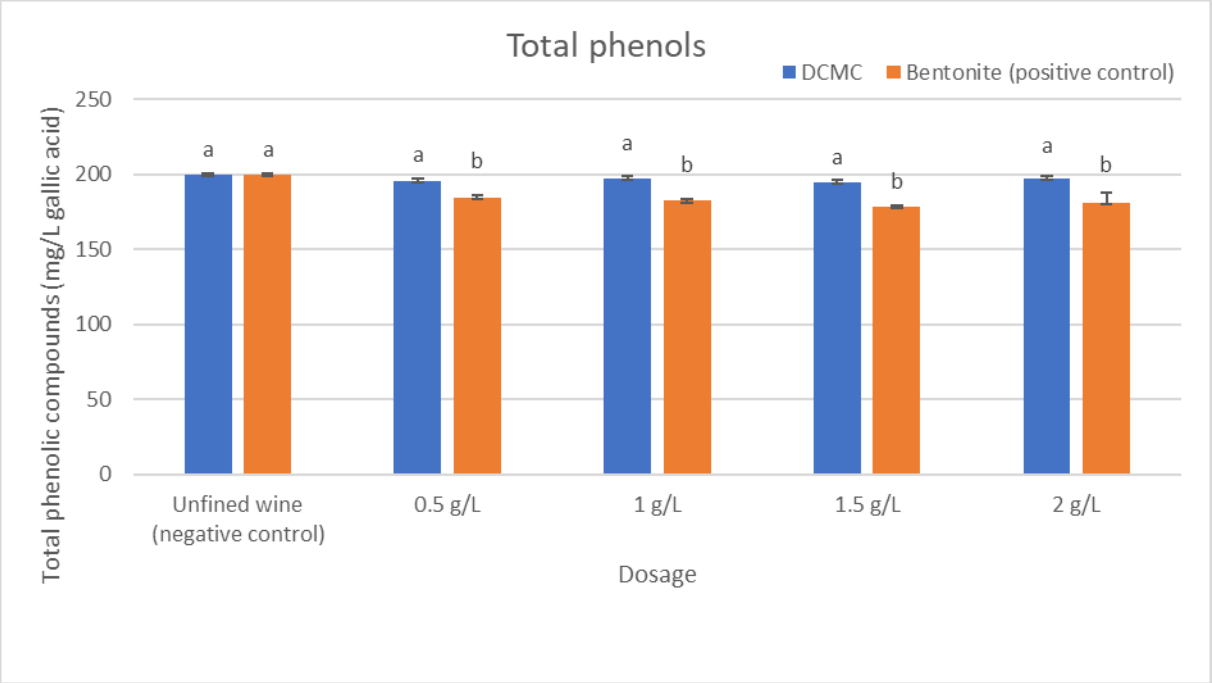


Figure 22. *Changes in amount of total wine phenols detected by the absorbance at 280 nm for increasing amounts of bentonite or DCMC in samples of Moscatel de Setúbal (a); Viosinho (b), Encruzado (c). Different letters represent different homogeneous subsets for p = 0.05.*

3.1.6 Effect of DCMC on the wine pH

For all wine samples treated with DCMC and bentonite, and on the controls, the pH was measured before and after treatment to see if there was any effect exerted by the DCMC and/or bentonite. In all trials, the maximum increase in pH, after the treatment, was of 0.1. The same trend was observed for the positive control (i.e. bentonite). In all 3 wines the DCMC had a small impact on the pH, indeed in almost all trials, the positive controls had a pH value significantly different than the sample treated with DCMC.

As shown in Fig. 22, the samples of the wines from the Moscatel de Setúbal variety, treated with 0.5 g/L of DCMC, were the only trials that had a pH slightly higher than to the positive control, and only in this case the positive control at 0.5 g/L wasn't statistically different when compared to the negative control. In fact, with 1 g/L of DCMC the pH was statistically different than its positive control due to lower pH of the samples treated. Regarding the samples treated with 1.5 and 2 g/L of DCMC fining and their positive controls, these were almost statistically identical between them. But they were statistically different when compared to the negative control.

