The mechanism of the protein haze formation in white wine.
The role of the sulfur dioxide

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Thank to all…

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Abbreviations

PR: pathogenesis-related

TLPs: thaumatin-like proteins

CHA: Chardonnay

Sab: Sauvignon Blanc

MW: molecular weight

pI: isoelectric points

kDa: kilodaltons

SAXS: small angle X-ray scattering

DSC*:* differential Scanning Calorimetry

DLS: dynamic light scattering

HIC: hydrophobic interaction chromatography

Abs: absorbance

PVPP: polyvinylpolypyrrolidone

IWP: isolated wine protein

WMS: wine model solution

I: ionic strength

DDT: dithiothreitol

TCEP: tris-(2-carboxyethyl)-phosphine

SCX: strong cation exchange chromatography

PCA: principal components analysis

HST: heat stability test

Abstract

The present work tries to identify the (bio)chemical mechanism underlying protein haze formation in wines, a common defect in many commercial wines. In particular, this work is centred on the role played by sulfur dioxide, an additive widely used in the oenological sector, in this phenomenon, as it was recently shown to be one of the multiple factors implicated of protein instability.

In order to define and interpret the interactions of sulphur dioxide with the wine proteins, the protein profiles of different varietal wines, prepared from Portuguese white grapes such as Alvarinho, Arinto, Encruzado, Macabeu, Moscatel Galego, Moscatel of Setúbal and Viosinho of two different vintages have been characterized.

The results show that wines produced with certain varieties are more prone to protein haze than others in the presence of sulfur dioxide, both for their protein content and for the protein classes contained in them.

Keywords: protein haze, sulfur dioxide, Portuguese wines, white wine, chromatography

Resumo

O presente trabalho tenta identificar o mecanismo (bio)químico subjacente à formação da turvação proteica nos vinhos, um defeito comum em muitos vinhos comerciais. Em particular, este trabalho está centrado no papel desempenhado pelo dióxido de enxofre, um aditivo amplamente utilizado no setor enológico, nesse fenómeno, pois recentemente mostrou-se ser um dos múltiplos fatores implicados na instabilidade proteica.

Para definir e interpretar as interações do dióxido de enxofre com as proteínas do vinho, foram caracterizados os perfis proteicos de diferentes vinhos varietais, preparados a partir de castas de uvas brancas Portuguesas como Alvarinho, Arinto, Encruzado, Macabeu, Moscatel Galego, Moscatel de Setúbal e Viosinho de dois *vintages* diferentes.

Os resultados obtidos mostram que os vinhos produzidos com certas castas são mais propensos à turvação proteica do que outros na presença de dióxido de enxofre, tanto pelo seu conteúdo proteico como pelas classes de proteínas neles contidas.

Palavras-chave: turvação proteica, dióxido de enxofre, vinhos Portugueses, vinho branco, cromatografia

Resumo alargado

O presente trabalho tenta identificar o mecanismo (bio)químico subjacente à formação da turvação proteica nos vinhos, um defeito comum em muitos vinhos comerciais. Em particular, este trabalho está centrado no papel desempenhado pelo dióxido de enxofre, um aditivo amplamente utilizado no setor enológico, nesse fenómeno, pois recentemente mostrou-se ser um dos múltiplos fatores implicados na instabilidade proteica.

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Em geral, tem sido observado como a tendência climática (principalmente a chuva) pode influenciar a composição proteica das diferentes variedades de uvas, com base no facto de que todos os vinhos analisados em 2017 têm menos proteína que os vinhos de 2018, produzidos com as mesmas variedades de uvas e colhidas das mesmas plantas. Além disso, a partir do presente trabalho, também se destaca que, além do teor em proteínas, o ano também influencia os perfis de proteínas, em termos das relações entre as diferentes classes de proteínas presentes. As proteínas do tipo taumatina constituem uma classe fortemente instável, especialmente se presente em concentração elevada e na presença de dióxido de enxofre.

Testes de estabilidade proteica foram realizados em vinhos sob condições reais, validando a ação do dióxido de enxofre como fator não proteico implicado na formação de turvação proteica.

Palavras-chave: turvação proteica, dióxido de enxofre, vinhos Portugueses, vinho branco, cromatografia

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1. Introduction

Wine, like most food products, contains different nitrogenous substances, the most important of which are proteins. Depending on the variety, vintage, weather conditions, fertilization or healthy status of the vines, wines most often present protein concentrations in the range between 15 and 230 mg/L (Ferreira *et al., 2002;* Waters *et al*., 2005).

Wine proteins assume a very important role on the technological and economical features since they can affect the clarity and stability of white wines. A wine that suffered protein precipitation will have a huge impact in the first impression on the consumer, who will repel the wine containing the aggregated proteins, regardless of how the wine tastes (Ferreira *et al*., 2004). This type of haze is one of the most common non-microbial defects of commercial wines (Bayly *et al*., 1967). Only in a few cases, the proteins have a positive role on wine quality, like for example in sparkling wines for the foam quality. In fact, wine proteins, especially when interacting with other components like mannoproteins, are fundamental for the foam formation and stability in sparkling wines (Briossonet *et al., 1993).* Grape cell wall glycoproteins and proteoglycans rich in arabinose and galactose (or arabinogalactan-proteins) can also have a positive effect in stabilizing the wine against heat-related protein instability, for this called non-haze-forming proteins (Van Sluyter *et al.*, 2015).

Most proteins present in wines derive from the grape pulp, but there are also some proteins that can be originated from the process of yeast autolysis (Feuillat *et al*., 1980). As said before, the grape variety, the growth environment, and the process of fermentation affects protein concentration and consequently the stability of the wine. The climate conditions are very important because they can affect not only the final protein concentration but also the type of proteins synthesized during grape development and maturity. According to Murphry *et al.* (1989) the soluble proteins found in the wine increase following the grape ripening. This aspect occurs because by decreasing the physical-mechanical defences following ripening, due to the degradation of the pectins, the chemical defences of berries increase. Grape constitutive pathogenesis-related (PR) proteins synthesis starts rapidly after veraison and their pattern of accumulation is parallel to that of sugars (Luis, 1983; Tattersall *et al*., 2001). It is important to underline that the concentration of protein found in the grape and in the final wine is very different, because most of them are lost during vinification (Ferreira *et al*., 2000). This decrease in protein content is explained thanks to the grape housekeeping protein degradation by proteolysis and denaturation, caused by proteases and changes of pH, rspectively. Some of the constitutive proteins (i.e. the grape PR proteins) resist at these adverse conditions in musts and wines and originate the subsequent problem of instability in white wines, a topic of great importance that has been studied over the last decades.

However, the mechanisms leading to haze formation are complex and not yet fully understood.

The proteins that 'survived' the unfavourable process of vinification, are the most abundant in the wine and comprise the plant defense family of proteins known as pathogenesis-related (PR) proteins, which include chitinases, osmotins, thaumatin-like proteins (TLP), invertases and β-glucanases (Robinson *et al.,* 2000). These different classes mentioned are shown in figure 1, that represents a typical electrophoretic profile of wine polypeptides. Infection with common grapevine pathogens before veraison, results in the increasing concentration of the inducible PR proteins in the grapes, which 'lived-through' to the wine, since these proteins are believed to exert biocontrol against fungal pathogens (Cilindre *et al.,* 2005). In addition, PR proteins are resistant to proteolysis and to low pH values (3.0 - 3.8), and have therefore been identified as the principle responsible for turbidity of white wines.

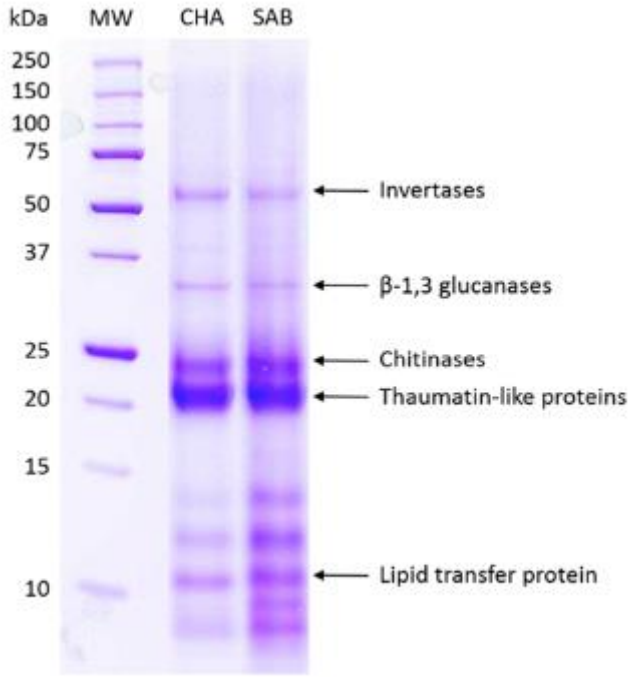


Figure 1. *Typical electrophoretic profiles of two unfined grape juices (CHA, Chardonnay; SAB, Sauvignon blanc), with polypeptide band identities assigned by proteomic analysis* (*Van Sluyter* et al., *2015*).

The presence of proteins in wine is certainly a pre-requisite for haze formation, so it is generally accepted (but not entirely correct) that the higher the concentration of proteins, the higher is the risk of wine instability (Mesquita *et al*., 2000). But focusing exclusively on the wine protein concentration is not enough to explain the complex mechanism of protein

instability. Indeed, Trivedi *et al.* (2009) divided the factors that influence wine protein stability in two categories:

1) Intrinsic factors: namely physico-chemical properties of the proteins themselves;
2) Extrinsic factors: all the external circumstances that affect the stability of the wine proteins, like temperature, pH, alcohol concentration, organic acids, sulfate and ionic strength.

Recently, an important role for another extrinsic factors was identified: sulfur dioxide. This effect was established by Chagas *et al*. (2016) proving that the wine, after heat stress, in presence of SO_2, became highly unstable regarding the protein stability, originating high turbidity when compared to the same wine in the absence of sulfur dioxide.

1.1 Mechanism of haze formation

The complex mechanism of the haze formation in the wine is not yet completely understood, even considering that according to several studies it consists of three important steps, as described in figure 2 (Van Stuyter *et al*., 2015). In the first stage, the wine proteins unfold (loss of tertiary structure) due to high temperatures, to which the wine is subject, that induces changes in the protein intramolecular interactions (Dufrechou *et al*., 2010). Once unfolded, the unstable proteins will aggregate through hydrophobic interactions and flocculate to form a visible haze, a colloidal aggregation derived from intermolecular interactions. During the heating stage, only a few proteins start to aggregate. In fact, the real aggregation and consequent haze formation takes place mainly during the cooling stage.

Each class of proteins exhibits a different behaviour in what haze forming potential is concerned, since they differ in their chemical-physical properties such as melting point and molecular weight, that affect the unfolding-aggregation model, making the haze formation even more complex and difficult to predict. In addition, as described before, there seems to be a considerable number of non-proteinaceous or extrinsic factors that can modulate the mechanism of the protein haze. For this reason, the different haze-proteins may follow various pathways of haze formation. However, it seems that the common point of instability for all proteins are the hydrophobic interactions: when proteins unfold, some hydrophobic sites, previously buried in the core of the soluble proteins, are exposed to the aqueous exterior and higher the extension of the hydrophobic sites, the greater the potential for haze formation. This point explains the increased instability induced by TLPs, which have a higher hydrophobic surface when compared with other wine protein classes (Marangon *et al*., 2014).

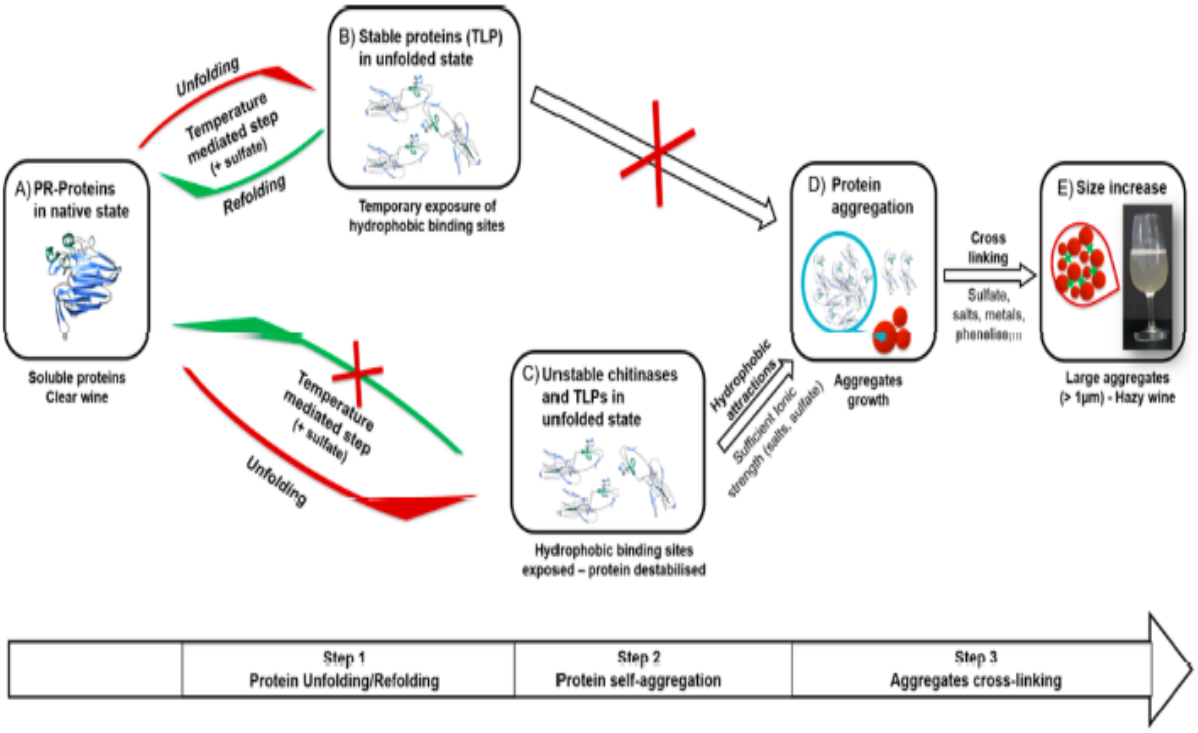


Figure 2. *Revised unfolding and aggregation mechanisms of heat-unstable proteins in wine* (*Van Sluyter* et al., *2015*)

1.2 Intrinsic factors influencing protein stability

The intrinsic factors that influence the stability of wines seem to be totally related to the features of the proteins and not to the environment where they are located. One of these features is certainly the protein molecular weight (MW), regarding which there are conflicting positions in the literature (Ferreira *et al*., 2002; Marangon *et al*., 2011). Most wine proteins show low molecular masses (20 - 30 kDa) and low isoelectric points (4.1 < pI < 5.8), having a positive net charge at the regular wine pH values (Ferreira *et al*., 2000). This aspect is fundamental for the stabilization of the wine. Usually, the most used coadjutant is bentonite which presents a negative net charge at the wine pH, thus allowing it to react electrostatically with the positively-charged wine proteins, inducing their flocculation (Hsu *et al*., 1987) and "theoretically" the stability of the wine. One of the resulting problems is that bentonite doesn't have the same effectiveness to remove all proteins from the wine. In fact, the work of Hsu and colleagues (1987) demonstrated that to remove the proteins in the range 20-30 kDa, thus including chitinases and TLPs, a high level of bentonite is necessary (until 80 g/hl), and that a part of these remain in the wine after the fining treatment. A more recent work (Sauvage *et al*., 2010) reported different results and conclusions. Glucanases, with an intermediate MW of 30-35 kDa, exhibited the highest affinity for the clay, while invertases, with high MW of 60-70 kDa, needed the highest bentonite doses to be removed. The lower molecular weight proteins, thaumatins and chitinases, exhibited intermediate behaviors, with a considerable fraction of the thaumatin (ca. 30%) remaining unaffected for bentonite doses lower than 150 g hl^{-1} (it must be noted that their adsorption by bentonite is achieved at a dose of 200 g hl^{-1}). These observations explain the importance of the molecular weight of proteins and highlight that the proteins with lower molecular weight (identified as TLPs) are responsible for the high instability of wines, but are also the most resistant to be removed by bentonite.

The isoelectric point is another important protein property since it affects both the solubility of the protein in wine and their propensity to be eliminated by clarifying agents, such as bentonite (Dawes *et al*., 1994). In the work of Hsu *et al*. (1987) the wine proteins isoelectric points (pI) correlated with the effectiveness of bentonite in the adsorption of proteins: first bentonite removes proteins with higher pI values (5.8 – 8.0), and only then those with lower pI values (4.1 – 4.8) (identified such as TLPs). This is in accordance with the observed efficiency of bentonite as a function of the wine protein MW and pI

1.3 Extrinsic factors

As said before the amount of proteins present and their physicochemical properties are fundamental to assess the potential of a wine to form haze, but at the same time, there are several non-proteinaceous factors implicated in the protein haze of wines. The most important are pH, temperature, alcohol concentration, organic acids, polyphenols, polysaccharides, ionic strength, sulfate and sulfur dioxide. For this reason, the phenomenon that leads to wine protein haze is described as a multi-factorial process (Batista *et al*., 2010).

1.3.1 pH

One important factor is the wine pH, which is closely related to the physical-chemical features of proteins. At the pH of wine, the proteins are positively charged (for their low pI, 4.1 < p*I* < 5.8) (Ferreira *et al*., 2000), and this allows intermolecular electrostatic repulsions among proteins that are thus expected to contribute to the wine colloidal stability, as well as electrostatic bonding to the negatively charged fining agent bentonite.

Dufrechou *et al.* (2012) studied the effect of the pH in the stability of proteins in a model wine solution and in real wine conditions. The authors used a pH range of 2.5 – 4.0 and showed that lowering the pH increased protein instability, especially for the proteins having molecular weights of 22-28 kDa, identified as thaumatin-like proteins and chitinases. This aspect is likely related to a two-step mechanism, involving conformational changes (pH unfolding) and formation of aggregation-prone intermediates, followed by colloidal aggregation. This aspect is also supported by Dufrechou *et al*. (2013), who showed that the aggregation of some wine proteins at pH below 3.0 at room temperature is triggered by conformational changes. The results of this latter work show, through small angle X-ray scattering (SAXS) analysis curves, the formation of protein aggregates at different pH values (figure 3). Specifically, two pH values were taken in consideration: pH 4, represented by the red line, and pH 2.5, represented by the blue line. In the first case the protein aggregation is not affected by the high pH; in contrast, at pH 2.5 there is a higher presence of protein precipitates. Moreover, the chitinase class of proteins were shown to be influenced by the low pH regarding aggregate formation, whereas TLPs and invertases were not involved in haze formation at this pH. From the results of this work, a lower pH value could be involved in protein instability, but the type of protein and more specifically the isoforms of some proteins play a big role in the instability.

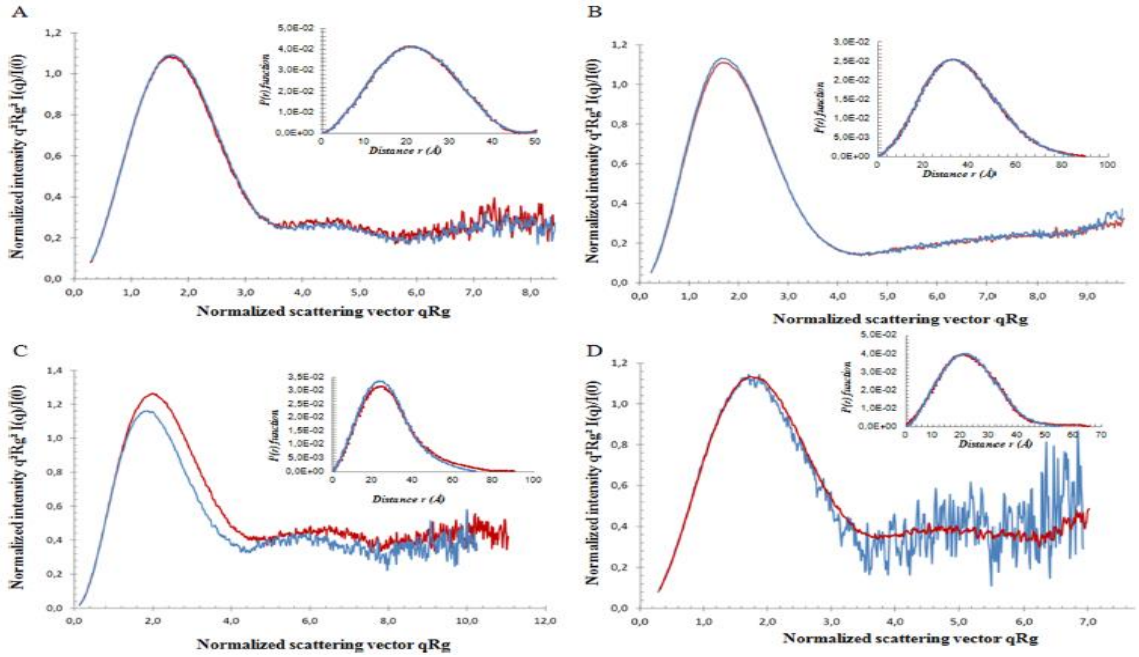


Figure 3. *SAXS curve analysis of (A) VVTL1, (B) invertase, (C) 27.5 kDa chitinase, and (D) 24.2 kDa chitinase. The measurements were performed at pH 4 (curves in red) and pH 2.5 (curves in blue) and superimposed for comparison. The normalized Kratky plot is used to compare the SAXS curves at the two pH values, with normalized scattering vector qRg on the horizontal axis and normalized intensity (qRg)2I(q)/I (0) on the vertical axis. From each SAXS curve at the two pH values, the corresponding autocorrelation function P(r) was calculated and superimposed (with the same color as above). The chitinases 27,5 kDa, in the c) graph are the most affected by the low pH, since the scattering is lower, demonstrating a greater aggregation. (Dufrechou* et al., *2013)*.