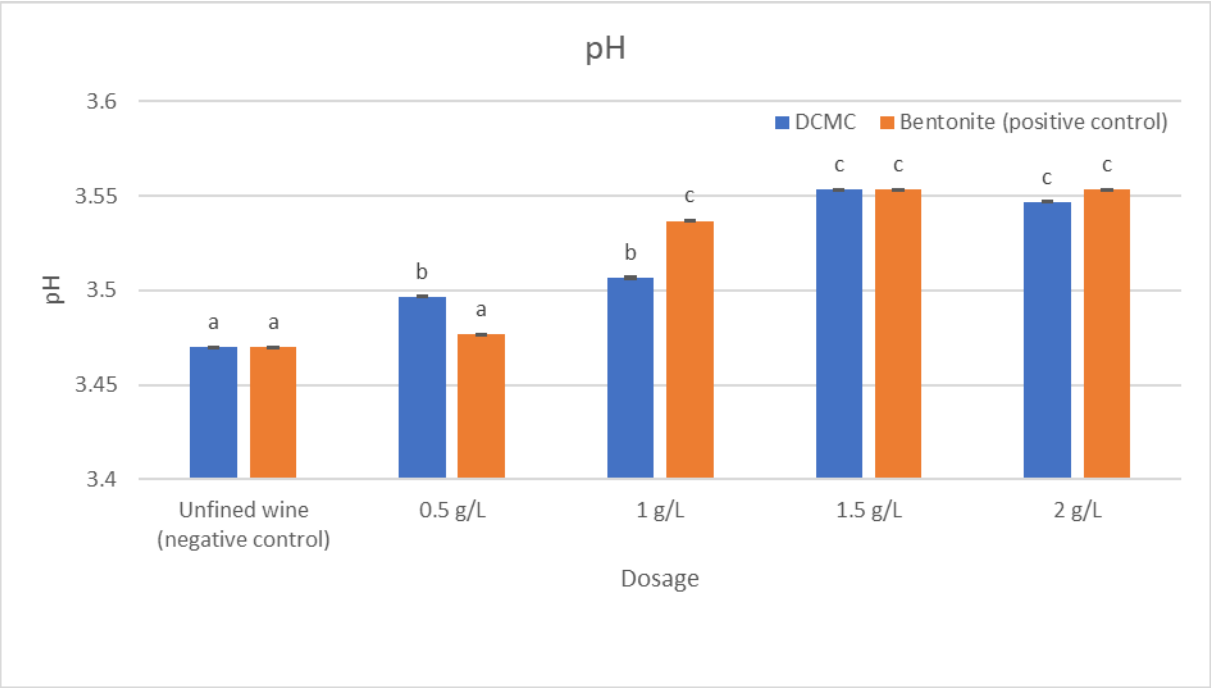
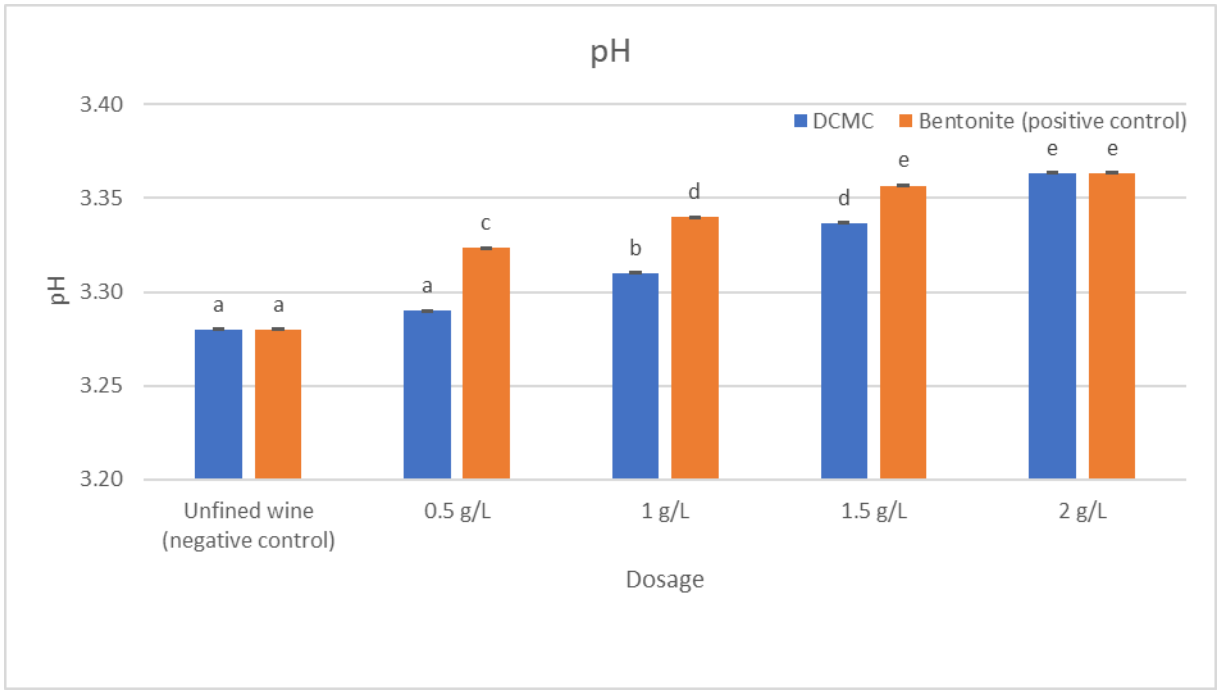