When decreasing the pH, proteins become more heavily charged and there are also more repulsive interactions. For the authors (Dufrechou *et al*., 2013) this latter point is responsible for the increase of instability at low pH: in fact, more repulsions could destabilize the folded conformation, which in turn provokes changes in the surface area and in the zones accessible for inter-molecular interactions.

In an additional work, Dufrechou *et al*. (2012) investigated another interesting aspect: increasing the melting point of the proteins as a result of the increase of the pH within the range 3.0 - 4.0 (figure 4). The authors showed that an increase in pH makes proteins less sensitive to thermal stress, especially for specific protein classes, such as chitinases and TLPs.

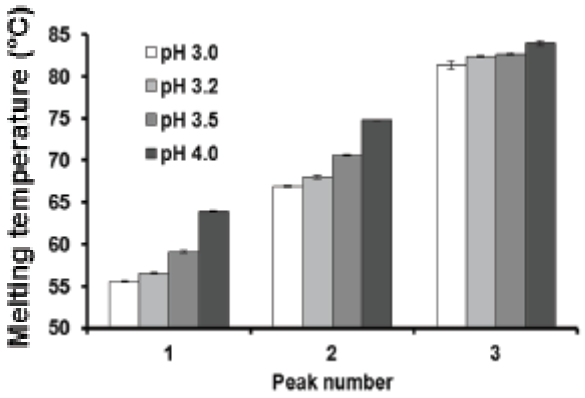


Figure 4. *Determination of wine protein melting temperature by DSC (Differential Scanning Calorimetry) at different pH values, 3.0, 3.2, 3.5 and 4.0. Peaks 1 and 2 represent respectively chitinases and thaumatin-like proteins, peak 3 invertases. Adapted from Dufrechou* et al. (*2012*).

If on one side the higher pH seems to stabilize the proteins, from the other it is also true that the proteins are near their isoelectric point at the higher pH values, therefore a lower net electrical charged leads to a quicker aggregation under heat stress, hindering the possibility of their refolding. For this reason, only at low pH values, as demonstrated in the study of Dufrechou *et al*. (2012), can some proteins re-fold, achieving their natural conformation, in a process outlined in figure 5. As stated above, there is no optimal pH value or range of pH values to reduce or limit protein haze. At each pH value, there are multiple factors involved in the stability/instability of proteins.

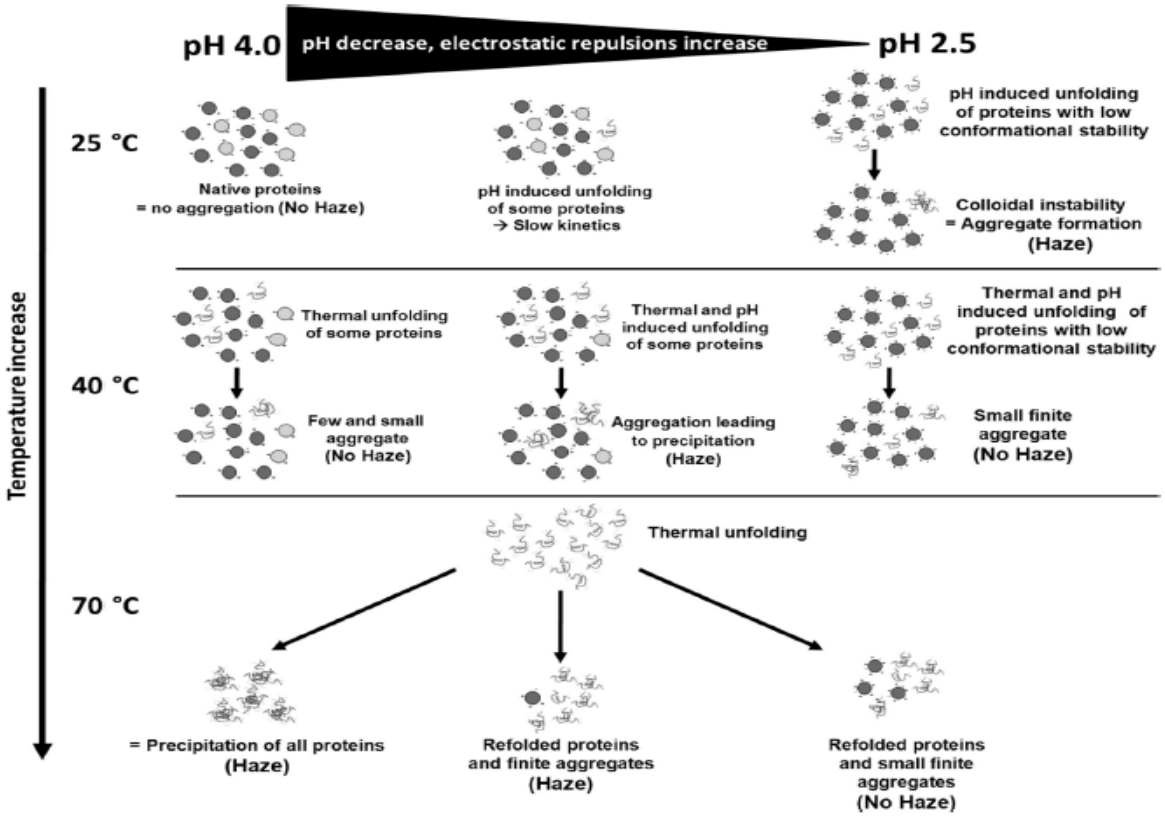


Figure 5. *Proposed mechanisms for protein aggregation depending on the pH and the heat treatment, illustrating the combined effect of pH and temperature on protein aggregation (Dufrechou* et al., *2012*).

1.3.2. Temperature

Temperature affects the wine composition and the equilibrium of its compounds, including proteins. In fact, the assessment of protein stability in a wine is based in the heat test. The role of this factor was deepened by Dufrechou *et al*. (2010), who used a huge range of temperatures (30 - 70 °C), screening the behavior of the proteins with different molecular weights present in the wine. The effect of the temperature is important because there are different classes of proteins with distinct melting points. Some proteins have a low melting temperature (40 °C), low enough to be reached even during transport of the wine in trucks or during an inadequate storage of the wine. The different destabilization of proteins was evaluated in term of aggregate precipitates through dynamic light scattering (DLS) in the work of (Dufrechou et al., 2010), allowing to correlate the diameter of the particles formed in relation to time (during the heat test, and subsequent cooling). The authors showed that a temperature of 60 °C is needed to induce the precipitation of proteins with a molecular weight of 60-70 kDa and that their precipitation remains moderate until 70 – 80 °C. This class of

proteins, identified as invertase, seems to be heat-resistant and is not primarily involved in haze development during wine transport and/or storage. In contrast, other classes of proteins are more sensitive. These heat-sensitive proteins include the ranges of 25-30 and 30-35 kDa, usually assigned to chitinases and β-glucanases. In fact, just a short exposure to moderate temperatures (40 °C) was enough to induce their aggregation and the formation of a visible haze (figure 6). β-Glucanases wre shown to be even more heat-unstable, being fully precipitated by 30 min at 40 °C after heat treatment (Sauvage *et al*., 2010). In contrast, class IV chitinases were studied by Falconer *et al.* (2010) and shown to be the least when compared to invertases and thaumatin-like proteins.

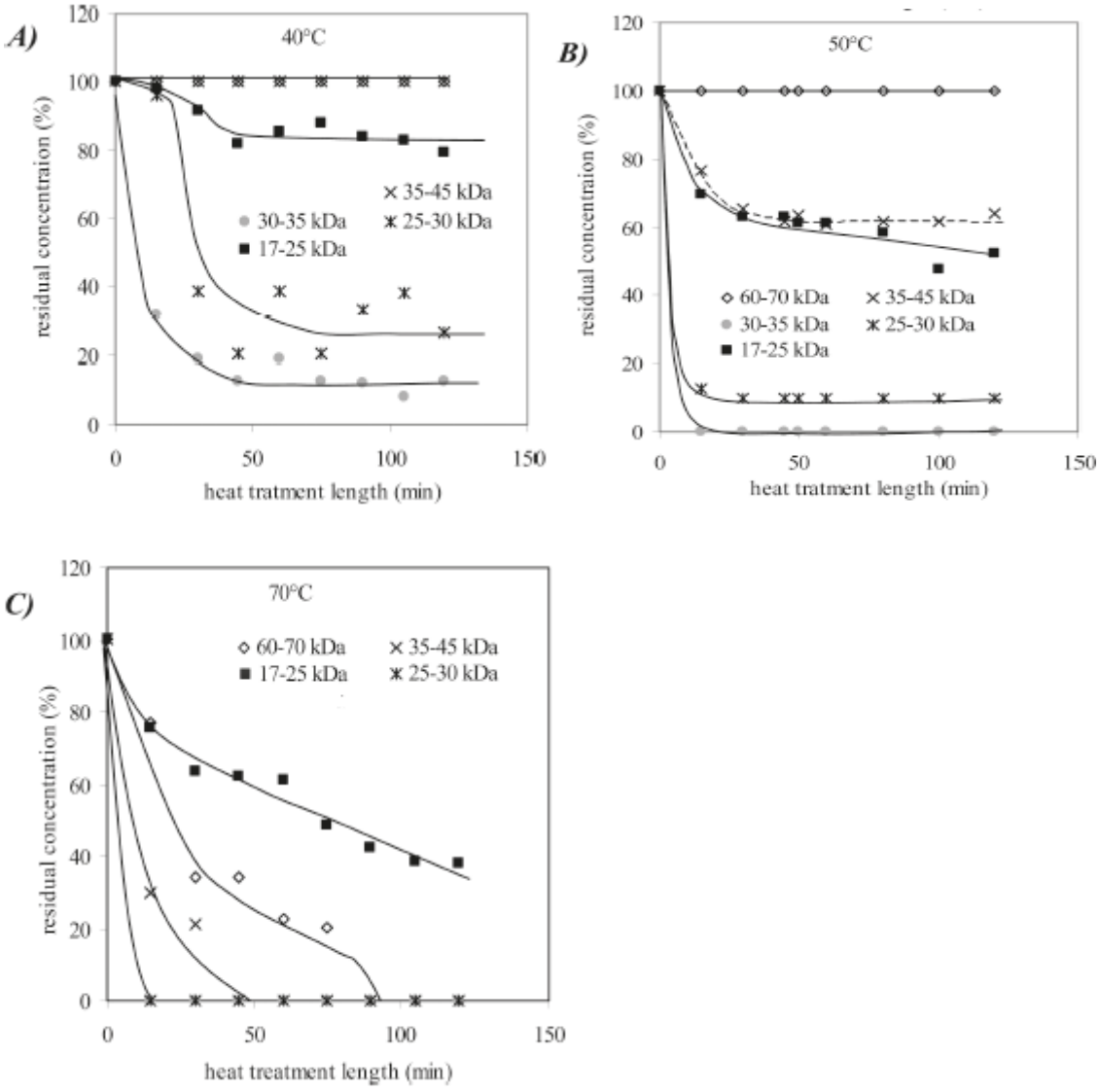


Figure 6. *Protein depletion in the Sauvignon Sa1 wine due to different heat-treatment durations at different temperatures. (A) 40 °C, (B) 50 °C, (C) 70 °C. Protein depletion was evaluated following 24 h cooling at room temperature and aggregate removal by centrifugation* (*Dufrechou* et al., *2010*)

Finally, it is important to explain the behavior of the proteins within the range of 17 - 25 kDa, comprising the most abundant fraction generally found in the wine, identified mainly as the thaumatin-like proteins. In this case, the authors claim a very different heat-sensitive fraction depending on the different samples of wine (Dufrechou *et al*., 2010). This aspect is justified by the existence of several classes of proteins within the same MW range and at the same time the presence of different isoforms, especially for the TLPs, with different conformation and consequently behavior in response to the heat stress.

1.3.3. Alcohol concentration

The alcohol content of white wines can be implicated in the stability of proteins, so much so that one of the tests to assess the potential of protein precipitation before bottling, is based on the addition of alcohol to a specific volume of wine (Boulton *et al*.,1999). This test is not very reliable, since the addition of alcohol causes the precipitation of other compounds, such as polysaccharides, overestimating the risk of the haze (Ribéreau-Gayon *et al*., 2007). Despite this, there are only a few studies which investigated the relationship between haze protein and alcohol content. In one of these reports (Mesquita *et al*., 2001) to assess the role of alcohol in protein haze formation, extra alcohol was added to white wine sample (0.5, 1 and 2% v/v). In this study, the addition of alcohol was not correlated with a corresponding increase of turbidity (evaluated at 540 nm), an observation that suggests that alcohol foes not interfe with the solubility of proteins in the range of concentrations tested. From what has been said, it seems that alcohol has no effect on the proteins, but from other studies (Achaerandio *et al*., 2001) the alcohol could have an indirect effect in the stability treatment. In fact, this later work reported that the higher the concentration of alcohol, the higher the swelling of bentonite, thus increasing the clay surface area in direct contact with the liquid. All this information translates into a higher deproteinizing action of bentonite and more stability in the finished wine at higher alcohol concentrations.

1.3.4. Organic acids

Organic acids are present in wines which derive not only from the grapes, such as tartaric, malic, citric and gluconic acids, but also from the alcoholic and malolactic fermentations, as succinic, lactic and acetic acids (Ribéreau-Gayon *et al.*, 2006). The most important in terms of quantity are tartaric, malic and citric acids, whose normal concentrations are, respectively, 3 to 5, 1 to 2 and 0.5 to 1 g/L depending on the grape's growth conditions and management of the vineyard. It is important to underline that organic acids are rather reactive, establishing bonds with other molecules. In the work of Batista *et al*. (2010) it was investigated and

established the stabilizing effect of the organic acids on the proteins, due to the electrostatically interactions established between the acids and the proteins, as shown in figure 7.

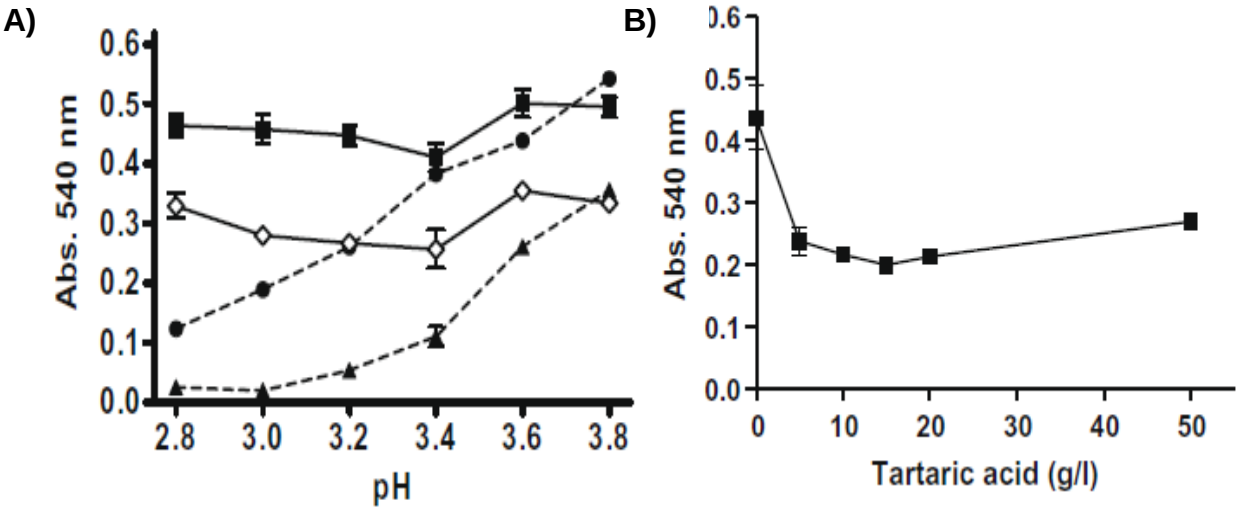


Figure 7. A) *Heat stability tests of Arinto wine (naturally containing 280 mg protein/L; ■), Arinto wine exogenously added with 20 mM tartaric acid (◊), an aqueous solution containing 280 mg/L of isolated Arinto wine proteins (●), and an aqueous solution containing 280 mg/L of Arinto wine proteins and 20 mM tartaric acid (▲). All experiments were performed in triplicate. Vertical bars represent plus or minus the standard deviation, shown when the bar is bigger than a symbol. B) Samples of Arinto wine, pH 2.8, were incubated for 24 h at 4 °C in the presence of exogenously added 0, 5, 10, 15, 20 and 50 g/L tartaric acid. The tartrate crystals were pelleted and collected by centrifugation, and the supernatant subjected to the heat stability test. Adapted from Batista* et al*. (2010).*

The work reported by Batista *et al*. (2010) showed that all the organic acids have a stabilizing effect, especially tartaric acid, because it is the most abundant and, having a low pk_{a1}, bears a higher proportion of negative charges, and is therefore able to interact with the positively charged proteins and stabilize them. In fact, at low pH values (2.8 – 3.0), the acids with a low pk_{a1} (such as tartaric and citric acids) exhibit a more intense protective effect. In contrast, the greater the values of the organic acid pk_{a1}, the greater is the repulsion between organic acids and wine proteins, and so the smaller the stabilizing effect. In an opposite way, at higher wine pH values (3.6 – 3.8), organic acids such as gluconic, succinic and malic participate due to stronger electrostatically interactions with the proteins, decreasing the haze formation, being more intense charged and proteins bearing no net charge or weak positive or negative charge. This work demonstrated that the protective effect of the organic acids is not related

to the number of carboxyl groups or to potential interaction sites with the proteins, but to the values of their pK_a, which directly influences their net electric charge. (Batista *et al.*, 2010).

Therefore, wines with a higher total acidity, expressed in g/L of tartaric acid, could be less prone to the formation of protein haze.

1.3.5. Polyphenols

During storage in the bottle, a wine can manifest haze due to the in-solubilization of its proteins. One cause of this phenomenon can be triggered by the presence of compounds naturally present in the wine called flavan-3-ols in the form of oligomers or polymers also known as procyanidins or tannins. These latter compounds are highly reactive with the proteins, inducing their aggregation and precipitation in the bottle. This is another possibility for protein haze, investigated in the work of Marangon *et al*. (2010). For the authors the protein-tannin interaction is mainly due to hydrophobic interactions.

The total wine proteins were fractionated in base of the hydrophobicity of their surface, via hydrophobic interaction chromatography (HIC) (Marangon *et al*., 2010). The proteins with medium to high hydrophobic surfaces were indeed the TLPs, represented by peaks H3 and H5 that were also the major peaks present as reported in table 1.

Table 1. *Percentage on the total peak area and conductivity (used as a putative index of protein surface hydrophobicity) of the fractions obtained by HIC of wine proteins. FT1, FT2, 1–5 indicate protein fractions as obtained by HIC separation (Marangon* et al*., 2010).*

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

To validate the role of tannins in the aggregation and instability of proteins, the authors performed a trial consisting of a heat stability test with and without tannins and correlated the results obtained with the development of turbidity, as represented in figure 8.

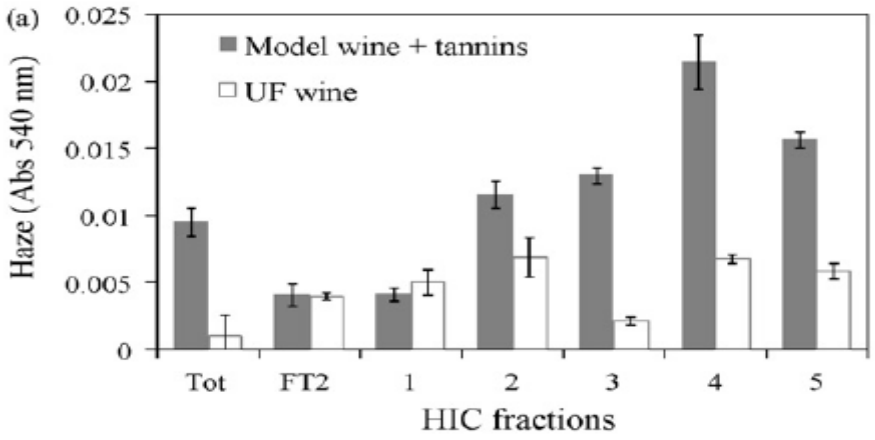


Figure 8. *Turbidity (Abs 540 nm) developed at room temperature (25 ˚C) immediately after mixing wine proteins (total and HIC fractions at 75 mgL^{-1}, final concentration) with wine tannins (at 50 mgL^{-1}) in the model (grey bars) and ultra-filtered (UF, 3 kDa, white bars) wines. Bars with different letters are significantly different at P ≤ 0.01(Student Newman–Keuls test) (Marangon* et al*., 2010).*

The results show that the highest instability is achieved when the tannins react with some specific classes of proteins, previously obtained through HIC, especially peaks 4 and 5 which represent TLPs, the most abundant and those with the higher hydrophobic surface.