Figure 23. *Changes in pH in samples of Moscatel de Setúbal wine treated with increasing amounts of bentonite or DCMC. Different letters represent different homogeneous subsets for p = 0.05.*

Concerning the wine samples of Viosinho and Encruzado varieties, the samples treated with DCMC were statistically different than their positive controls, and only with doses of 2 g/L there weren't statistically significant differences between the samples treated with DCMC and their positive control. Besides, in both cases, with doses of 0.5 g/L of DCMC, the fining agent did not produce a statistically significant effect on pH (Fig. 23 a, b).

As a whole, we may conclude that in contrast to bentonite fining, treating wines with DCMC is effective at very low doses (e.g. 0.5 g/L) in removing proteins, withdraws only residual amounts of phenolic compounds, and leads to a minor increment in the wine pH.

(a)



(b)

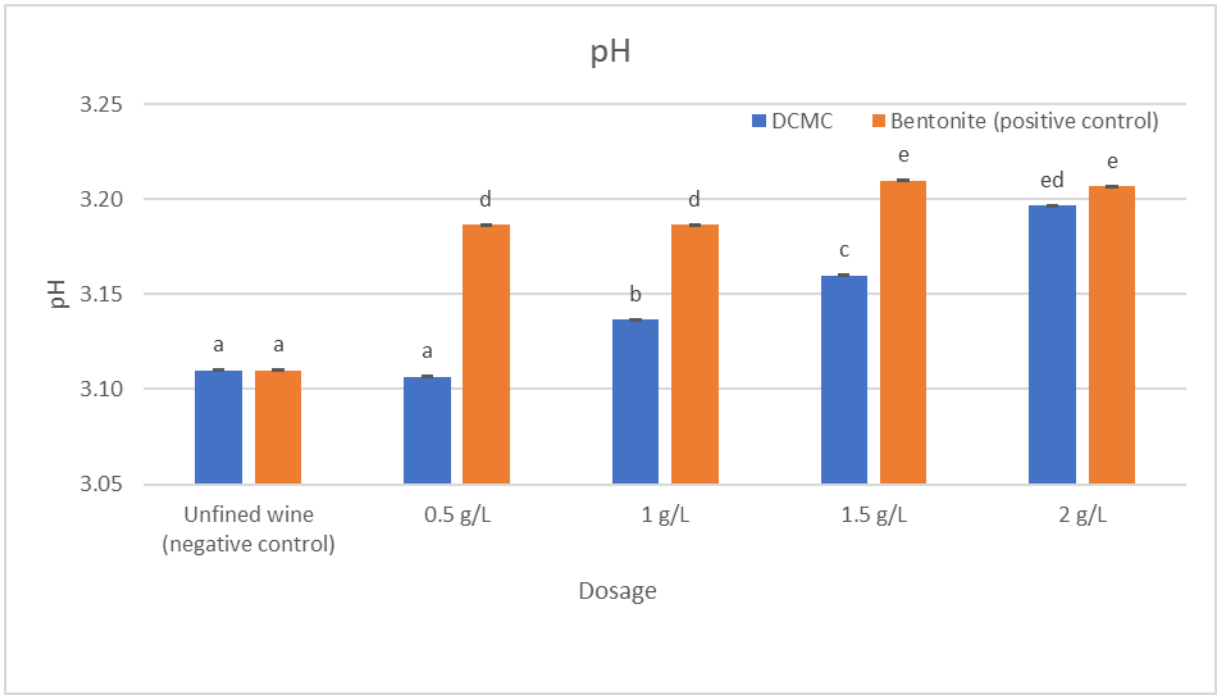


***Figure 24**. Changes in the treated wine pH with increasing amounts of bentonite or DCMC in samples of Viosinho wine (a), and Encruzado wine (b). Different letters represent different homogeneous subsets for p = 0.05.*