White wines with a high tannin content could more easily originate protein turbidity. Supporting this last consideration Pocock *et al*. (2006) showed that polyvinylpolypyrrolidone (PVPP) fining of commercial wines (used to reduce oxidized and oxidizable phenolic compounds) results in a reduction in protein haze, which suggests that phenolic compounds play a modulating role in wine haze formation. Regarding this point, another work was performed by Esteruelas *et al.* (2011) who studied the phenolic compounds present in a natural haze, showing that all phenolic compounds detected in the wine were also present in the natural protein precipitate, specifically tyrosol, *trans*-*p*-coumaric acid, and *trans*-caffeic acid. An important aspect of this work was that when the natural precipitate was submitted to acid hydrolysis (Bate-Smith), new compounds were identified: cyanidin and quercetin. For the cyanidin, its appearance is explained by the degradation of the procyanidins. These data confirmed the presence of the procyanidins in the natural protein precipitate, as it has been described previously by other authors (Waters *et al.,* 1995). To deepen the knowledge on the phenolic compound interaction in the protein haze, Chagas *et al*. (2017) examined the role of caffeic acid, the major compound present among the low molecular weight molecules in the wine precipitate. Even though these results show that caffeic acid is present in considerable amounts in wine protein precipitate, it does not seem to be involved in triggering or

participating in the haze protein mechanism after/during heat treatment, as reported in table 2.

Table 2. *Changes in turbidity of wine and wine model solutions after heat stability test measured at 540 nm. IWP + <3 kDa: <3 kDa wine fraction supplemented with 190 mg/L of isolated wine protein; 140 mg/L SO_2: isolated wine protein in wine model solution supplemented with 140 mg/L of total SO_2 added as sodium hydrogen sulphide; IWP + WMS: isolated wine protein (190 mg/L) in wine model solution; CA: caffeic acid (1.1 or 5 mg/L) added to IWP in wine model solution; CTA: caftaric acid (1.1 or 5 mg/L) added to IWP in wine model solution. The pH of all samples was adjusted to 3.2. Different letters represent distinct homogeneous subsets for p = 0.05 (ANOVA, Tukey HSD) (Chagas* et al*., 2017).*

Sample	Absorbance at 540 nm
Control wine	0.233 ± 0.023^{a}
IWP + <3 kDa	0.182 ± 0.004^{b}
140 mg/L SO_2	0.294 ± 0.007^{c}
IWP + WMS	0.004 ± 0.001^{d}
1.1 mg/L CA	0.005 ± 0.001^{d}
5 mg/L CA	0.012 ± 0.003^{d}
1.1 mg/L CTA	0.013 ± 0.001^{d}
5 mg/L CTA	0.013 ± 0.001^{d}

1.3.6. Polysaccharides

Polysaccharides are compounds with a high molecular weight (50 to 560 kDa) and are present in wines within a range of 150 to 600 mg/L. They derive essentially from the grape berry (pectic polysaccharides) and from yeast cell walls (mannoproteins) (Dufrechou *et al*., 2015).

Wine usually contains between 15 and 230 mg/L of proteins. Therefore it can be assumed that polysaccharides may interact with the proteins, affecting as consequence their aggregation (Jaeckels *et al*., 2016). In fact, such was demonstrated by Ribéreau-Gayon *et al.* (2007): the polysaccharides, especially mannoproteins can stabilize the wine, shielding the charge of the proteins and tannins, thus inhibiting the growth of the particles by adsorption phenomena. In this way protein aggregation and precipitation is avoided. For this reason, mannoproteins have been termed “colloidal protector“. However, the work of Dufrechou *et al*. (2015) gave a contrasting point of view. Indeed, these authors performed two trials in the presence of a pool of polysaccharides and proteins, and specific polysaccharides and

proteins, to verify the aggregation complexes at room temperature; they did not find a relationship or correlation between the presence of polysaccharides and the reduction of turbidity. Moreover, other trials were performed in a wine model system with only the addition of proteins or with proteins and polysaccharides together - also here without significant results obtained (table 3). Their results show that polysaccharides are implicated in the interaction with proteins, forming sometimes (depending on the pH and the type of polysaccharides, neutral or acidic) a stable complex, but a decreased aggregation size and growth did not necessarily result in the lower formation of haze.

Table 3. *Impact of polysaccharides on turbidity estimated by measurements of the absorbance at 720 nm after 15 days of storage at 20 °C. Model systems containing protein or protein/polysaccharide mixtures were compared at different ionic strengths (0.02 and 0.15 M) and pH values (2.5 to 4.0). A visual haze is observed for a value higher than 0.01 a.u. (Dufrechou* et al*., 2015)*.

Ionic strength	Model system	pH 2.5	3.0	3.2	3.5	4.0
0.02 M	Proteins	0.031 ± 0.002	0.029 ± 0.001	0.021 ± 0.001	*0.006 ± 0.002*	*0.003 ± 0.000*
	Prot/Polysacc.	0.031 ± 0.003	0.028 ± 0.001	0.021 ± 0.002	*0.006 ± 0.001*	*0.008 ± 0.001*
0.15 M	Proteins	0.037 ± 0.005	0.027 ± 0.001	*0.002 ± 0.003*	*0.002 ± 0.000*	*0.002 ± 0.001*
	Prot/Polysacc.	0.044 ± 0.003	0.016 ± 0.000	*0.006 ± 0.001*	*0.006 ± 0.001*	*0.003 ± 0.001*

According to the authors, wine polysaccharides cannot be considered as having a determinant effect on protein stability in properly stored wines. This position is in agreement with the work of Gazzola *et al*. (2012) that studied the influence of the polysaccharides in the wine submitted at heat stress (figure 9). In a particular way, in this work compared the effect of the interaction of the polysaccharides and polyphenols with the proteins, chitinases, and TLPs, featuring the size and concentration of the protein aggregates formed upon heating and after cooling.

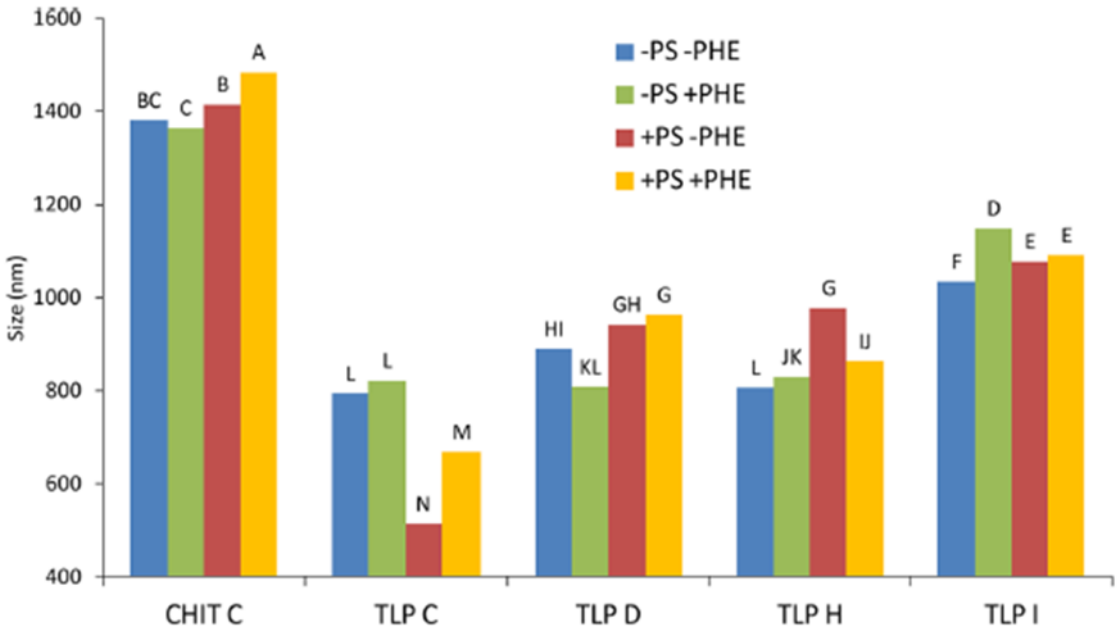


Figure 9. *Effect of protein and treatment (interaction protein × treatment) on the size (nm) of the aggregates formed upon heating/cooling cycles. Effect of the treatments (no addition of polysaccharides and phenolics, -PS -PHE; addition of phenolics, -PS +PHE; addition of polysaccharides, +PS -PHE; addition of polysaccharides and phenolics, +PS +PHE) (main effect treatment) on the particle size (nm) of the aggregates formed upon heating of the samples (Gazzola* et al*., 2012).*

From these results, chitinases originated the larger protein aggregates with respect to TLPs, whereas polysaccharides did not limite or decrease protein aggregation during and after the heat treatment, either for chitinases or TLPs.

1.3.7. Ionic strength and sulfate

Some authors have studied the influence of the ionic strength (*I*) and sulfate concentration on wine haze protein turbidity. Ionic strength reflects the effect of charges and interionic interactions on electrolyte activities, and hence on ionic activity coefficients (Solomon, 2001).

Under normal non-membrane cell conditions, proteins are stabilized and maintained in their native conformation essentially by hydrophobic interactions. In wines, most proteins carry a net positive electrical charge, thus causing a repulsive interaction, that avoids the interaction among proteins and consequently their aggregation. Marangon *et al*. (2011) demonstrated that an increment of *I* reduces the electrostatic interactions (in this case repulsion), thus favoring the aggregation of the proteins after heat stress as shown in the figures 10 and 11.

The wine has typically a range of ionic strengths between 10 and 100 mM. Marangon *et al*. (2011) used a broader range, from 20 to 500 mM (an extreme of the natural values found in wines) in a model wine solution, using additional doses of NaCl.

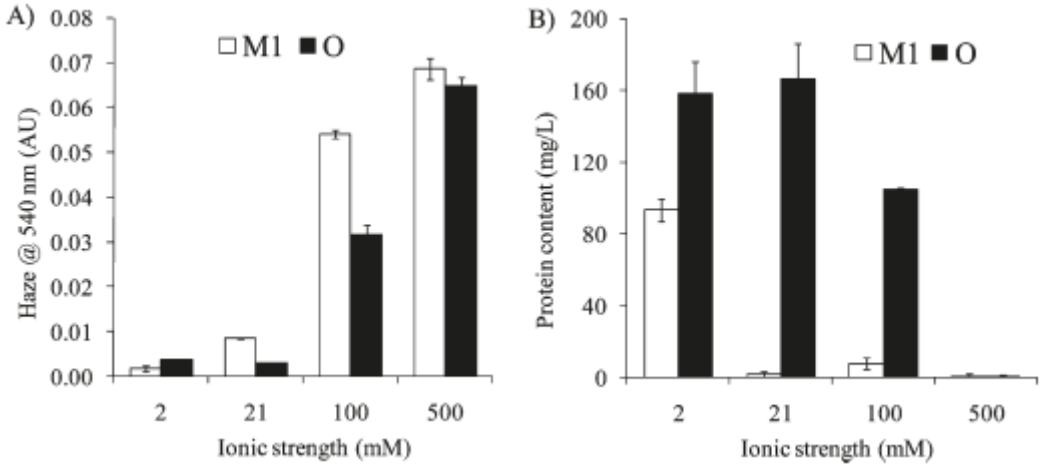


Figure 10. *Chitinases were dissolved in a model wine solution containing increasing dosages of NaCl to obtain I levels of 2 mM (no salt), 21 mM (1.23 g/L NaCl), 100 mM (5.85 g/L NaCl), and 500 mM (29.25 g/L NaCl): (A) haze (at 540 nm) of samples after heat test (analyses were performed after samples were cooled for 20 h at 25 °C); (B) protein content (measured by EZQ, a protein quantification kit) in the supernatant obtained from centrifugation of samples (21000 g, 15 min, 15 °C) after heat test. Protein contents in the untreated samples were 93.2 ± 12.4 mg/L and 158.2 ± 35.8 mg/L for M1 and O, two different isoforms of IV class chitinases, respectively (Marangon* et al*., 2011).*

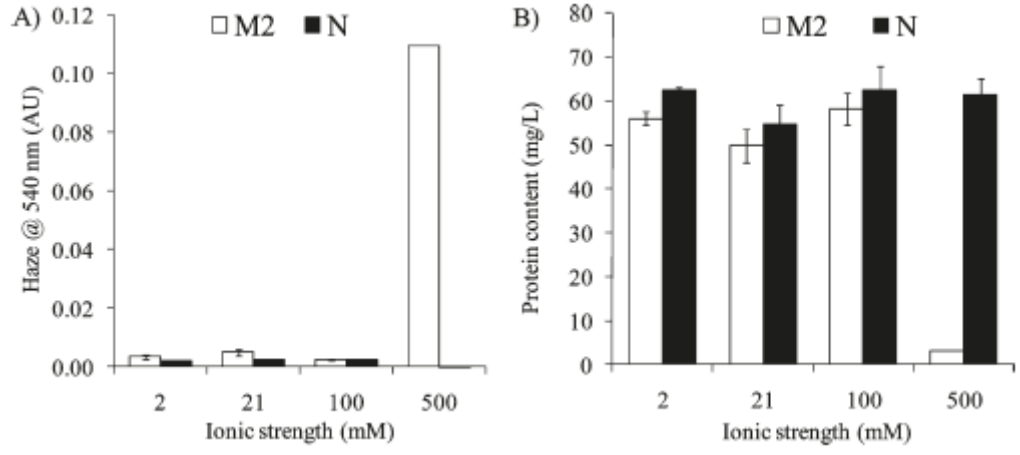


Figure 11. *TL-proteins were dissolved in model wine solution containing increasing dosages of NaCl to obtain I levels of 2 mM (no salt), 21 mM (1.23 g/L NaCl), 100 mM (5.85 g/L NaCl), and 500 mM (29.25 g/L NaCl): (A) haze (at 540 nm) of samples after heat test (analyses*

were performed after samples were cooled for 20 h at 25 C); (B) protein content (measured by protein quantification kit called EZQ) in the supernatant obtained from centrifugation of samples (21000g, 15 min, 15 °C) after heat test. Protein contents in the untreated samples were 56.1 ± 2.9 and 62.5 ± 1.4 mg/L for M2 and N, TL-protein and putative TL-protein, respectively (Marangon et al., 2011).

Under these conditions, chitinases appear more sensitive to induce the haze in comparison to the thaumatin-like proteins; according to the authors, the TLPs being less charged than chitinases, are less sensitive at changing of *I* of the medium.

In the work of Pocock *et al.* (2007), sulfate is considered the possible factor responsible for wine protein haze. Is also important to underline that an increase of sulfate corresponds to an increment of the ionic strength. Therefore, Marangon *et al*. (2011) studied if the aggregation/precipitation of TLPs and chitinases was induced by *I* or if the sulfate had a specific role in this instability. To evaluate the role of sulfate increasing doses up to 4 g/L of Na_2SO_4 were used (figure 12), an extreme value quite above the maximum concentration allowed by the OIV when compared to those generally observed in the wine (the maximal concentration for the legal threshold is 2 g/L).

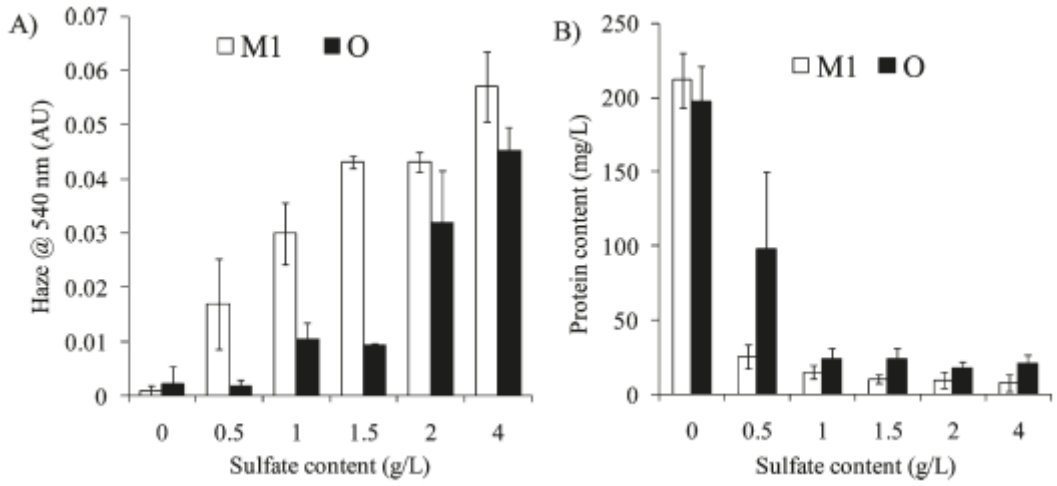


Figure 12. *Chitinases were dissolved in model wine solution containing increasing dosages of Na_2SO_4 (from 0 to 4 g/L): (A) haze (at 540 nm) of samples after a heat test (analyses were performed after samples were cooled for 20 h at 25 °C; the ionic strength given by 1 g/L of Na_2SO_4 is 21 mM); (B) protein content (measured by a protein quantification kit, EZQ) on*

supernatant obtained from centrifugation of samples (21000g, 15 min, 15 °C) after a heat test (Marangon et al., 2011).

The data illustrated in figure 12 show that with 0.5 g/L Na_2SO_4 (equal to 10 mM) the chitinases are highly unstable, instead before with the addition of NaCl to achieve the same instability it was necessary 100 mM (5.85 g/L NaCl). In the same way, as well as the trials with NaCl, also after addition of Na_2SO_4, the thaumatins were less affected than chitinases. In fact, chitinases start their aggregation during the heating stage, giving as consequence more turbidity when the wine is cooled down. In contrast, for the TLPs, aggregation started only during the cooling stage and to a lower extent. In conclusion, the study shows that sulfate is implicated in the aggregation and precipitation of proteins during a heat stress, especially for the chitinases. This effect of sulfate can be found also at concentrations commonly found in the wine (e.g. 0.5 g/L K_2SO_4). The authors concluded the sulfate and its correlation with ionic strength should be included in the X-factor of the complex mechanism of wine protein haze. These results agree with the work of Pocock *et al.* (2007), which considered sulfate as a modulator of the protein haze. Sulfate mediates the aggregation of unfolded chitinases, probably through a cross-linking action, during heating and/or cooling. Is also important to underline that this work (Marangon *et al*., 2011) was done in a model wine, where the conditions are strikingly different from those in a real wine. For this reason, not all proteins are precipitated at 70 °C in the model wine solution. Therefore, there are additional factors implicated in the pathway of in-stabilization of wine protein (Marangon *et al*., 2011).

1.3.8. Sulfur dioxide in the wine

One of the oldest additives used in winemaking is certainly sulfur dioxide, which until today remains the most used for its multifunctional effects, since no appropriate substitute has been found to this date. Among its actions and properties considered most important, there are the antiseptic, antioxidant and antioxidasic activities (Ribéreau-Gayon *et al.,* 2007). Generally, total sulfur dioxide in the wine is divided into free acid (active or molecular SO_2), having the properties above mentioned, and bisulfite (HSO_3^-), which altogether represent the free sulfur dioxide. Increasing the value of pH decreases the proportion of free acid. In fact as the wine pH increases from 3 to 4, the proportion of SO_2 decreases sharply from 6.06% to 0.64%, explaining the need for more substantial sulfiting when the pH, of must or wine, is high (Ribéreau-Gayon *et al*., 2007). Another fraction is the combined sulfur dioxide, that doesn't have, or is very insignificant, the antiseptic and antioxidant properties. The sum of

these fractions, free and combined, gives the total sulfur dioxide in the wine (figure 13). In a sulfited wine, an equilibrium exists between free sulfur dioxide and bound sulfur dioxide. Therefore, addition of sulfur dioxide to a wine results in the combination of a part of this added sulfur dioxide. Conversely, a decrement of free sulfur dioxide by oxidation results in a decrease of the bound fraction. From the oxidation of free sulfur dioxide SO_4^{2-} is obtained. This last, as stated before, plays a role in the complex of wine protein haze formation.