3.1.7 Effect of DCMC on calcium and sodium

The DCMC has a high ability to remove positively charged molecules from wines, therefore it can carry out a cation exchange involving the removal of proteins. Thanks to this

mechanism, the DCMC, which has sodium ions in its structure, is also able to remove calcium resulting in an increase in the sodium content in the wine due to its release.

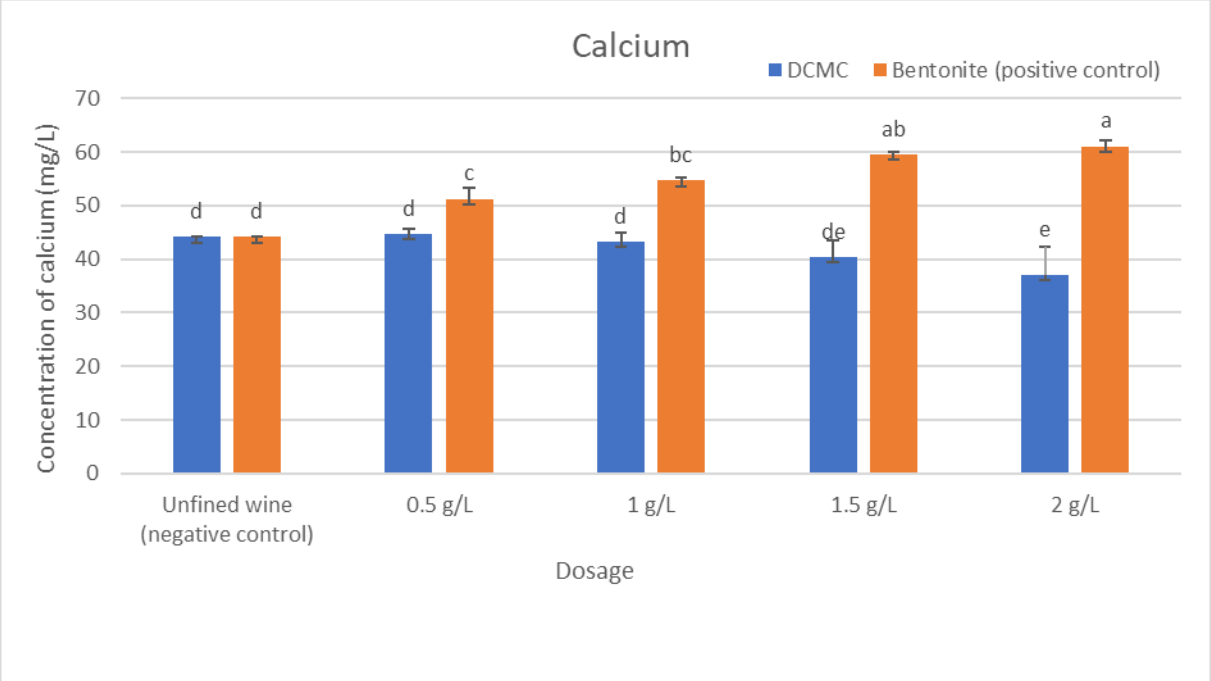
Calcium ion is one of the most important factors that can lead to tartaric instability, being 10 times less soluble than potassium bitartrate (Ribéreau-Gayon *et al.,* 2006), so its decrease makes the wine even more stable to tartaric instability.

For all three wines analysed, DCMC produced significant differences on the decrease of calcium content when compared to the positive control (Fig. 24 a, b and c) resulting in an increase in sodium content and producing significant differences when compared to negative and positive control. (Fig. 25 a, b and c)