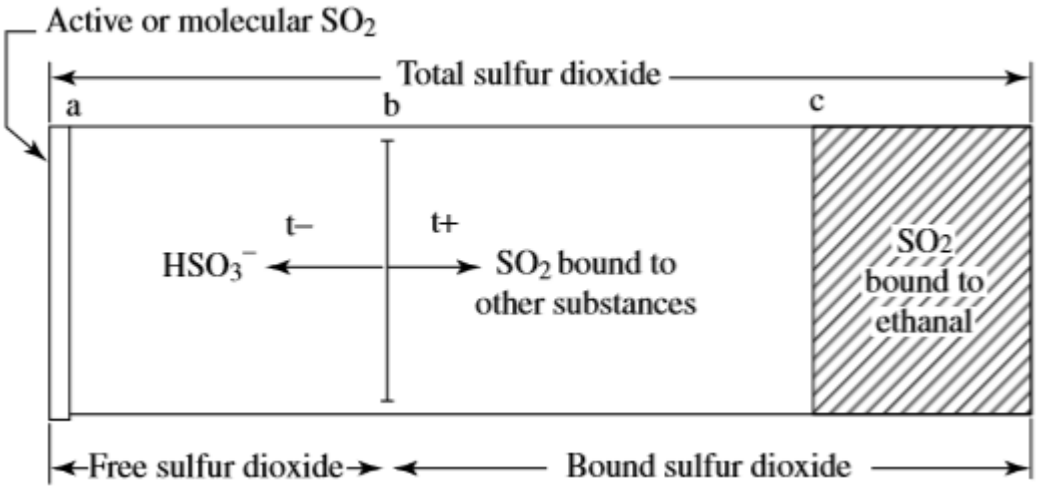


Figure 13. The different states of sulfur dioxide in wine: active SO_2 is located on the left; as already addressed, its separation (a) with HSO_3^- varies essentially according to the pH. On the extreme right, sulphurous aldehydic acid represents the SO_2 fraction combined with ethanal. The (c) separation line is definitive. On the other hand, the (b) separation between sulfur dioxide free and sulfur dioxide combined with other substances varies, moving in one direction or the other according to temperature and free SO_2 concentration (*Ribérau-Gayon* et al., *2007*).

Sulfur dioxide and its derivatives can cause various detrimental problems to human health, including the triggering of anaphylactic reactions, as well as a wide range of symptoms, including dermatitis, urticaria, flushing, hypotension, abdominal pain and diarrhoea, although most of the reports described concern asthmatic patients (Laura Di Renzo, 2012). For these reasons, researchers have been searching a new additive to eliminate sulfur dioxide use: unfortunately until today without results. This explains the undesirable presence of SO_2 in almost all commercial wines.

In the work of Maragon *et al*. (2010), protein haze related at the presence of a reductive compound able to cleave the intramolecular disulfide bonds that stabilize the protein's structure has been evaluated. Only in this way it is possible to hinder the unfolding of the native structure, expositing new hydrophobic sites, before buried inside the protein, that can interact with the hydrophobic sites on other proteins or of tannin, destabilizing the wine. This

reduction compound of non-protein nature was termed the X-factor by Pocock *et al*. (2007). Recently the effect of sulfur dioxide on the wine protein instability was studied. To fully understand the potential role of SO_2 in the mechanism of aggregation and precipitation of proteins, it is fundamental to clarify the structure of these proteins, responsible for the haze, and how are different chemical structures directly related at the reaction with this additive and their instability (Marangon *et al*., 2014; Chagas *et al*., 2016). Chitinases have been shown to have lower heat stability than TLPs, possibly as a result of an irreversible unfolding behavior. In contrast, TLPs have a partially reversible unfolding behavior and usually return to the native state after cooling. Nevertheless, the available literature has identified TLPs as the most responsible for the wine protein instability. This contradiction, as explained by Marangon *et al*. (2014), could be overpass only at the presence of different TLPs having distinct haze forming behaviors. Highlighting this point, Marangon *et al*. (2014) studied the conformational and 3D structure of proteins, comparing three different isoforms of TLP classes VVTL1 (F2/4JRU, I/4L5H and H2/4MBT). Thanks to their different times of elution during hydrophobic interactions chromatography (HIC), the authors understood the different hydrophobicity of the proteins, hypothesis later supported by their 3D reconstruction. In a particular way, the F2/4JRU, a TLP whose structure is depicted in figure 14, presents major hydrophobic regions, between β-strand 9 and 10, being more hydrophobic than I/4L5H and H2/4MBT. In the picture, the eight F2/4JRU disulfide bridges are represented in yellow.

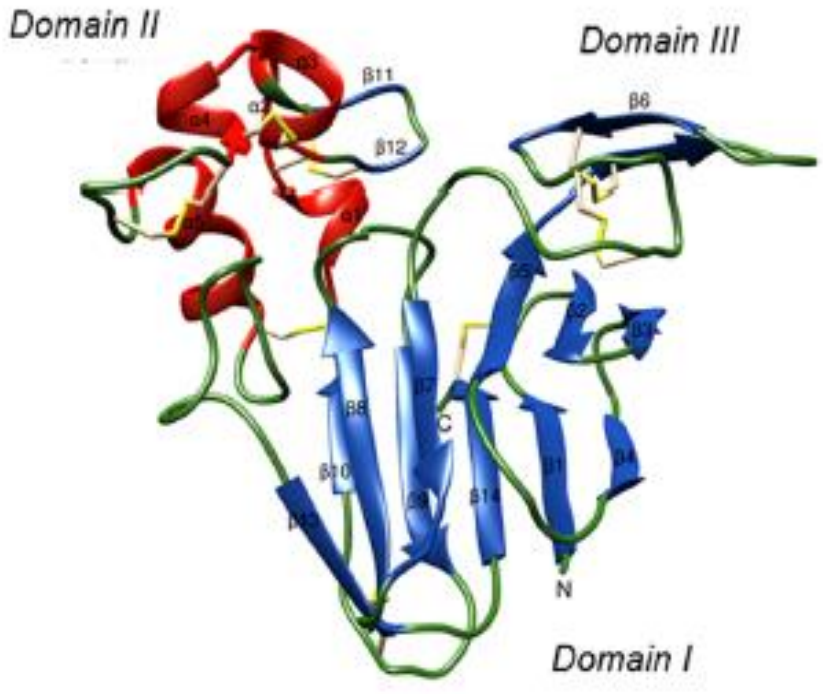


Figure 14. *Ribbon diagram of the overall three-dimensional structure of protein F2/4JRU*, one of the TLPs commonly found in wines*. The protein consists of three structural domains:*

a central core domain I built from a β-sandwich of two sheets of six (front) and five (back) β-strands, flanked by one shorted domain II (where the 5 α-helixes and β-strand 11 and 12 are located) and one shorted domain III (where two β-strands - 5 and 6 -and a turn form two looping regions). The eight disulfide bridges are shown in yellow (Marangon et al*., 2014).*

One major consequence of this structural conformation of the isoform F2/4JRU is that upon unfolding, it can expose more hydrophobic regions, thus favoring its aggregation via hydrophobic interactions. In this way, the refolding of F2/4JRU would be hindered, aggregation would take place, and haze in wine would be formed. This explained the different behavior observed for the proteins, in terms of classes and inside the classes among the different isoforms of the same proteins. In fact, despite the similarities in terms of sequence, the three grape TLPs have exhibited very different haze develop and turbidity of wine potentials, as shown in figure 15. The differences between the three isoforms are also supported by differences in their chromatographic behavior, a feature that allowed their separation and that also indicated that they possess some differences in physical properties.

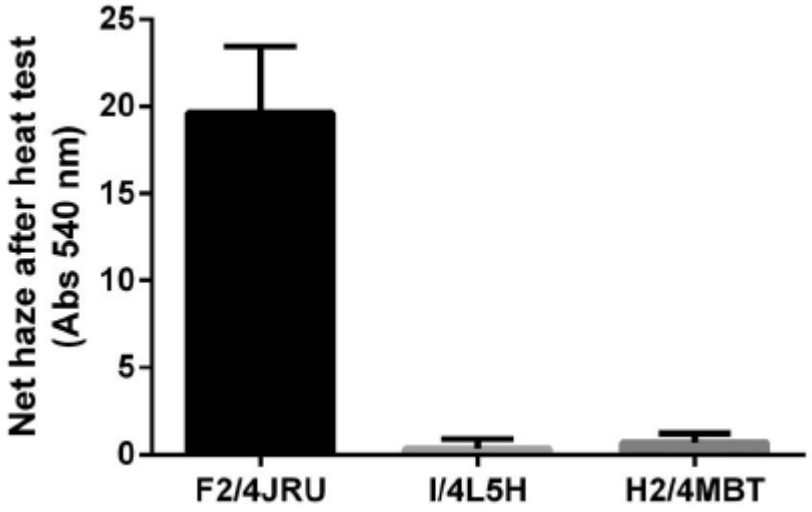


Figure 15. *Haze developed upon heat test of wine TLPs F2/4JRU, I/4L5H and H2/4MBT. Haze was measured after treatment at 80 ˚C for 2 h, followed by 4 ˚C for 2 h. Assays were carried out at 50 mg/L in a model wine solution (Marangon* et al*., 2014).*

Moreover, the wine *TLPs* contain disulfide bridges in their hydrophobics regions, thus stabilizing the different domains of the protein. The authors, also supported by later work (Chagas *et al*., 2016), believe that these disulfide bridges, especially that between residues Cys140 and Cys213, are the key of the refolding/unfolding of the TLPs. This last point is closely correlated with the use of sulfur dioxide during winemaking: SO_2 seems to be able to

cleave the S-S bonds, particularly for the F2/4JRU protein, thus allowing the aggregation formation to take place (Marangon *et al*., 2014).

The effect of the sulfur dioxide was studied by Chagas *et al*. (2016), using a model wine solution supplemented with 100 mg/L of proteins, previously extracted with a strong cation exchange chromatography. A heat stability test was performed using an increasing SO_2 concentration (0, 25, 90 and 250 mg/L), and to assess the instability, the absorbance at 540 nm was used. In their work, the results demonstrate a correlation between the higher concentration of SO_2 and increasing of turbidity (higher values at 540 nm), highlighting the role of this additive in the destabilizing of proteins (figure 16).

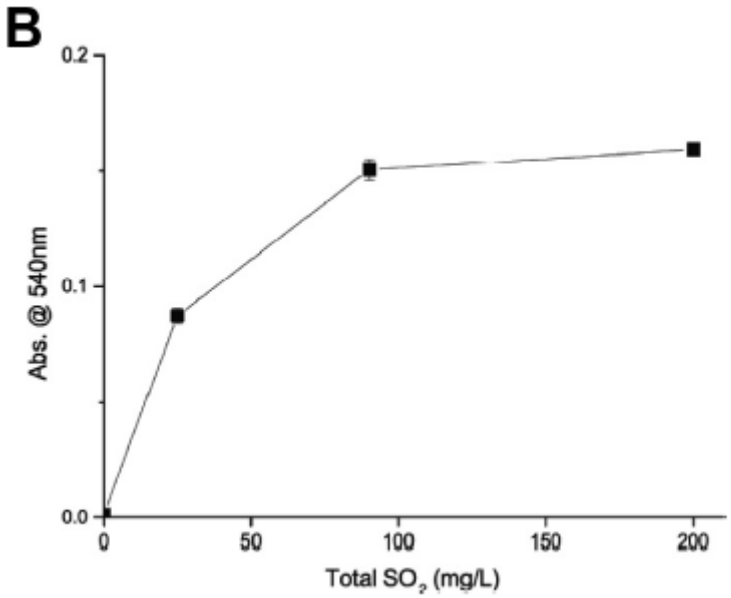


Figure 16. *SO_2 haze inducing effect (at different concentrations) after heat stability test upon a model wine solution, consisting of 100 mg/L previously isolated total wine protein, 12% (v/v) ethanol, 5 g/L tartaric acid, pH 3.2. The turbidity was measured at 540 nm (Chagas* et al*., 2016).*

Other important points of this work was the fractionation of the proteins and the evaluation, for each fraction, of their stability/instability in the presence of SO_2, as shown in figure 17. The fraction called H5 in the work represents the TLPs fraction. In the control (without sulfur dioxide), TLPs did not produce a visible haze after heat test. In contrast, the same sample with fraction H5 and added SO_2, made the wine highly unstable, underlining the high instability of TLPs especially in the presence of SO_2 and thus reinforcing the hypothesis of Marangon *et al*. (2014) on the conformation of this protein class and consequent instability related to it.

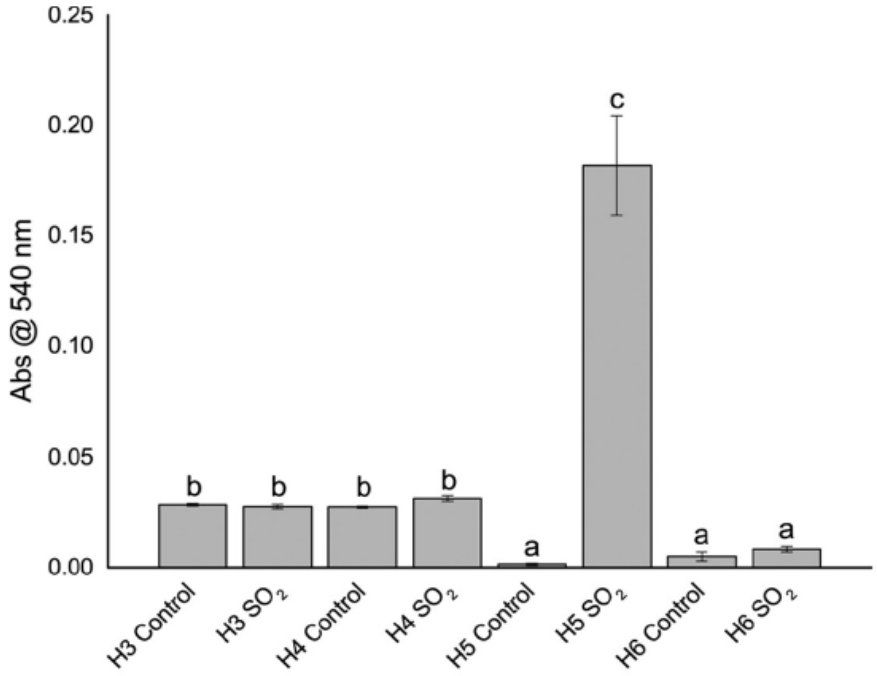


Figure 17. *Haze development after heat stability test of the isolated HIC protein fractions (adjusted to 100 mg/L protein) in two model wine solutions. Control: no added SO_2. SO_2: model wine solution containing 120 mg/L total SO_2 (added as $NaHSO_3$). H3–H6 indicate the number of the protein fractions obtained by HIC separation (Chagas* et al*., 2016).*

Unlike other studies, where the reducing substance used was dithiothreitol (DDT; which does not function well as an antioxidant at the low wine pH; Maragon *et al*., 2011), Chagas *et al*., (2016) used tris-(2-carboxyethyl)-phosphine (TCEP; which functions well as an antioxidant at the low wine pH) and observed that SO_2 leads to a significant haze formation. This effect is due to the fact that sulfur dioxide breaks the disulfide bridges at the low wine pH, and after induces new cysteine-derived bonds. This process starts with the thiosulfonation of proteins, that hinders or delays thiol-disulfide exchange during the protein interactions (an important point upon cooling, during which the proteins would 'like' to return to his native status) (Zhang & Sun, 2008).The formation of a 'wrong' pattern of disulphide bonds leads to a new conformational change, and as a result some hydrophobic sites, previously buried inside the protein, become exposed to the exterior and could react with one another non-polar sites, of tannin or other protein, inducing aggregation and precipitation (Chagas *et al*., 2016). A schematic process of wine haze formation is shown in figure 18.

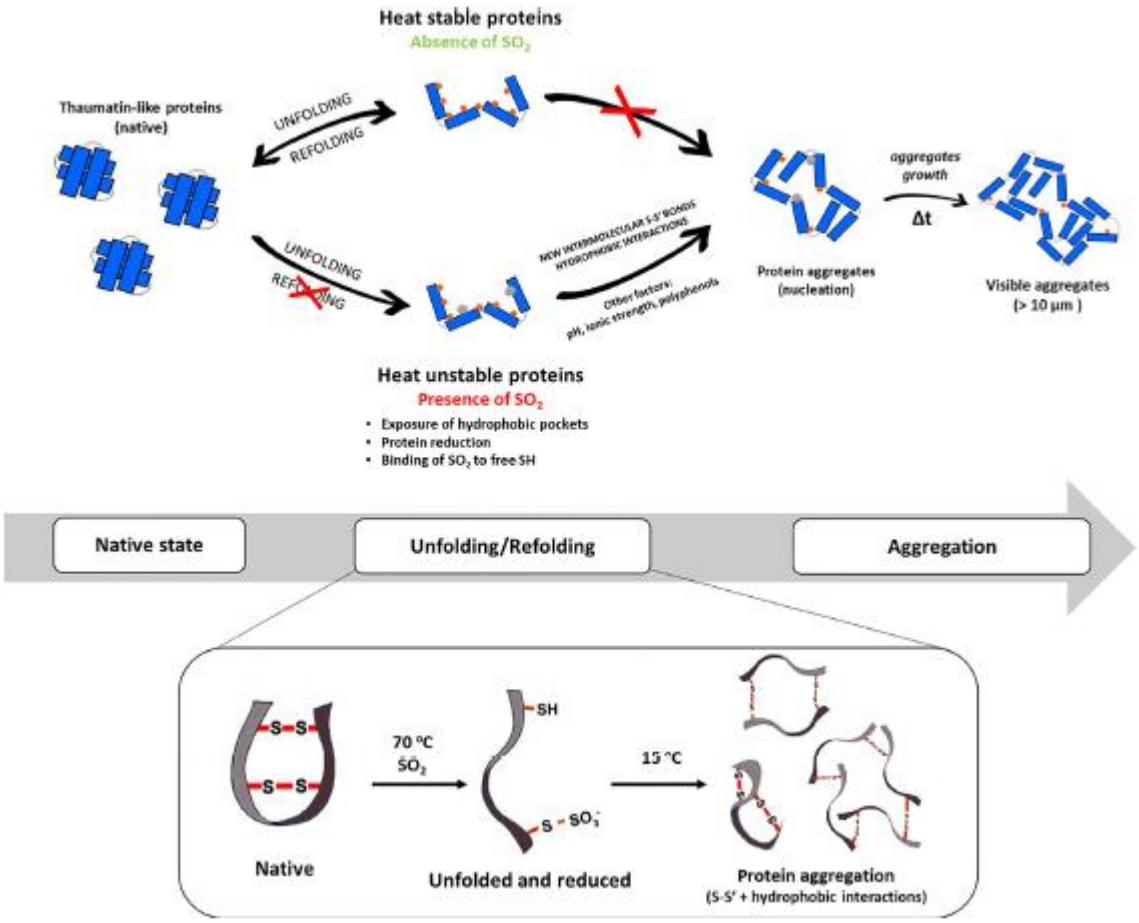


Figure 18. *Updated model for the protein haze development mechanism in white wine (Chagas* et al.*, 2018).*

1.4. Aim of the project

Based on the interaction between sulfur dioxide and wine proteins, the goal of the current work is to understand if the real instability of the wine in presence of the thaumatin-like proteins (TLP) and SO_2, can be demonstrated in a variety of wines. In this study fourteen wines will be considered. For each one, sample routine analysis, quantification of proteins and their fractionation will be performed to characterize each sample.

Subsequently two wines will be selected to assess the role of the sulfur dioxide on their protein instability. To evaluate the role of SO_2, a different doses will be added to wines, and after that the heat stability tests will be performed to demonstrate the role of SO_2 in the haze formation.

The general idea is to obtain a correlation to understand at which concentration of specific proteins and SO_2, can the wines be considered stable. Therefore, this work investigates the impact of sulfur dioxide on protein instability in the wines prepared from different grape varieties.

2. Materials and methods

The wine samples here analysed were produced at the Instituto Superior de Agronomia winery. The selected varietal wines were from the varieties Encruzado, Alvarinho, Viosinho, Arinto, Moscatel Galego, Macabeu and Moscatel de Setúbal, from the vintages 2017 and 2018.

The bottles of wine were stored at 4 °C until needed and the headspace filled with nitrogen.

2.1. Proteins stability test

The protein stability test was performed following the method of de Bruijn *et al*. (2009) with some modifications (Chagas *et al*., 2016). Five mL of each sample were placed into a screw capped test tube and heated at 80 °C for 2 h in a water bath. Following heating, the samples were subsequently cooled down for 2 h in ice to 0 ºC. The samples were left to reach room temperature (25 °C) and the turbidity was evaluated measuring the absorbance at 540 nm. If the difference of absorbance, before and after the heat treatment, exceeds the threshold of 0.02 absorbance units the wine was considered unstable (Pocock and Waters, 2006). All measurements were performed in triplicate.

2.2. Proteins stability test with tannins

To properly assess the results of the first protein instability test, it was necessary to use another method that involves the addition of exogenous tannins. Twenty mL of wine, previously centrifuged at 10,000 g for 5 min, was used for each replicate with the addition of 200 mg of ascorbic acid and 1 mL of tannin solution. Following thoroughly mixing, the samples were incubated in a water bath at 80 °C for 10 min, followed by the spectrophotometry measurement at 650 nm. The differences between the absorbance of the heat-treated samples with and without tannins represents the protein stability. In this case, wine showing an absorbance higher than 0.1 were considered as unstable. All measurements were performed in triplicate

2.3. Sulfur dioxide quantification

Total sulfur dioxide was determined by potentiometric titration with the iodide/iodate solution after alkaline hydrolysis. The semiautomatic apparatus Sulfilyser was used to measure free and total SO_2 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.

2.3.1. Free SO_2

A burette was filled with the solution iodide/iodate N/64. In a 50 mL beaker, 25 mL of wine and 5 mL of sulfuric acid diluted 1:3 (v/v) were added. The titration starts with pouring of the oxidizing solution of iodine into the wine and ends when the latter oxidizes all the sulfur present in the wine, a time indicated by the permanence of the red light on the machine. The volume of solution iodide/iodate used in the burette is called $V_{F,}$ and the concentration of the free SO_2 is expressed by the formula:

Concentration (free SO_2) = V_F × 20 (expressed in mg/l).

2.3.2. Total SO_2

To 10 mL of wine, 2 mL of NaOH 2 N are added. Following 5 min of alkaline hydrolysis, 20 mL of acid sulfuric diluted 1:10 (v/v) are added. Upon homogenization of the sample, titration starts by pressing the apparatus dispenser button at a rate of one pulse per second till the red led remains lighted, indicating total oxidation of the sulfur dioxide by the oxidizing solution of iodine. In this case, the volume of solution iodide/iodate used from the burette is called $V_{t.}$ Total SO_2 concentration was then given with the following formula:

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

2.4. Volatile acidity

The volatile acidity of all samples was analysed following the method OIV-MA-AS313-02. Volatile acids are separated from the wine by steam distillation and titrated using standard sodium hydroxide. Carbon dioxide must 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. Twenty mL of sample, without $CO_{2,}$ are taken into the flask, and about 0.5 g of tartaric acid added. During the distillation process, at least 250 mL of sample was collected. The latter is titrated with the sodium hydroxide solution, 0.1 M, using phenolphthalein as indicator. “n” mL is the volume of sodium hydroxide used.

The acidity of free and combined sulfur dioxide in wine distilled under these conditions should be subtracted from the acidity of the distillate, to avoid overestimating the volatile acidity.

After addition of four drops of hydrochloric acid, diluted 1:4 with distilled water, 2 mL of starch solution, 5 g/L, and a few crystals of potassium iodide, the free sulfur dioxide was titrated with the iodine solution, 0.005 M. “n'” mL is 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, 0.005 M. “n”' mL is the volume used.

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

All measurements were performed in duplicate.

2.5. Total acidity

The total acidity of our samples was analysed according to the method OIV-MA-AS313-01. Carbon dioxide was removed from the samples by placing 50 mL of wine in a vacuum flask and applying vacuum with a pump for one to two min while shaking continuously. Twenty-five 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 (0.1 M) was added until the color 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 a volume of the sample equal to 10 mL of wine. Sodium hydroxide solution (0.1 M) was added until the same color was obtained as in the preliminary test above. “n” mL was the volume of sodium hydroxide solution (0.1 M) added.

The total acidity is expressed in milliequivalents per litre of tartaric acid and is obtianed by A = 10 n. It is recorded to one decimal place. The total acidity expressed in g of tartaric acid per litre is given by:

A' = 0.075 x A. The result is quoted to two decimal places.

All measurements were performed in duplicate.

2.6. Alcohol strength

The alcoholic strength is calculated by ebulliometry (Zoecklein *et al.,* 1995). First, the boiling point of water is determined, for the calibration of the instrument. After this, the instrument was cooled down. The wine to be analyzed was used to rinse the instrument. Approximately

50 mL of wine was loaded into the boiling chamber. The condenser is filled up with cold water. The thermometer was placed inside the instrument and the latter is placed on the source. When the thermometer reaches a stable point, the temperature is taken. The boiling point of the wine is located on the inner “Degrees du thermometer” scale and the corresponding alcohol point is recorded (% v/v) on the outer scale. Only one measurement was taken.

2.7. pH

The pH of all samples was measured according to the method OIV-MA-AS313-15. Before use the pH meter must be calibrated at 20 °C using standard buffered solutions. The pH values selected for the calibration must encompass the range of pH values encountered in musts and wines. After calibration, the electrode is dipped directly in the sample, that was previously degassed in vacuum, having a temperature between 20 and 25 °C and as close as possible to 20 °C. After some min the pH values were directly recorded off the scale. At least two determinations on the same sample were carried out.

2.8. Reducing sugars

Reducing substances that comprise all the sugars exhibiting free ketonic and aldehydic functions were quantified following the method OIV-MA-AS311. Depending on the density of the wine, it may have to be diluted: if it is less than 0.997 the sample should not be diluted. Fifty mL of the wine was placed in a 100 mL volumetric flask; 5 mL of solution I, potassium ferrocyanide and water (150 g of potassium ferrocyanide and water made to 1000 mL), and 5 mL of solution II, zinc sulfate and water (300 g of zinc sulfate and water made to 1000 mL) were added. After stirring, the solution was made up to the mark of the volumetric flask with water and was left to rest 10 min before filtration (1 mL of filtrate contains 0.50 mL of dry wine). The reagents used were alkaline copper salt solution (25 g copper sulfate, 50 g citric acid monohydrate, 388 g crystalline sodium carbonate and taken at volume 1 L with water), potassium iodide solution 30% (w/v), sulfuric acid 25% (w/v), starch solution 5 g/L, sodium thiosulfate solution 0.1 M and inverted sugar solution, 5 g/L, to be used for checking the method of determination.

Twenty-five mL of the alkaline copper salt solution, 15 mL water and 10 mL of the clarified solution were collected into a 300 mL conical flask. This volume of sugar solution must not contain more than 60 mg of inverted sugar. Some small pieces of pumice stone were added, and the mixture boiled for 10 min. The flask was cooled down under cold running water. When completely cool, 10 mL potassium iodide solution, 30% (w/v), 25 mL sulfuric acid, 25%(w/v) and 2 mL starch solution, 5 g/L were added. The solution was titled with sodium

thiosulfate solution, 0.1 M. Let ‘n’ be the number of mL used. At the same time, a blank titration was carried out, in which the 25 mL of sugar solution was replaced by 25 mL of distilled water. Let ‘n’’ be the number of mL of sodium thiosulfate used. The quantity of sugar expressed as inverted sugar, contained in the test sample is given in table 4 below as a function of the number (n' - n) of mL of sodium thiosulfate used. The sugar content of the wine is 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

Table 4. *A table giving the relationship between the volume of sodium thiosulfate solution: (n'-n) mL, and the quantity of reducing sugars expressed in mg/L.* (Source: OIV-MA-AS311)

Table giving the relationship between the volume of sodium thiosulfate solution: (*n*'-*n*) mL, and the quantity of reducing sugar in mg.					
$Na_2S_2O_3$ (ml 0.1 M)	Reducing sugars (mg)	Diff.	$Na_2S_2O_3$ (ml 0.1 M)	Reducing sugars (mg)	Diff.
1	2.4	2.4	13	33.0	2.7
2	4.8	2.4	14	35.7	2.8
3	7.2	2.5	15	38.5	2.8
4	9.7	2.5	16	41.3	2.9
5	12.2	2.5	17	44.2	2.9
6	14.7	2.6	18	47.2	2.9
7	17.2	2.6	19	50.0	3.0
8	19.8	2.6	20	53.0	3.0
9	22.4	2.6	21	56.0	3.1
10	25.0	2.6	22	59.1	3.1
11	27.6	2.7	23	62.2	
12	30.3	2.7			

2.9. Density

The density of our samples was measured via the method OIV-MA-AS2-01B. Density is the mass per unit volume of wine or must measured at 20 °C. Two hundred and fifty mL of the prepared sample was poured into a measuring cylinder; the hydrometer and thermometer were inserted. The sample was mixed, and 1 min must be waited to allow for temperature equilibration; the temperature was taken. The thermometer was removed and after a further 1 min, the apparent density was taken.

The measurement is taken only once.

2.10. Total dry matter

The total dry extract of wine samples was calculated indirectly from the specific gravity of the alcohol-free wine, according to the method OIV-MA-AS2-03B. The specific gravity of the wine was determined. The specific gravity of the "alcohol-free wine" was obtained through the formula:

dr = dv - da + 1.000 where:

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

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.

In the end, from the values of the specific gravity of the alcohol-free wine and using table 5 it is possible to calculate the total dry matter expressed in g/L.

Table 5. *The relationship between the specific gravity and the total dry matter of alcohol-free wines expressed in g/L*. (Source: OIV-MA-AS2-03B)

Density to 2 decimal places	3rd decimal place									
	0	1	2	3	4	5	6	7	8	9
	Extract g/L									
1.00	0	2.6	5.1	7.7	10.3	12.9	15.4	18.0	20.6	23.2
1.01	25.8	28.4	31.0	33.6	36.2	38.8	41.3	43.9	46.5	49.1
1.02	51.7	54.3	56.9	59.5	62.1	64.7	67.3	69.9	72.5	75.1
1.03	77.7	80.3	82.9	85.5	88.1	90.7	93.3	95.9	98.5	101.1
1.04	103.7	106.3	109.0	111.6	114.2	116.8	119.4	122.0	124.6	127.2
1.05	129.8	132.4	135.0	137.6	140.3	142.9	145.5	148.1	150.7	153.3
1.06	155.9	158.6	161.2	163.8	166.4	169.0	171.6	174.3	176.9	179.5
1.07	182.1	184.8	187.4	190.0	192.6	195.2	197.8	200.5	203.1	205.8
1.08	208.4	211.0	213.6	216.2	218.9	221.5	224.1	226.8	229.4	232.0
1.09	234.7	237.3	239.9	242.5	245.2	247.8	250.4	253.1	255.7	258.4
1.10	261.0	263.6	266.3	268.9	271.5	274.2	276.8	279.5	282.1	284.8
1.11	287.4	290.0	292.7	295.3	298.0	300.6	303.3	305.9	308.6	311.2
1.12	313.9	316.5	319.2	321.8	324.5	327.1	329.8	332.4	335.1	337.8
1.13	340.4	343.0	345.7	348.3	351.0	353.7	356.3	359.0	361.6	364.3
1.14	366.9	369.6	372.3	375.0	377.6	380.3	382.9	385.6	388.3	390.9
1.15	393.6	396.2	398.9	401.6	404.3	406.9	409.6	412.3	415.0	417.6
1.16	420.3	423.0	425.7	428.3	431.0	433.7	436.4	439.0	441.7	444.4
1.17	447.1	449.8	452.4	455.2	457.8	460.5	463.2	465.9	468.6	471.3
1.18	473.9	476.6	479.3	482.0	484.7	487.4	490.1	492.8	495.5	498.2
1.19	500.9	503.5	506.2	508.9	511.6	514.3	517.0	519.7	522.4	525.1
1.20	527.8	-	-	-	-	-	-	-	-	-

Looking at table 6, considered the 4th decimal of the density, the value corresponding to the dry extract is summed to the number previously found in table 5.

Table 6. *Correspondence with the 4th decimal place.* (Source: OIV-MA-AS2-03B)

4th decimal place	Extract g/L	4th decimal place	Extract g/L	4th decimal place	Extract g/L
1	0.3	4	1.0	7	1.8
2	0.5	5	1.3	8	2.1
3	0.8	6	1.6	9	2.3

The calculation was done only once taken the average value of the factors considered.

2.11. Color intensity

The color intensity is measured according to the method OIV-MA-AS2-07B. When a wine is cloudy, this must be clarified by centrifugation prior to the trial. The optical path b of the glass cell used must be chosen so that the measured absorbance A, falls between 0.3 and 0.7. The spectrophotometric measurements are taken using distilled water as the reference

liquid, in a cell of the same optical path b, to set the zero on the absorbance scale of the apparatus at the wavelengths of 420, 520 and 620 nm. Using the appropriate optical path b, the absorbencies are read off at each of these three wavelengths for the wine.

The color intensity “I” is conventionally given by:

I = A420 + A520 + A620

and is expressed to three decimal places.

In our case, working only with white wines was not necessary to read the values at 520 nm and 620 nm, but only at 420 nm. Absorbance values at 520 nm and 620 nm are taken into consideration for red wines, to evaluate the evolution of color. The absorbance at 420 nm is important for the white wines since it provides some information about the state of wine, especially in what concerns the oxidation and its evolution; in fact, the higher is the A_{420} value, the higher is the oxidation process of the wine.

The values were taken in triplicate.

2.12. Total phenols

The determination of total phenols firstly requires a dilution of wine with distilled water followed by the absorbance reading at 280 nm in the spectrophotometer (Somers and Evans, 1977). In the first trial a dilution 1:10 was performed and, in this way, the absorbance values were too high, therefore dilutions were increased to 1:20. Specifically 2.5 mL of wine and 47.5 mL of water were used. The method follows a direct spectrophotometric reading of absorbance at 280 nm, the absorption wavelength of total phenols. The final value of absorbance is multiplied by 20 because of the previous dilution factor and transformed using the calibration curve in mg/L of gallic acid equivalents. The values were taken in triplicate.

2.13 Non-flavonoids and flavonoids

The quantification of non-flavonoids phenols in wine is based on the determination of the phenolic content before and after the precipitation of the flavonoids through the reaction with formaldehyde, as under certain conditions (low pH, room temperature, darkness) non-flavonoid phenols do not precipitate. Following the method of Kramling and Singleton (1969)**,** 10 mL of wine sample, 10 mL of HCl diluted 1:4, and 5 mL formaldehyde were mixed in a test tube. After 24 h incubation in the dark, a dilution with distilled water (1:10) was carried out and the absorbance read at 280 nm in the spectrophotometer. Quantification of the

flavonoid phenols was obtained from the difference between total phenols and non-flavonoid phenols. The values were taken in triplicate and transformed using the calibration curve expressed in mg/L of gallic acid equivalents.

2.14. Sulfates

Wine sulfates were determined according to the method OIV-MA-AS321-05A, comprising a gravimetric determination following precipitation of barium sulfate. The reagents used were hydrochloric acid 2 M and barium chloride solution 200 g/L. Forty mL of sample is place into a 50 mL centrifuge tube, and 2 mL hydrochloric acid and 2 mL of barium chloride solution were added. After homogenization, the solution was centrifuged for 5 min at 10,000 *g*, and the supernatant carefully removed. The barium sulfate precipitate was then rinsed with 10 mL hydrochloric acid, and centrifuge again for 5 min always at 10,000 *g*. The supernatant was once more carefully removed. The washing step of the pellet was repeated twice as before using 15 mL distilled water each time. The precipitate was quantitatively transferred, with distilled water, into a tared platinum capsule and placed over a water bath at 100 °C until dryness. The dried precipitate was calcined several times briefly over a flame until a white residue was obtained. It was then left to cool in a desiccator and weighed. Let m = mass in mg of barium sulfate obtained.

The sulfate content, expressed in mg/L of potassium sulfate, is given by 18.67 x m

The sulfate content in musts or wines is expressed in mg/L of potassium sulfate, to the nearest whole number. All analyses were done in triplicate.

2.15. Chloride

This parameter was quantified following the method OIV-MA-AS321-02, determined directly in the wine by potentiometry using an Ag/AgCl electrode. The apparatus used is a pH/mV meter graduated at intervals of at least 2 mV, provided by an Ag/AgCl electrode with a saturated solution of potassium nitrate as the electrolyte. The reagents were standard chloride solution: 2.1027 g of potassium chloride, KCl (max. 0.005% Br), dried before use, by leaving in a desiccator for several days; it was dissolved in distilled water and made up to 1 L; one mL of this solution contains 1 mg Cl^-. Another reagent was silver nitrate solution: 4.7912 g of analytical grade silver nitrate, dissolved in an ethanol solution, 10% (v/v) and made up to 1 L; oner mL of this solution corresponds to 1 mg Cl^-. The last reagent was nitric acid, not less than 65% (ρ_{20} = 1.40 g/mL). The procedure of the method starts with 5.0 mL of standard chloride solution poured into a 150 mL cylindrical vessel placed on a magnetic stirrer and diluted with distilled water to approximately 100 mL and acidified with 1.0 mL of

nitric acid. 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, successive incubations of approximately 30 s were done before reading the corresponding mV values. 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:

20 X *n* expressed as mg Cl/L. All measurements were performed in triplicate.

2.16. Tartaric stability test

For tartaric acid determination, 100 ml of wine is placed in a backer with a magnetic stirrer and the temperature adjusted to 0 °C by incubation in an ice bath. One g of potassium hydrogen tartrate powder was added to the wine, and the conductivity value measured in a conductivimeter each 1-2 min for 5-10 min. The conductivity value is taken until the measure remains stable for at least three successive intervals. The final value of conductivity corresponds to that which the wine is stable. If, in the 5-10 min after seeding, the drop in the conductivity is no more than 5% of the wine's initial conductivity (measured before adding potassium bitartrate) the wine is generally considered stable, although wineries often use a more stringent criterion of 3% for the white wine (Angele, 1992; Ribérau-Gayon *et al*., 2006).

2.17. Minerals

2.17.1. Copper

Copper is determined according to method OIV-MA-AS322-06. Twenty mL of sample is placed into a 100 mL volumetric flask and made up to 100 mL with double-distilled water. The dilution must be modified if necessary, to obtain a response within the dynamic range of the detector, an atomic absorption spectrophotometer. The absorbance is measured at 324.8 nm. The zero is set with double distilled water. Different volumes of the copper solution (0.5, 1 and 2 mL) are pipetted into each of three 100 mL volumetric flasks and made to the volume with double distilled water; the solutions contain 0.5, 1 and 2 mg of copper per litre respectively. The absorbance values of the standard solutions are measured. A graph is plotted to show 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 is read off. If F is the dilution factor, the concentration of the copper present is given in milligrams per litre by F x C.

2.17.2 Iron

Iron was determined according to the method OIV-MA-AS322-05A. The reagent used was a standard iron solution containing 100 mg iron, Fe (III), per litre. 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). Makeup to the original volume with distilled water. If necessary, dilute prior to analysis with distilled water. The calibration of the atomic absorption spectrophotometer was carried out in the following way: place 1, 2, 3, 4 and 5 mL of the solution containing 100 mg iron per litre into 100 mL volumetric flasks and makeup to 100 mL with distilled water. The solutions prepared in this way contain 1, 2, 3, 4 and 5 mg of iron per litre. These solutions should be stored in polyethylene bottles. For the determination, the absorption wavelength is set to 248.3 nm. Zero the absorbance scale using distilled water. Aspirate the diluted sample directly into the spectrophotometer, followed in succession by the five standards and record the absorbance vaues. A graph is plotted to give the variation in absorbance as a function of the iron concentration in the standard solutions. Record the mean value of the absorbance obtained with the diluted wine sample on this graph and read its iron concentration C. The iron concentration in mg/L of the wine to one decimal place is given by:
F x C, where F is the dilution factor.

2.17.3 Potassium

The potassium was determined following the method OIV-MA-AS322-02A. The reagents are a solution containing 1 g of potassium per litre, a matrix model solution and caesium chloride solution containing 5% (w/v) caesium. Two and a half mL of wine (previously diluted 1/10) are pipetted into a 50 mL volumetric flask, with 1 mL of the caesium chloride solution and made up to the mark with distilled water.

The calibration of the atomic absorption spectrophotometry was carried out in the following way: introduce 5.0 mL of the matrix solution into each one of five of 100 mL volumetric flasks and add 0, 2.0, 4.0, 6.0 and 8.0 mL of the 1 g/L potassium solution (previously diluted 1/10). Add 2 mL of the caesium chloride solution to each flask and make up to 100 mL with distilled water. The standard solutions contain 0, 2, 4, 6 and 8 mg of potassium per litre and each contains 1 g of caesium per litre. The absorbance wavelength is set to 769.9 nm. Zero the

absorbance scale using the zero-standard solution. Aspirate the diluted wine directly into the spectrophotometer followed in succession by the standard solutions.

A graphic is plotted to show the variation in absorbance as a function of potassium concentration in the standard solutions. Record the mean absorbance obtained with diluted wine on this graph and determine its potassium concentration C in milligrams per litre. The potassium concentration expressed in milligrams per litre of the wine to the nearest whole number is F x C, where F is the dilution factor.

2.17.4 Calcium

Calcium was determined according to the method OIV-MA-AS322-04. One mL of wine and 2 mL of the lanthanum chloride solution are placed in a 20 mL volumetric flask, which are then filled up to the mark with distilled water. The diluted wine containing 5 g lanthanum per litre, and 0, 5, 10, 15 and 20 mL of diluted standard calcium solution are placed 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 litre, and each also 5 g of lanthanum per litre. These solutions were stored in polyethylene bottles. The absorbance wavelength of the atomic absorption spectrophotometry was set at 422.7 nm. The absorbance scale was calibrated using the zero standards. 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 to show the variation in absorbance as a function of the calcium concentration in the standard solutions. The mean value of the absorbance obtained with the sample of diluted wine on this graph was recorded and its calcium concentration C was determined. The calcium concentration in mg/L of the wine to the nearest whole number is given by 20 x C.

2.17.5 Sodium

Sodium was analyzed according to the method OIV-MA-AS322-03A. Two and a half mL of wine were pipetted into a 50 mL volumetric flask, 1 mL of the caesium chloride solution was added, and the flask was made up until the mark with distilled water. For the calibration, 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 of a 1:100 dilution of the 1 g/L sodium solution were added. 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 litre respectively and each contained also 1 g of caesium per litre. These solutions were taken in polyethylene bottles. The absorbance wavelength of the atomic absorption spectrophotometer was set at 589.0 nm. The absorbance scale was calibrated 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 values 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 wine was then calculated by F x C, expressed to the nearest whole number, where F is the dilution factor and C the sodium concentration.

2.17.6 Magnesium

Magnesium was determined according to method OIV-MA-AS322-07. The wine was diluted 1/100 with distilled water. Different volumes of diluted standard magnesium solutions (5, 10, 15 and 20 mL) were placed into each one of a set of four 100 mL volumetric flasks and 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 litre, respectively. These solutions were kept in polyethylene bottles. The absorption wavelength was set to 285 nm. The absorbance scale was calibrated at zero using distilled water. The diluted wine was directly sucked into the atomic absorption 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.