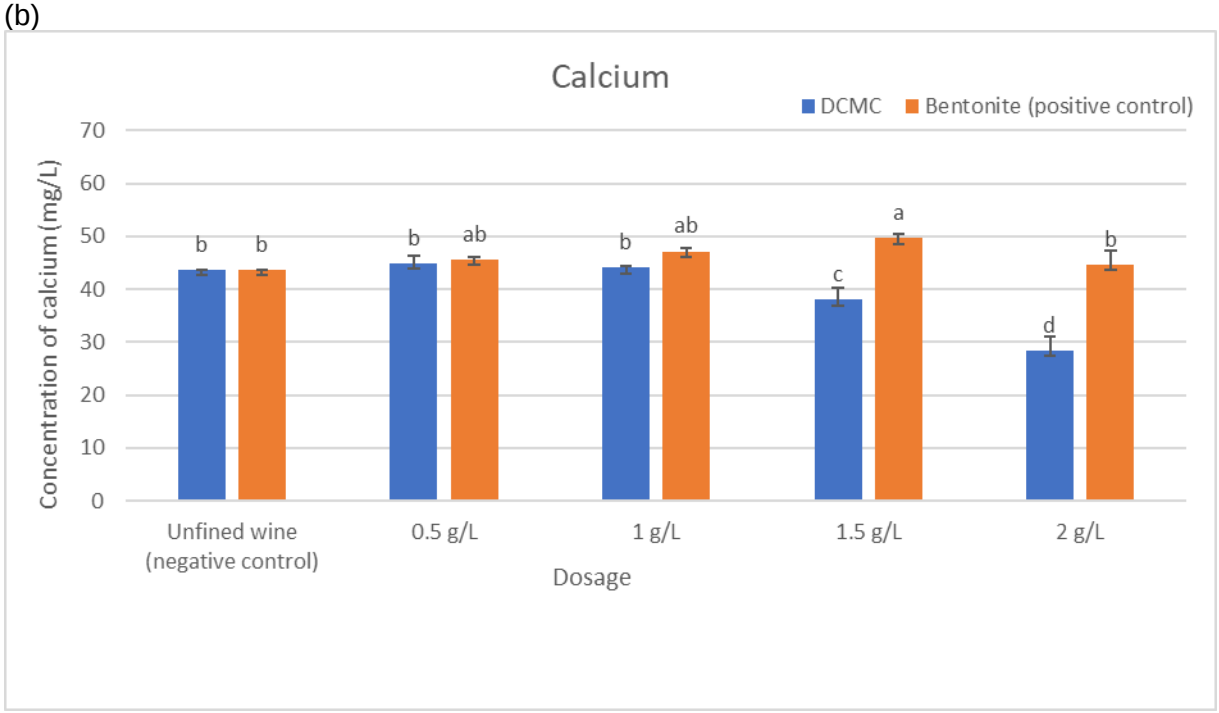
In any case, wines treated with DCMC didn't need of high quantities of DCMC to be stabilized, so the content of sodium in the higher quantities is not a problem.

On the contrary, wines treated with bentonite had always an increase in the calcium content, and an increase in the sodium content even if lower compared to wines treated with DCMC.

(a)



(b)



(c)