2.18. Protein quantification: Bradford method

The Bradford assay is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins (Bradford 1976), that was used here with some modifications. Under acidic conditions, the dye is predominantly in the protonated red cationic form (A_{max} = 470 nm). However, when the dye binds to the protein, specifically to the amino acid residues and their aromatic bases, it is converted to a stable unprotonated blue form (A_{max} = 595 nm) (Groth *et al*., 1963, Reisner *et al*., 1975, Sedmak *et al*., 1977). It is this

blue protein-dye form that is detected at 595 nm in the assay using a spectrophotometer or a microtitre plate reader. 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 protein concentration.

A calibration line was prepared with bovine serum albumin (BSA) solution at different concentrations: 0 µg/mL, 2.5 µg/mL, 5 µg/mL, 7.5 µg/mL, 10 µg/mL, 15 µg/mL and 20 µg/mL.

One mL cuvette assay was used to perform the method, in which 400 µL of each sample, 400 µL of water and 200 µL of the Bradford reagent were introduced (Gazzola *et al*., 2014). Some wines were diluted, to allow absorbance values to fall within the BSA calibration curve, using from 1:2 to 1:10 dilutions. The reaction was allowed to take place for 10 min in the dark and the absorbance at 595 nm was measured. The protein concentration was then calculated by the absorbance results obtained.

2.19. Total wine protein isolation: strong cation exchange chromatography (SCX)

The wine total proteins were isolated following the method of Van Sluyter *et al*. (2009) with some modifications (Chagas *et al*., 2016). The wine sample was adjusted to pH 3.0 with HCl and treated with 30 g/L polyvinylpolypyrrolidone (PVPP) overnight at 4 ºC. The wine was vacuum-filtered through three layers of Miracloth (Merck) and then through 0.45 µm and 0.2 µm polyethersulfone (PES) filter. All chromatographic steps were executed at room temperature. Using an AKTA Pure pump (Amersham Biosciences), the filtered wine was loaded at 2.5 mL/min on a cation exchange column (RESOURCE S, 6 mL bed volume, Amersham Biosciences) previously equilibrated with 30 mM sodium citrate, pH 3.0. The column was then connected to an AKTA Pure chromatography system with a UV detector (Amersham Biosciences) and washed at 2.5 mL/min with the buffer solution of 30 mM sodium citrate, pH 3.0 (buffer A). Bound proteins were eluted at 2.5 mL/min with 30 mM sodium citrate containing 1 M NaCl, pH 3.0 (buffer B) using the following step gradient: 0 min, 0% B; 25 min, 100% B and held. The protein fraction was collected and desalted into water twice on gel filtration columns (PD-10, GE Healthcare), and the subjected to lyophilisation for two days. The isolated proteins were stored at -20 ºC until necessary.

2.20. Fractionation of isolated proteins by hydrophobic interaction chromatography (HIC)

The total wine proteins isolated by SCX were fractionated by HIC. First, the samples were dissolved in 50 mM sodium citrate buffer, containing 1.25 M ammonium sulfate, pH 7.0, and loaded on a Phenyl Superose HR 5/5 HIC column (GE Healthcare). The column was connected to an AKTA Pure chromatography system with a UV detector (Amersham Biosciences). After each injection, the column was washed with 6 mL of 50 mM sodium citrate buffer, pH 7.0, containing 1.7 M ammonium sulfate and the bound proteins were subsequently eluted at 0.5 mL/min with a linear, 30 mL gradient to 50 mM sodium citrate buffer, pH 7.0.

2.21. Addition of different concentrations of sulfur dioxide and proteins to wines

The standard solutions of sulfur dioxide and proteins were prepared to be added to the wines in order reach different desired SO_2 and protein final concentrations.

To prepare the standard protein solutions, to be subsequently added to the wine, the pure proteins previously isolated were dissolved in a small aliquot of water. Then, they were added to different volumes of wine to achieve protein solutions with different protein concentrations, precisely 50 mg/L and 100 mg/L proteins solutions.

For the sulfur dioxide solution, the potassium metabisulfite salt was used, dissolved directly in water, in which it is highly soluble. Several volumes of this solution were then added to the wines to obtain the desired concentrations of total sulphur dioxide. In this work the concentrations of sulfur dioxide in the different trials were 120, 150 and 200 mg/L of total SO_2.

The wines used were then centrifuged at 10,000 *g* for 10 min and then filtered to make sure that the wines were free of coarse particles in suspension.

2.22. Statistical analysis

To study the correlation between the different factors affecting the protein instability of wines, PCA (principal components analysis) (Hotelling, 1933) was used, using OriginPro 2018 64-bit.

To compare the different chromatograms, obtained from the hydrophobic interaction chromatography (HIC) analyses, Peak analyzer of OriginPro 2018 64-bit was used.

The influence of the addition of SO_2 in two different varieties of wines, an analysis of variance (ANOVA) and a comparison of treatment means (Tukey's test) were performed using RStudio version 3.4.4. 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 analysis

3.1.1. Protein stability test

The protein heat stability test was performed on fourteen wines, to analyse the difference about the protein instability among the different varieties and vintages. In fact, a wine produced from the same variety but from different vintages may have different stability, probably based on the climatic trend of the year in which the grapes were produced. Due to the environmental factors, the final protein concentration can be quite different, and this can impact the protein stability (Mesquita *et al*., 2000). For this reason, varieties such as Encruzado, Moscatel Galego and Viosinho originated stable wines in the 2017 vintage but unstable in the 2018 vintage. Moscatel of Setúbal was an exception, since according to other authors (Chagas *et al*., 2016) it is common to wines from this variety to be highly unstable. Table 7 shows the results of both heat stability tests made in this work, with (HST+T) and without tannins (HST). It is important to underline that the heat test with tannins modifies the natural composition of the wine, leading to extreme turbidity values. This test was performed in the present work with the objective of having a comparison in terms of absorbance values with the other test. Regarding the HST, a wine is considered unstable if the absorbance at 540 nm is higher than 0.02 AU (Pocock and Waters, 2006). According to the HST+T, a wine is considered unstable if the absorbance at 650 nm is higher than 0.1 AU.

Tab. 7. *Protein instability test with tannins (HST+T) and without tannins (HST). In orange the unstable values of wines. 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	stable
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. Quantification of proteins by the Bradford method

Quantification of total wine proteins from all fourteen samples under study was performed using the Bradford protein assay (Bradford, 1976). From the values reported in table 8 it is possible to ascertain that some grape varieties are more predisposed to present high protein content, like for example Moscatel of Setúbal and Viosinho, on top of "the effect vintage" which is also widely known to influence the protein content (Monteiro *et al.*, 2003). The

increment in proteins can be induced by fungal attack, specially *Botrytis cinerea*, by wounding and there is also a correlation with high rain and accumulation of proteins in grapes: a wet growing season is usually indicative of intense fun gal attacks. The importance of the vintage is supported by the fact that all the wines of 2017 have lower protein contents than those of 2018.

Table 8. *Quantification of the total wine protein by the Bradford method. Protein concentration is expressed in mg/L. Values are mean ± SD (n = 3).*

Wine	Protein concentration (mg/L)
Encruzado 2017	*45.5 ± 1.9*
Alvarinho 2017	*42.5 ± 3.0*
Viosinho 2017	*61.2 ± 4.4*
Arinto 2017	*50.9 ± 4.1*
Moscatel Galego 2017	*54.7 ± 4.1*
Macabeu 2017	*27.2 ± 1.1*
Moscatel de Setúbal 2017	*94.6 ± 7.6*
Encruzado 2018	*80.7 ± 8.6*
Alvarinho 2018	*61.9 ± 2.8*
Viosinho 2018	*87.8 ± 6.9*
Arinto 2018	*73.1 ± 3.4*
Moscatel Galego 2018	*122.2 ± 6.1*
Macabeu 2018	*39.4 ± 2.6*
Moscatel de Setúbal 2018	*218.7 ± 10.0*

The amount of proteins in the wine is obviously a fundamental pre-requirement for protein haze formation - In fact, increasing their concentration also increases the risk to have this defect in the wine (Mesquita *et al*., 2000).

As stated above the mechanism(s) of protein haze formation is a multifactorial process, where several proteinaceous and non-proteinaceous compounds are known to be involved (Travedi *et al*., 2009).

3.1.3. Wine routine analyses

For all the fourteen wines under study, different parameters were analysed, following routine analysis to discriminate their composition. These parameters are part of the extrinsic factors that can modulate the complex process of protein haze formation in the wine. The most important factors are pH, polyphenols, sulfate, ionic strength and organic acids (Waters *et al*., 2005; Marangon *et al*., 2014; Dufrechou *et al*., 2012). Another important factor to take into consideration is the sulfur dioxide concentration: its role was recently studied (Marangon *et al*., 2014; Chagas *et al.,* 2016). According to these authors, the co-presence of the SO_2 and specific proteins in the wine, such as thaumatin-like proteins, could give a correlation with the mechanism of the haze formation. Table 9 shows the routine analysis of the fourteen wines under study.

Table 9. *Routine analyses of the fourteen wines from 2017 and 2018*; *values indicated represent the mean ± SD (n = 3).*

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	6.61 ± 0.11	0.32 ± 0.01	14.1 ± 0.0	0.8 ± 0
Alvarinho 2017	10 ± 0	50 ± 0	3.30 ± 0	7.52 ± 0.12	0.27 ± 0.01	14 ± 0.0	0.6 ± 0
Viosinho 2017	9 ± 0	57 ± 0	3.44 ± 0	6.3 ± 0.12	0.33 ± 0.03	15.4 ± 0.0	0.73 ± 0.01
Arinto 2017	8 ± 0	60 ± 0	3.32 ± 0	7.43 ± 0.10	0.28 ± 0.03	13.1 ± 0.0	1.01 ± 0.01
Moscatel Galego 2017	16 ± 0	80 ± 0	3.45 ± 0	7.22 ± 0.09	0.44 ± 0.02	15.8 ± 0.01	0.72 ± 0.02
Macabeu 2017	20 ± 0	90 ± 0	3.24 ± 0	7.72 ± 0.08	0.23 ± 0.02	12.3 ± 0.02	0.5 ± 0
Moscatel of Setúbal 2017	12 ± 0	85 ± 0	3.39 ± 0	6.84 ± 0.09	0.29 ± 0.01	13.2 ± 0.02	0.31 ± 0.01
Encruzado 2018	25 ± 0	90 ± 0	3.12 ± 0	8.42 ± 0.11	0.28 ± 0.03	13.5 ± 0.01	0.1 ± 0
Alvarinho 2018	32 ± 0	100 ± 0	3.3 ± 0	8.11 ± 0.11	0.2 ± 0.02	14 ± 0.01	0.71 ± 0.02
Viosinho 2018	28 ± 0	82 ± 0	3.28 ± 0	7.44 ± 0.06	0.4 ± 0.01	16 ± 0.02	1.22 ± 0.01
Arinto 2018	25 ± 0	95 ± 0	3.2 ± 0	7.83 ± 0.07	0.29 ± 0,01	14.5 ± 0.01	1.5 ± 0
Moscatel Galego 2018	20 ± 0	85 ± 0	3.4 ± 0	7.82 ± 0.08	0.55 ± 0.02	16.3 ± 0.02	1.61 ± 0.01
Macabeu 2018	24 ± 0	75 ± 0	3.19 ± 0	6.35 ± 0.12	0.26 ± 0.02	12.7 ± 0.02	0.41 ± 0.03
Moscatel of Setúbal 2018	21 ± 0	80 ± 0	3.47 ± 0	6.10 ± 0.11	0.32 ± 0.01	14.1 ± 0.0	0.43 ± 0.02

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	68.1 ± 2.6	121.58 ± 1,2	11.01 ± 0.11	0.1 ± 0
Alvarinho 2017	20.6 ± 0.5	0.184 ± 0	263.5 ± 3.5	100.3 ± 2	163.17 ± 1.5	7.12 ± 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	8.71 ± 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	9.42 ± 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	18.11 ± 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 ± 0.13	0.3 ± 0.04
Encruzado 2018	22.2 ± 0.18	0.067 ± 0	192.6 ± 1.4	67.8 ± 1.4	124.8 ± 1.1	8.12 ± 0.12	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

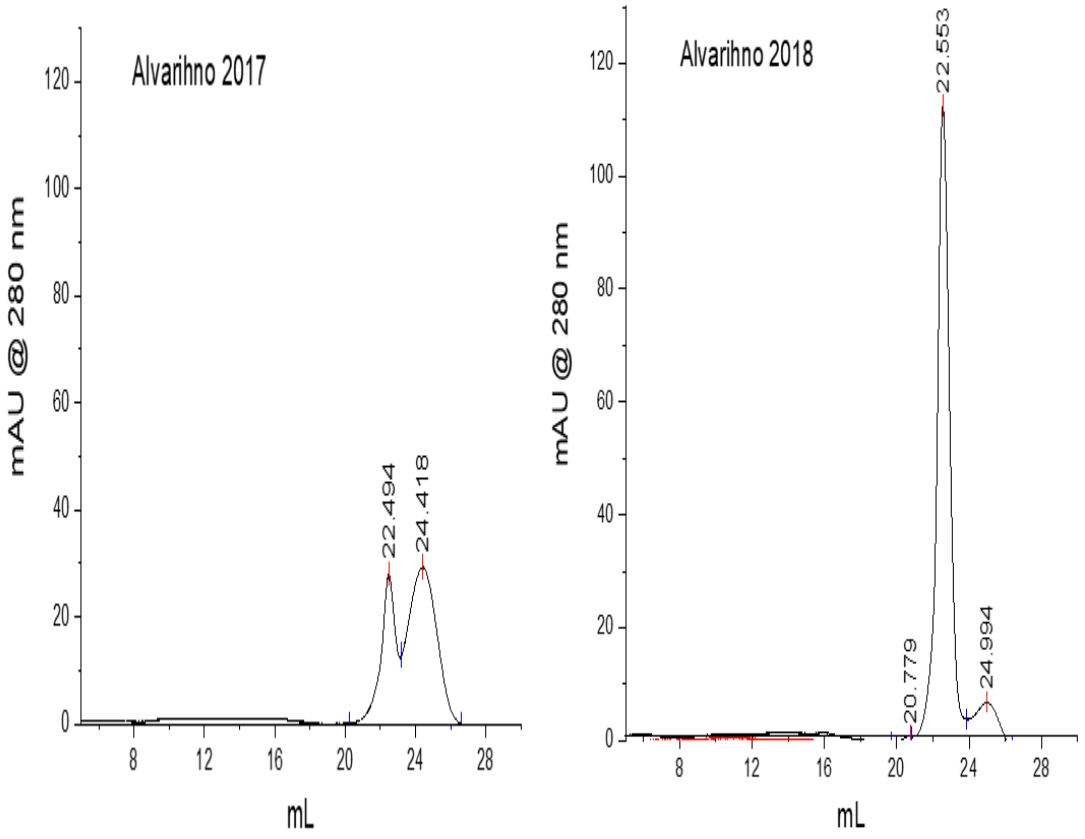
Parameters	Tartaric stability (%)	Cu (mg/L)	Fe (mg/L)	Ca (mg/L)	Mg (mg/L)	Na (mg/L)	K (mg/L)
Encruzado 2017	7.8 ± 1.3	0.06 ± 0	0.6 ± 0,02	42.78 ± 0.4	74.5 ± 1.2	7.63 ± 0.3	808.6 ± 4
Alvarinho 2017	11.0 ± 1.1	0.16 ± 0	1.05 ± 0,02	90.32 ± 2.6	73.7 ± 1.5	10.51 ± 0.2	748.23 ± 3.2
Viosinho 2017	6.3 ± 1.4	0.15 ± 0	1.32 ± 0,01	54.52 ± 1.8	72.3 ± 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	59.6 ± 1.7	11.61 ± 0.15	885.88 ± 4.3
Moscatel Galego 2017	5.9 ± 1.1	<0.01 ± 0	1.16 ± 0.02	58.48 ± 1.3	94.1 ± 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	51.0 ± 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	51.0 ± 1.3	5.66 ± 0.15	726.74 ± 4.2
Encruzado 2018	10.7 ± 1.1	<0.01 ± 0	0.75 ± 0.01	55.59 ± 1	68.4 ± 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	101.7 ± 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	87.4 ± 0.8	9.85 ± 0.25	525.04 ± 1,5
Arinto 2018	9.51 ± 0.05	<0.01 ± 0	0.64 ± 0	61.71 ± 1.2	72.5 ± 0.9	6.08 ± 0.1	739.9 ± 4.6
Moscatel Galego 2018	2.11 ± 0.07	<0.01 ± 0	0.79 ± 0,02	38.17 ± 0,3	91.8 ± 1.2	8.77 ± 0.2	653.25 ± 3.4
Macabeu 2018	10.32 ± 0.09	<0.01 ± 0	0.52 ± 0	57.00 ± 0.6	51.0 ± 1.1	5.66 ± 0.3	726.74 ± 3.7
Moscatel of Setúbal 2018	7.83 ± 0.07	0.062 ± 0	0.60 ± 0.01	42.78 ± 0.45	74.5 ± 0.5	7.63 ± 0.25	808.60 ± 4.1

3.2. Wine protein profile: hydrophobic interaction chromatography

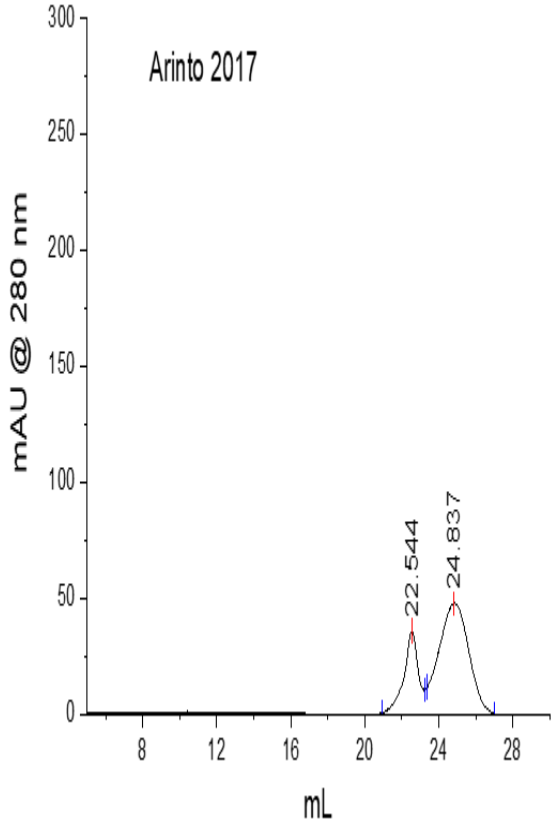
The total wine protein profile of the fourteen wines was analyzed by hydrophobic interaction chromatography (HIC), according to the method described by Marangon *et al.* (2009), with some modifications (Chagas *et al*., 2016). This technique allows to separate the proteins based on their hydrophobicity, which can be correlated with different protein classes (Marangon *et al*., 2009). In fact, each class of protein has a distinct degree of hydrophobicity, which in turn determines the time of its elution from the HIC column during the chromatographic run. This time may then be correlated with a specific class of proteins.

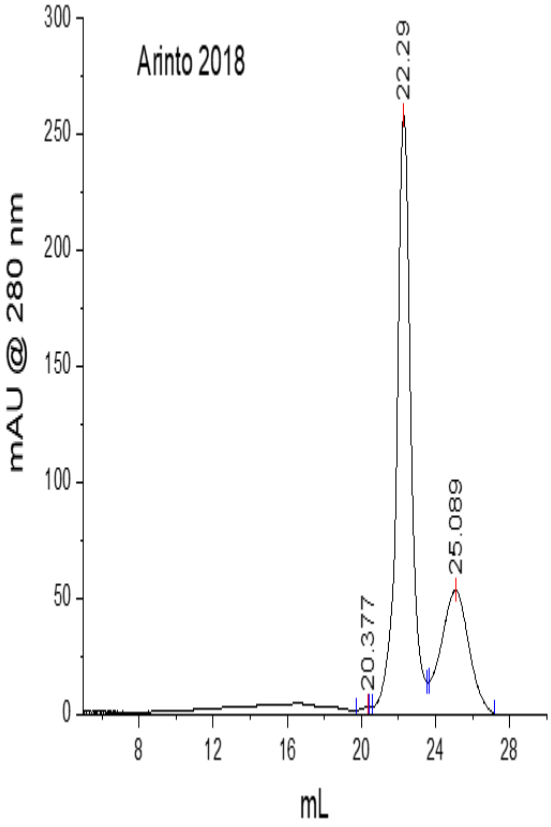
Before protein fractionation by HIC, the isolation of positively charged proteins using SCX was performed following the method of Van Sluyter *et al*. (2009), with some modifications (Chagas *et al*., 2016), since is necessary the injection of pure proteins to be able to have a high resolution. In this way, there is more certainty that the same peaks represent the proteins, and not some other interference present in the wine. Figure 19 shows the results of the fractionation of the total proteins of all the samples by HIC.

A)

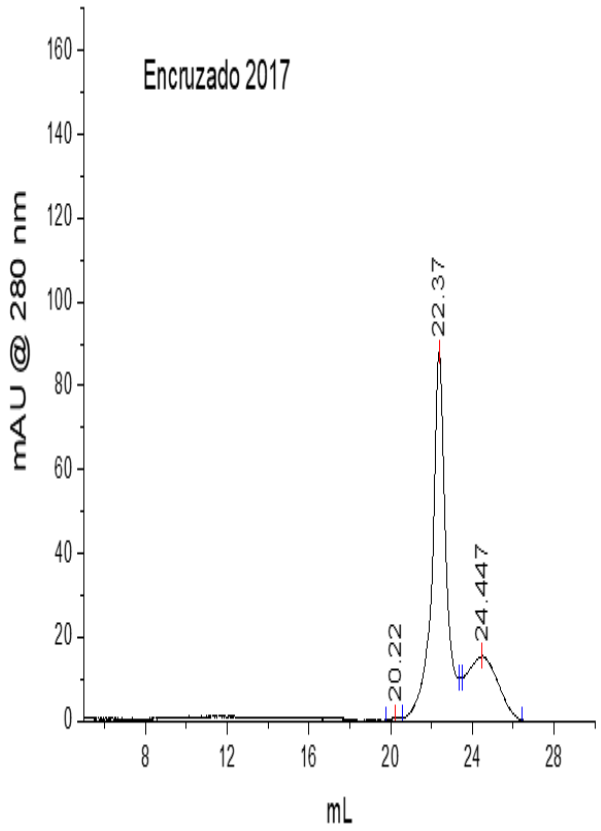


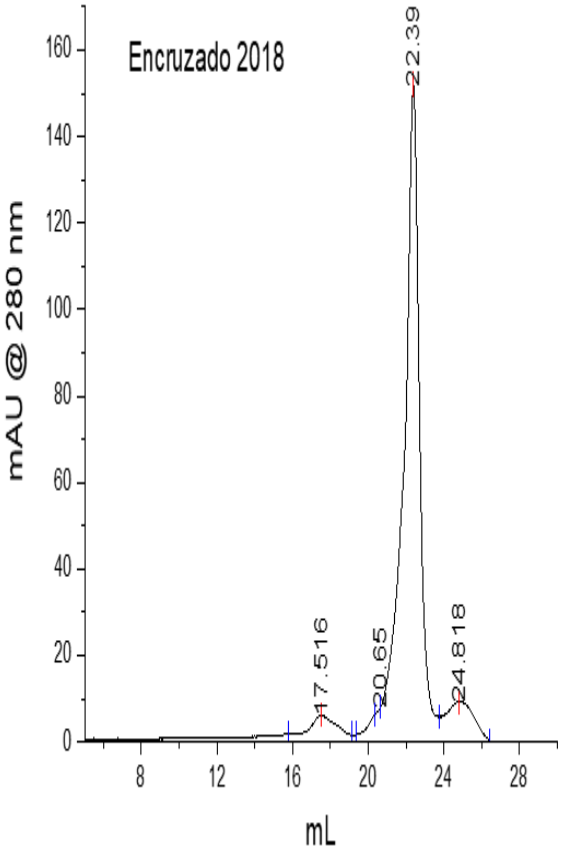
B)


Arinto 2017
mAU @ 280 nm
mL
22.544
24.837

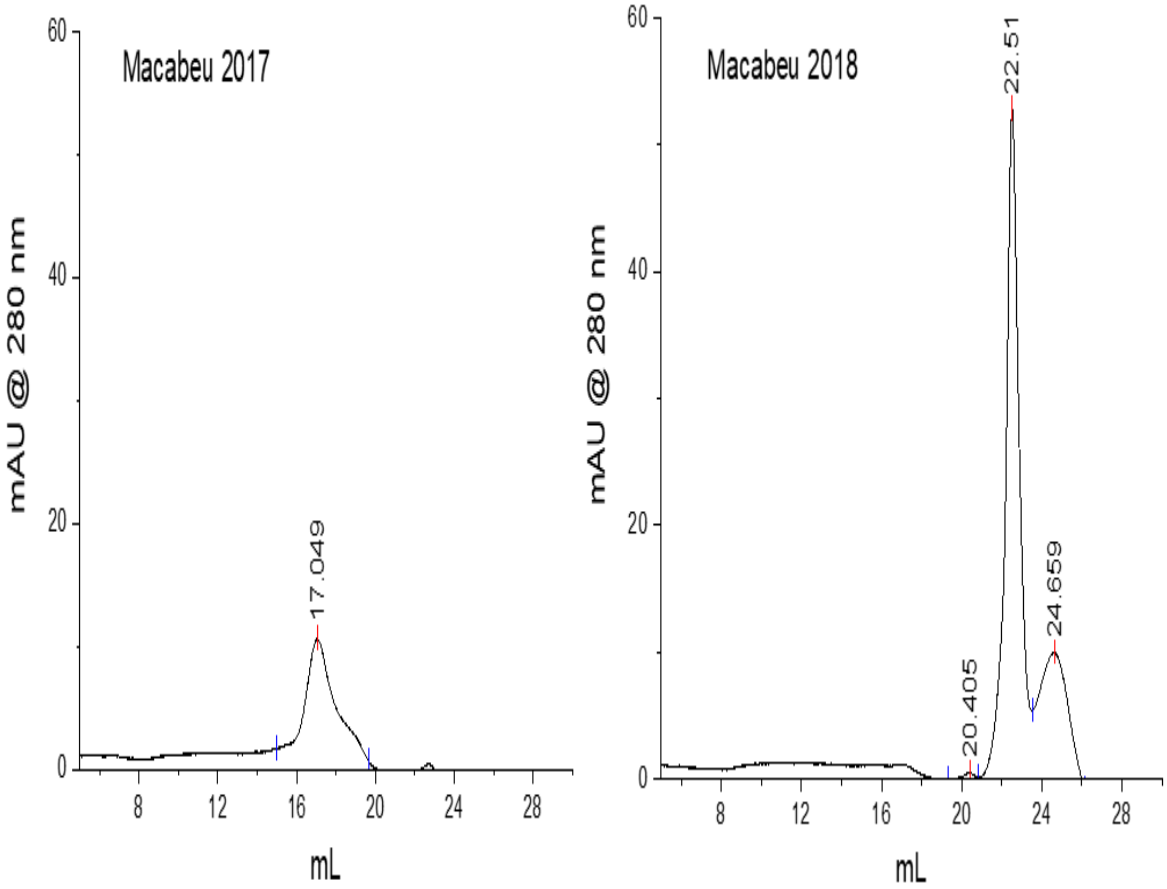

Arinto 2018
mAU @ 280 nm
mL
20.377
22.29
25.089

C)

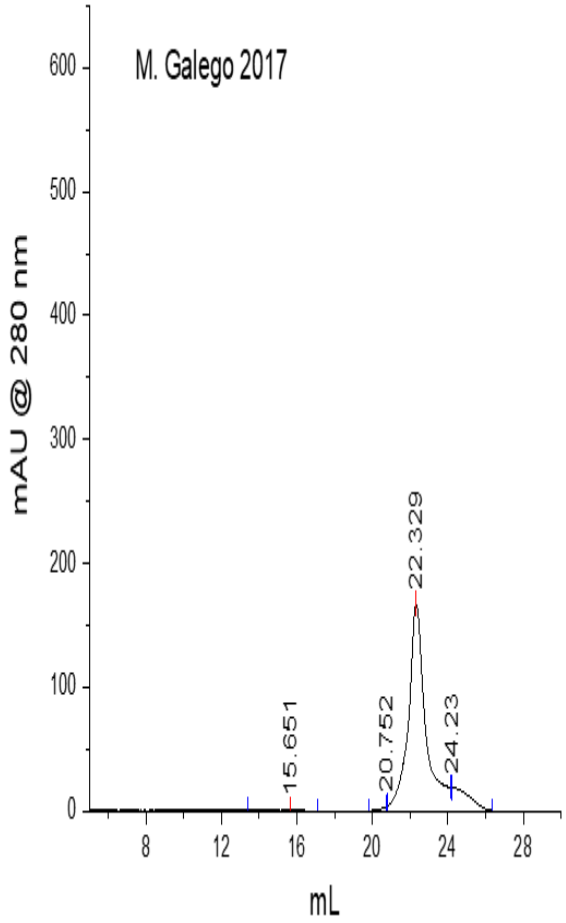

Encruzado 2017
mAU @ 280 nm
mL
20.22
22.37
24.447

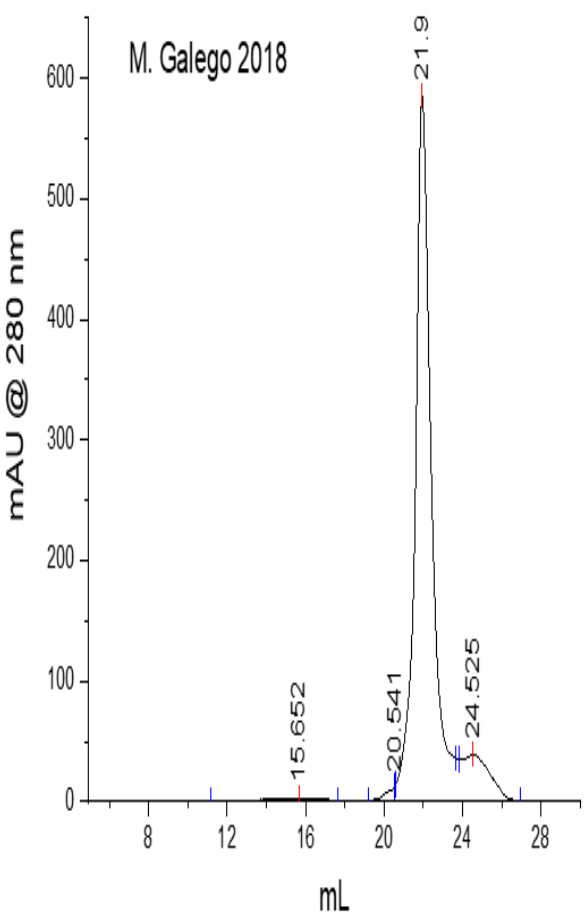

Encruzado 2018
mAU @ 280 nm
mL
17.516
20.65
22.39
24.818

D)

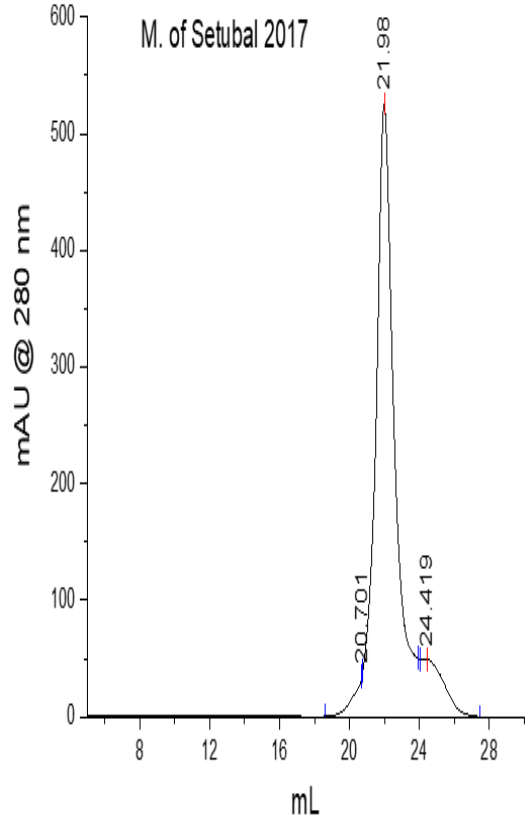

Macabeu 2017
mAU @ 280 nm
mL
17.049
Macabeu 2018
22.51
24.659
20.405

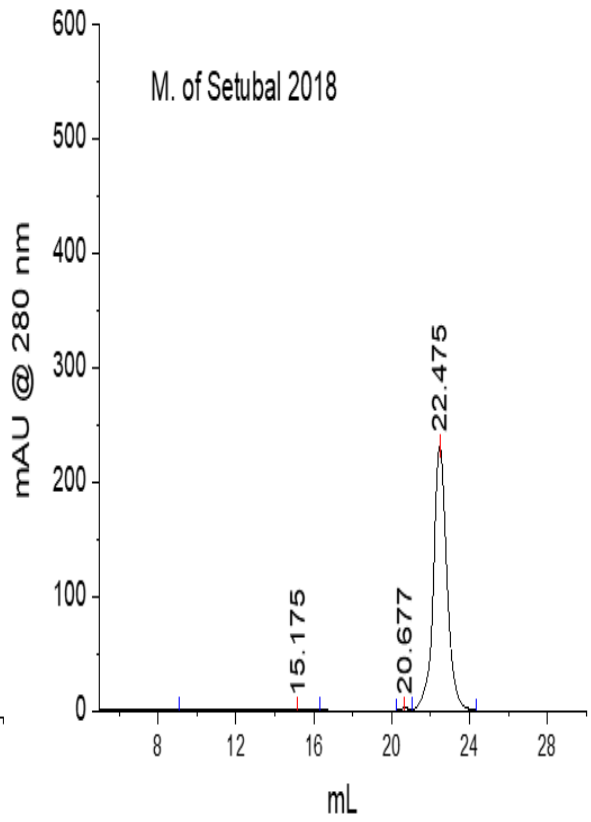
E)


M. Galego 2017
mAU @ 280 nm
mL
15.651
20.752
22.329
24.23


M. Galego 2018
mAU @ 280 nm
mL
15.652
20.541
21.9
24.525

F)


M. of Setubal 2017
mAU @ 280 nm
mL
20.701
21.98
24.419


M. of Setubal 2018
mAU @ 280 nm
mL
15.175
20.677
22.475

G)

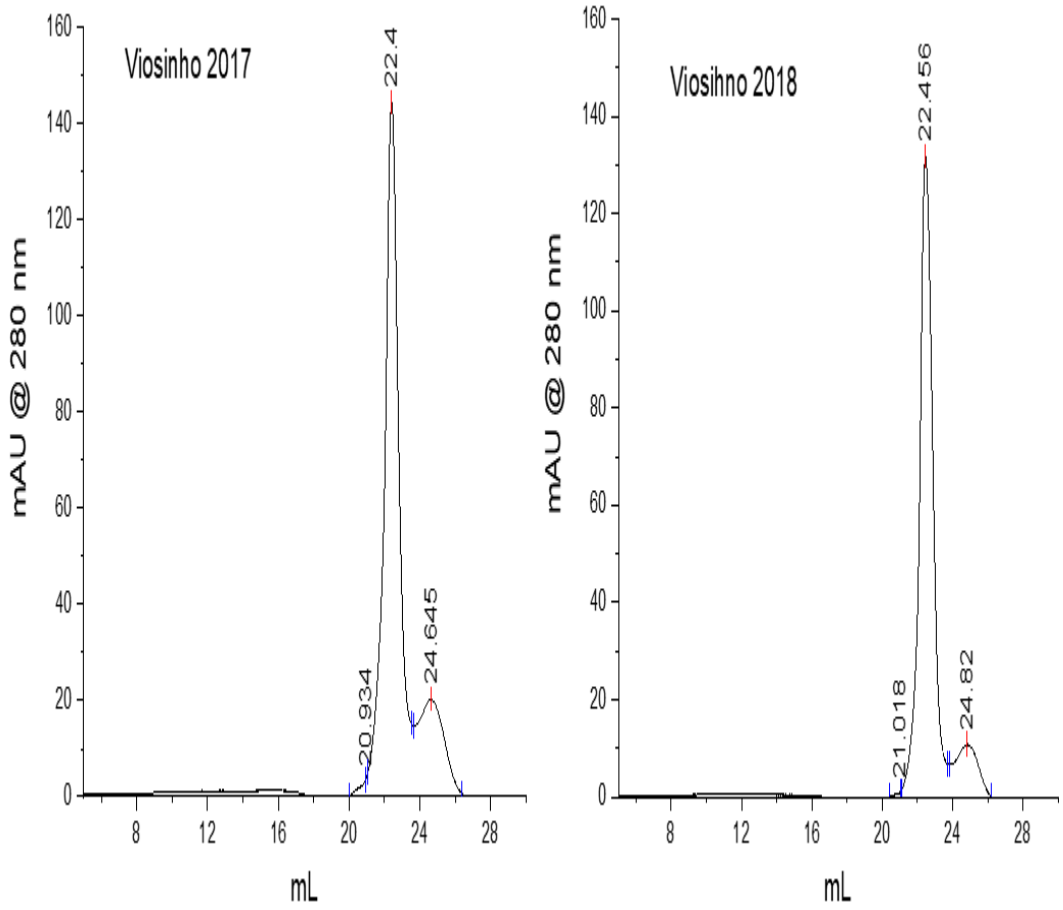


Figure 19. *Chromatograms obtained from the HIC runs of all fourteen samples: A) Alvarinho 2017 and 2018, B) Arinto 2017 and 2018, C) Encruzado 2017 and 2018, D) Macabeu 2017 and 2018, E) Moscatel Galego 2017 and 2018, F) Moscatel de Setúbal 2017 and 2018, G) Viosinho 2017 and 2018.*

In the case of Macabeu 2017, there is no sufficient protein concentration to ensure a good signal to noise in order to interpret the results. Therefore, it's not possible to discriminate a protein profile for this sample. The cause of this is to be found in the low concentration of total protein that this wine sample presented. To obtain a good profile, it was necessary to have higher quantity of wine sample which was not available.

For each chromatogram of figure 19, the principal peaks and corresponding areas were calculated. Using these results, it was possible to calculate the ratios between the different peaks and to match each peak to previously identified proteins, in particular to the presence and the impact in each wine of the TLPs. Among the grape PR-proteins, the thaumatin-like proteins (TLPs), and also the chitinases, can undergo changes in structural integrity. These changes lead to protein unfolding, resulting in the exposure of amino acid side chains that are normally hidden in the hydrophobic core of the protein. In their 'attempt' to avoid the

contact with the polar water molecules, the newly exposed side chains are then free to associate with other proteins or with wine components to form aggregates, that ultimately result in visible haze or precipitates in the bottles (Marangon *et al*., 2014). In fact, the biggest peak observed in each chromatogram in figure 20, corresponds to thaumatin-like proteins, eluted in all the wine samples between the 21.9 and 22.9 mL. This conclusion was validated by HIC runs of previously isolated TLP from Moscatel de Setúbal. However, in this work the isolation and identification of the different peaks was not possible to fulfil and therefore the putative identification of the different fractions was performed by comparison with the results obtained in the available literature.

It is important to underline that for the same class of proteins and for identical chromatographic conditions (e.g. equilibration and elution buffers as well as the gradients utilized), the time of elution from the HIC column must be the same. In this work slight differences were introduced when compared to the data in the available literature: this is due to the concentration of the equilibration solution. Depending on the wine, the yield of proteins during the isolation by SCX was not always the same, due to the different concentration of proteins in wines under study. This has resulted in a different amount of injected proteins, and consequently slight fluctuation in the elution time. This aspect is clearly visible in figure 20.

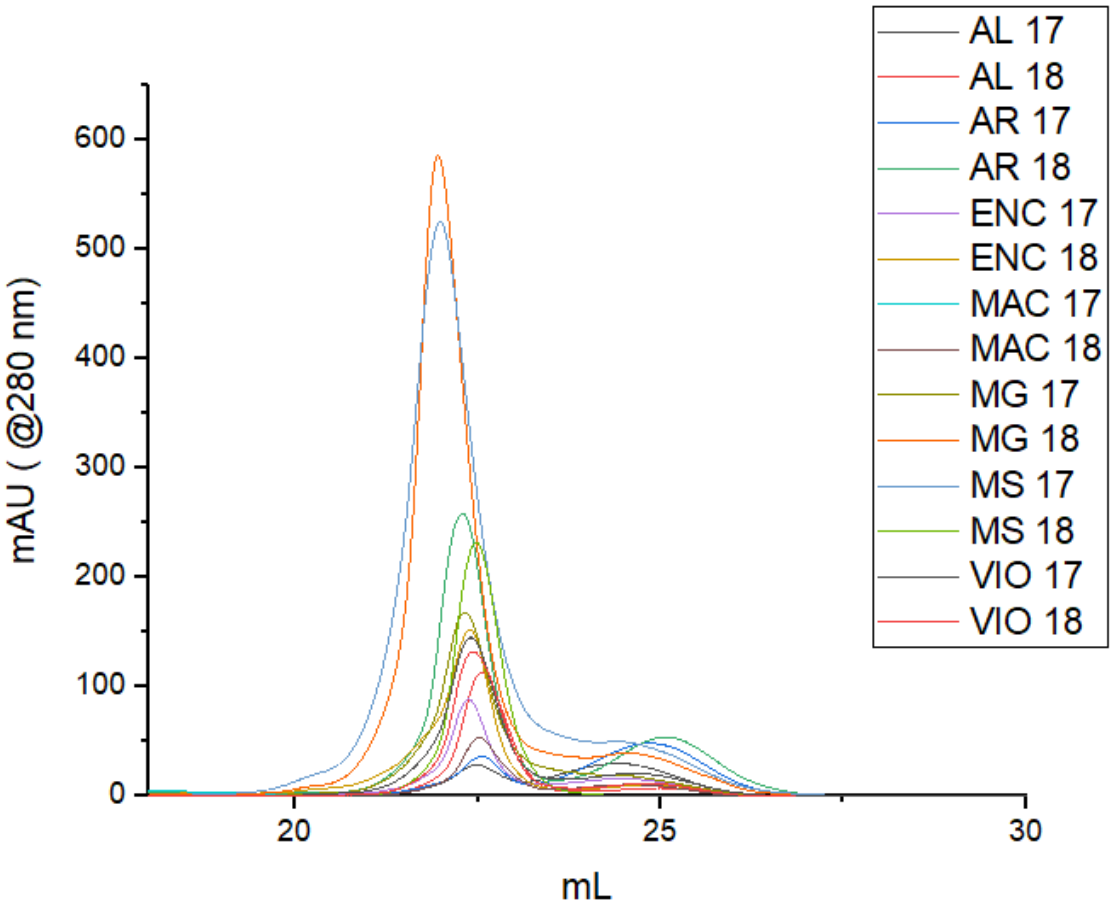


Figure 20. *Comparison and overlapping of total proteins after the fractionation of all fourteen wine samples: Alvarinho 2017 and 2018 (AL 17, Al 18), Arinto 2017 and 2018 (AR 17, AR 18), Encruzado 2017 and 2018 (ENC 17, ENC 18), Macabeu 2017 and 2018 (MAC 17, MAC 18), Moscatel Galego 2017 and 2018 (MG 17, MG 18), Moscatel de Setúbal 2017 and 2018 (MS 17, MS 18), Viosinho 2017 and 2018 (VIO 17, VIO 18).*

Certainly, the amount of proteins injected affected the behavior of specific proteins during the fractionation. According to Marangon *et al*. (2009) such differences observed during the fractionation may be due to the presence of enzymes with proteolytic activity in the musts (Cordonnier *et al*., 1968). Although these proteins are typically quite resistant to proteolysis, this enzymatic activity could modify some peptides in the protein chain, resulting in a slightly altered behavior of proteins during the chromatographic run. The changes in some peptides could be translated into a different hydrophobicity of the protein surface. Despite this, in this work the variability observed between the injected proteins was mainly due to the method used, in particular to the mass of the proteins used for each run and not to the variability among samples.