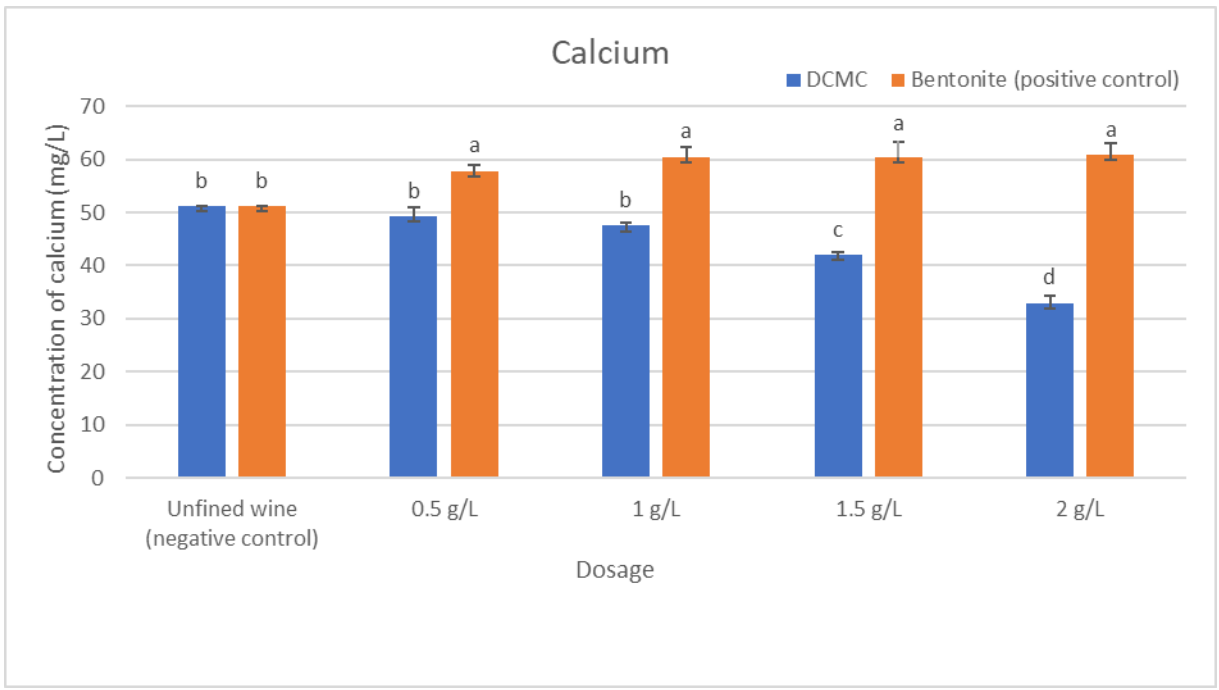
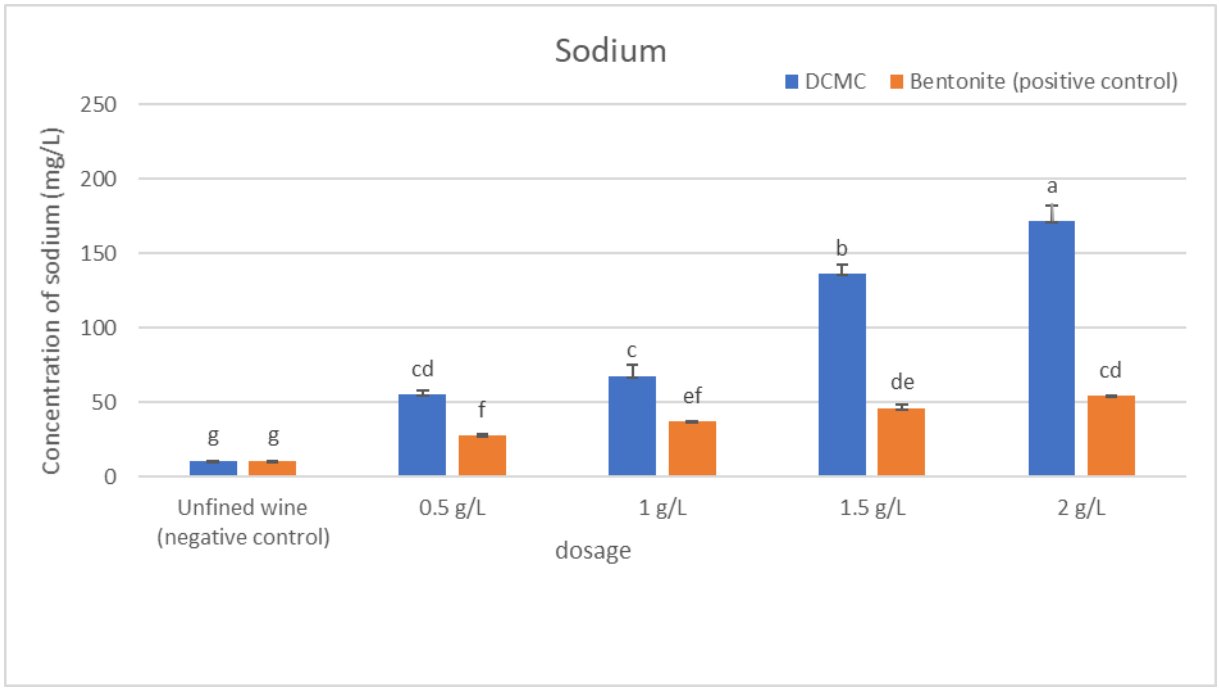
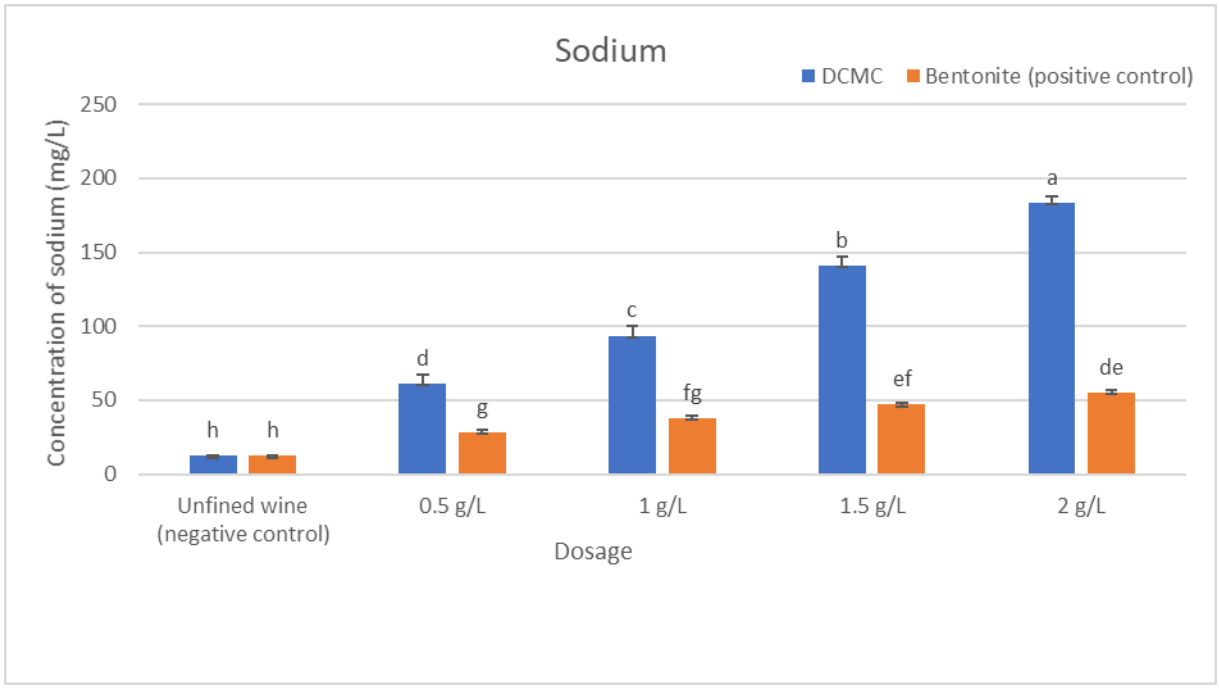


Figure 25. *Changes in amount of calcium detected with increasing amounts of bentonite or DCMC in samples of in samples of Moscatel de Setúbal (a); Viosinho (b), Encruzado (c). Different letters represent different homogeneous subsets for p = 0.05.*

(a)


Sodium
DCMC
Bentonite (positive control)
Concentration of sodium (mg/L)
250
200
150
100
50
0
g
g
cd
f
c
ef
b
de
a
cd
Unfined wine (negative control)
0.5 g/L
1 g/L
1.5 g/L
2 g/L
dosage

(b)


Sodium
DCMC
Bentonite (positive control)
Concentration of sodium (mg/L)
250
200
150
100
50
0
h
h
d
g
c
fg
b
ef
a
de
Unfined wine (negative control)
0.5 g/L
1 g/L
1.5 g/L
2 g/L
Dosage

(c)

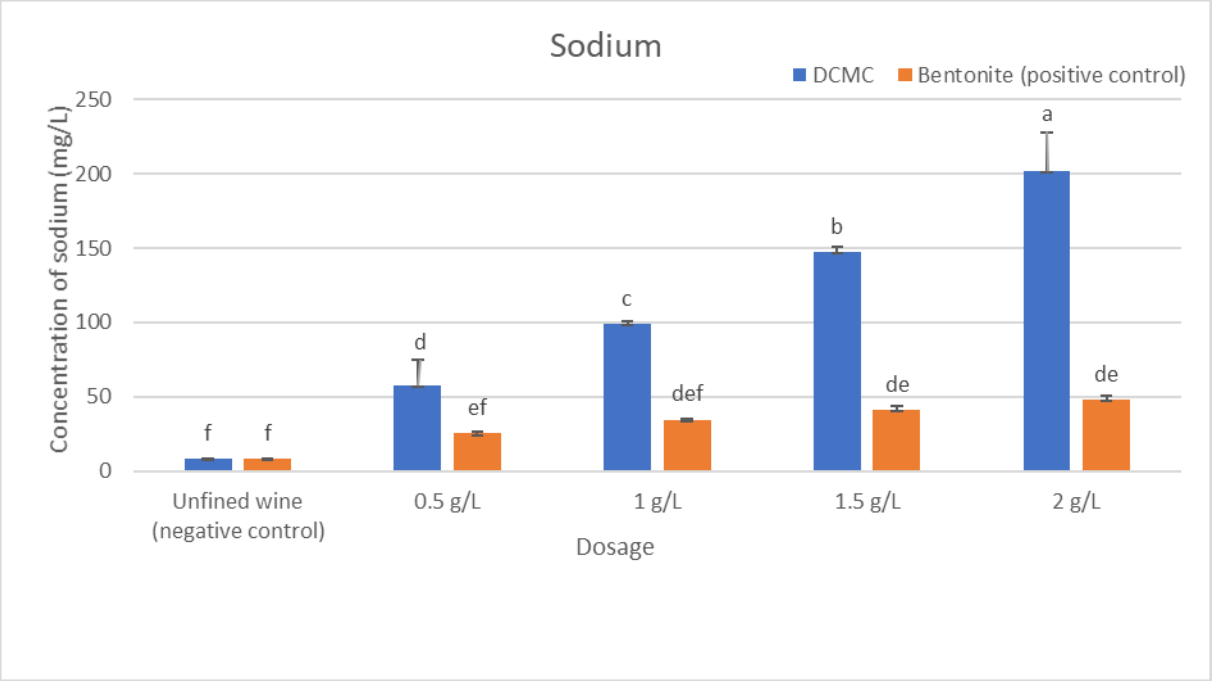


Figure 26. *Changes in amount of sodium detected with increasing amounts of bentonite or DCMC in samples of in samples of Moscatel de Setúbal (a); Viosinho (b), Encruzado (c). Different letters represent different homogeneous subsets for p = 0.05.*

4 Conclusion

In all trials, the DCMC-treated wines produced a significant effect on the removal of proteins and on the haze formation. Particularly, it was observed that there was always a decrease on the protein concentration of at least 50% when compared to the negative control. Moreover, with a low dose of 0.5 g/L DCMC, the protein concentration was always lower than the samples treated with the same amount of bentonite. Indeed, DCMC seem to have a strong effect on the protein concentration especially at low doses, attenuating its effect with increasing doses and thus leading to minimal further reductions in the protein concentration. Only in the trials with the high protein concentration Moscatel de Setúbal varietal wine there was a slight scalar reduction with increasing polymer doses. For the Viosinho and the Encruzado varietal wines, the wine protein content after the clarification was about equal to all the doses (i.e. between 0.5 and 2.0 g/L). In contrast, the treatment with bentonite involved an approximately linear decrease in the amount of wine proteins when the doses increased, reaching very low values for the highest doses, as previously shown from Vincenzi *et al.* (2015). Anyway, except the trials with the wine from Moscatel de Setúbal varietal wine, which had, after heat stability test, a higher turbidity value than the pass-fail point in protein stability tests suggested by Pocock and Waters (2006) , the other tests with the other two wines became almost fully stable after the treatment with the polymer.

The difference between clarification with bentonite and clarification with DCMC consists mainly in the different doses necessary to achieve stability. In fact, the DCMC may not remove so much protein as bentonite, but it is important to note that since the accuracy of the Bradford method to quantify proteins, as with any other protein quantification method that we may apply to wines, is far from desirable, we are not sure if the final values of protein detected after the HST are really protein. Furthermore, stability or near stability seems to be reached with low levels, when compared to bentonite, of DCMC.

Even on total phenols and on pH there were differences between the two treatments. The DCMC did not shown a significant effect on removal of total phenols, thus demonstrating the selectivity of the DCMC in removing almost only proteins.

In the contrary, as shown also by Main and Morris (1994), bentonite has a very significant effect on the removal of total phenols.

The treatment with bentonite could have involved a greater reduction of the wine proteins in all the tests compared to the treatment with DCMC because the proteins, as demonstrated Siebert *et al.* (1997), could be bound to polyphenols. Consequently, with the removal of polyphenols by bentonite, a small fraction of protein could be eliminated indirectly, or other way around, with the removal of proteins by bentonite, a small fraction of polyphenols could be eliminated indirectly.

Also, for the pH, the treatment with the polymer affected less the raising in the treated wine pH than the clarification with bentonite.

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