The fractionation of the total wine proteins by HIC shows, as reported in figure 20, the presence of different protein peaks. Until now it was described the largest peak, a putative TLP, present in all the samples. The other peaks, as suggested by Maragon *et al*. (2009), could be isoforms of TLPs that have different hydrophobicity surface, and consequently different retention time, or other classes of proteins, such as chitinases. According to the same authors, their results showed that chitinases tended to behave in a less hydrophobic way than TLPs. Therefore, in this work, the peak or peaks eluted after the putative TLPs could be tentatively identified as the chitinases or, alternatively, they could be other TPLs, isoforms with different characteristics.

Regarding the differences among samples, all varieties from the 2017 vintage presented a lower percentage of putative TLPs with respect to the 2018 vintage, as shown below. The only exception observed was for the Arinto and the Moscatel Galego wines: these varieties maintained constant the proportions of TLPs in both vintages 2017 and 2018 but changed the total amount of proteins from the two vintages. The Arinto 2018 had a slight increase of proteins in the 2018 wine, despite being stable as the 2017 vintage. Instead, the Moscatel Galego maintained constant the proportion of TLPs in the two years but showed a higher increase in the amount of protein in 2018, changing from a very stable wine in 2017, to one of the most unstable in 2018.

The proportion of TLPs relative to the total winer protein is very important and affects the stability of the wine: under the conditions of this work, it seems that the highest protein instability in wines is achieved when the samples contain a high proportion of TLPs in the protein profile and a high concentration of total protein simultaneously.

3.3. PCA analysis

PCA analysis was performed to correlate different variables, and to discriminate the wines in terms of their distribution in the plot shown in figure 21. This analysis was performed using a matrix of data that represents the average of replicates for each parameter taken into consideration. The number of selected variables considered were five in this case.

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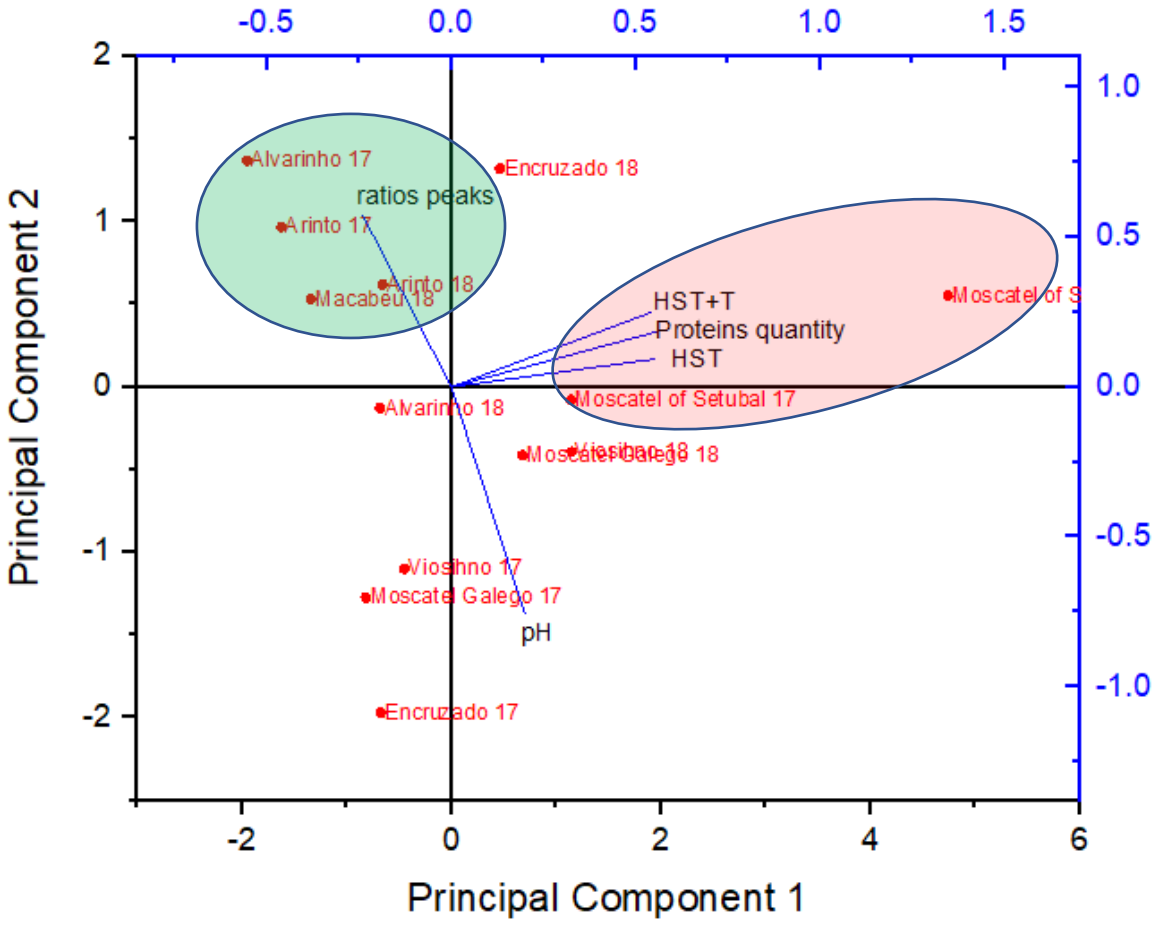


Figure 21. *PCA of all wines analysed with some selected variables: HST and HST+T, protein quantity, pH and ratio of HIC peaks of all samples. The cumulative percentage of the variance of PC1 and PC2, in this case, is 81.28 %.*

In the analysis represented in figure 21, the results of all samples of the heat stability tests, reports of protein peak integrals, the protein quantity and the pH were taken into consideration. In the picture, there is a clear subdivision between the stable and unstable wines. The Moscatel de Setúbal 2017 and 2018, identified in the plot with the pink ellipse, are the wines closer to the variable protein quantity and HST, since they are the most unstable and with a higher concentration of proteins. In the same area, there are also the

Viosinho and Encruzado 2018, that represent the other two unstable wines. On the opposite side, all the remaining stable wines are placed.

Regarding the pH variable, there there seems to be no correlation with the distribution of the wines and their instability. The ratio of the HIC protein peaks is obtained from the integrals of the different individual peaks, obtained with the HIC: in this way the protein profiles of the wines are normalized and it is possible to evaluate how protein variability can affect protein stability. In the green area four wines are delimited, very close to the “ratio peaks”; they are Arinto 2017 and 2018, Macabeu 2018 and Alvarihno 2017. These wines like the others that are located in the negative part of the plot are stable, but these four are the wines with the lowest percentage of TLPs. The results confirm again the importance of the presence and concentration of the TLPs in the wine stability, as stated above.

3.4. Assessment of the role of the SO_2 on the HST

Once all the wines were characterized, thanks to all the parameters described above, to evaluate the impact of sulfur dioxide on protein instability, two of the fourteen wines were selected. An unstable wine, Moscatel de Setúbal 2018, the highest for instability and for protein concentration, and Macabeu 2017 that in the opposite way is stable, were selected. In figure 22, there is a 3D comparison of all protein HIC profiles of all samples, useful for selecting the samples on which to assess the impact of the sulfur dioxide in the HST.

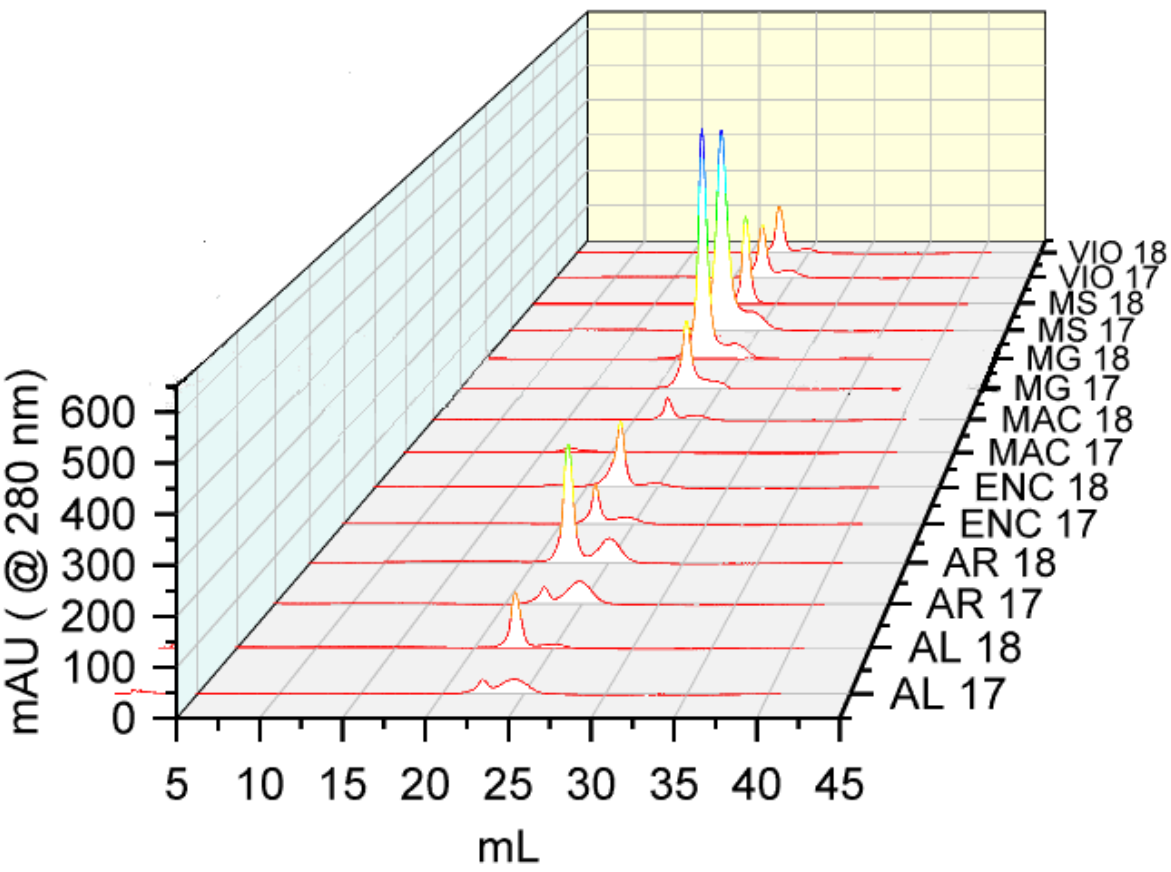


Figure 22. *Chromatograms obtained by HIC of the 14 wines under study: Alvarinho 2017 and 2018 (AL 17, Al 18), Arinto 2017 and 2018 (AR 17, AR 18), Encruzado 2017 and 2018 (ENC 17, ENC 18), Macabeu 2017 and 2018 (MAC 17, MAC 18), Moscatel Galego 2017 and 2018 (MG 17, MG 18), Moscatel de Setúbal 2017 and 2018 (MS 17, MS 18), and Viosinho 2017 and 2018 (VIO 17, VIO 18). The main peak corresponds to the TLPs (with a retention time between 21.9 and 22.9 min, depending on the sample).*

After choosing the wines, a test to evaluate the role of sulfur dioxide on protein haze formation in the white wines was performed.

To better evaluate the increase in turbidity after the heat stability test, it was necessary to have the selected wines stabilized; therefore, Moscatel de Setúbal 2018 was treated with bentonite. The stable wine (i.e., Macabeu 2017) instead was not treated with bentonite, since it was already stable. Regarding the Moscatel de Setúbal, enough bentonite was added until the wine could be considered stable. To stabilize the wine it was necessary to use 1.5 g/L of bentonite. The amount of proteins left in the sample, after this fining treatment, was checked with the Bradford method, to know the real protein concentration of the treated wine. At the same time, the analysis of total and free sulfur dioxide were performed in the treated wine.

The trial, explained in figure 23, was divided in three steps: first, performed for both stable wines, was to evaluate their turbidity after the heat test with different doses of SO_2: 120, 150 and 200 mg/L of total sulfur dioxide. The addition of the different doses of sulfur dioxide in the different sample was performed 48 h before the HST. For the Moscatel de Setúbal 80 mg/L of total SO_2, is the concentration present in the wine without any addition, called the experiment control. For the Macabeu the experiment control has 75 mg/L of total SO_2

In the second step, the turbidity after the HST was estimated at increasing concentrations of sulfur dioxide, but in this case there was also an addition of 50 mg/L of proteins. For the Moscatel de Setúbal 2018, previously isolated 50 mg/L of proteins from the same wine were back-added. Instead, in the Macabeu 2017, proteins from Viosinho 2018 were back-added. The proteins of this last, unstable wine, with a protein profile similar to that of Moscatel de Setúbal 2018, were chosen to evaluate the results in terms of turbidity, using proteins other than those of Moscatel de Setúbal 2018.

The third and last step consisted on the HST but with an addition of 100 mg/L of proteins at a different doses of sulfur dioxide.

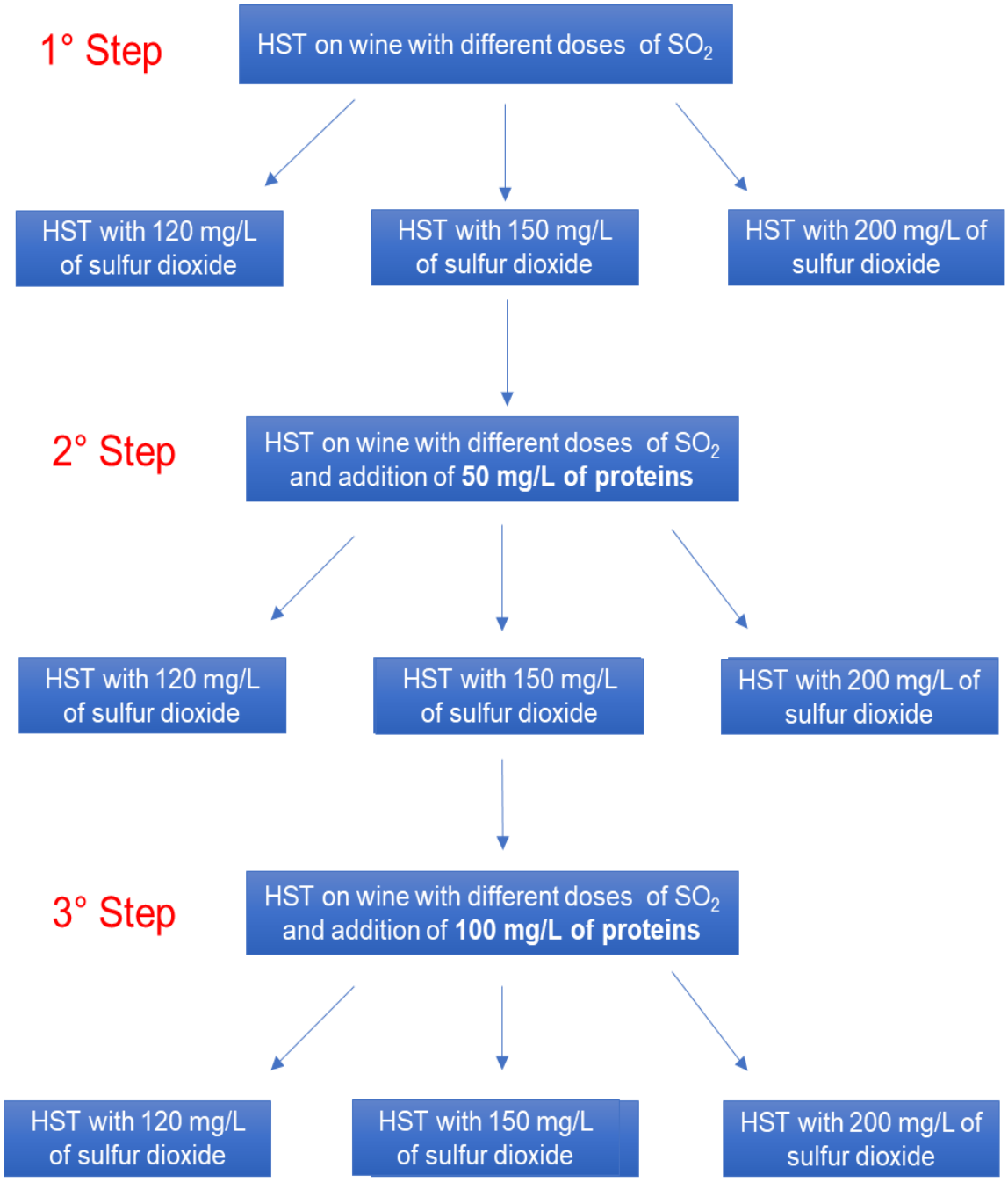
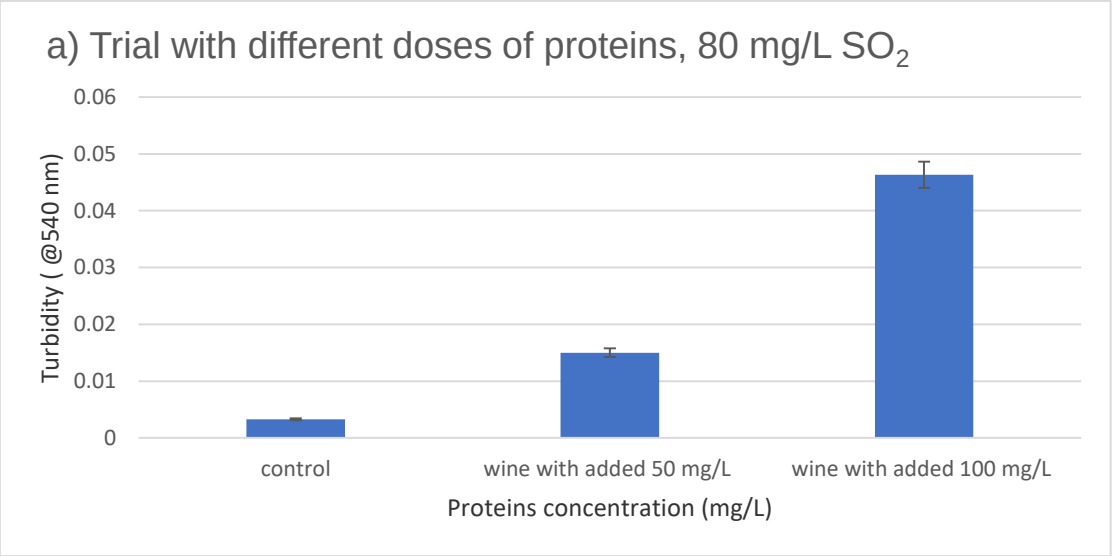


Figure 23. *Different steps carried out to test the effect the sulfur dioxide on the HST in wines with different concentration of proteins.*

In figure 24 the results of the influence of the increment in protein concentration on the turbidity for both the wines is shown, with constant concentration of total sulfur dioxide: 80 mg/L for the Moscatel de Setúbal, 75 mg/L for the Macabeu. In both cases, a protein concentration increase corresponded to a statistically significant increase in protein turbidity. This point is also supported by the work of McRae *et al*. (2018), in which an increment of proteins had a positive correlation on the protein stability of wines.



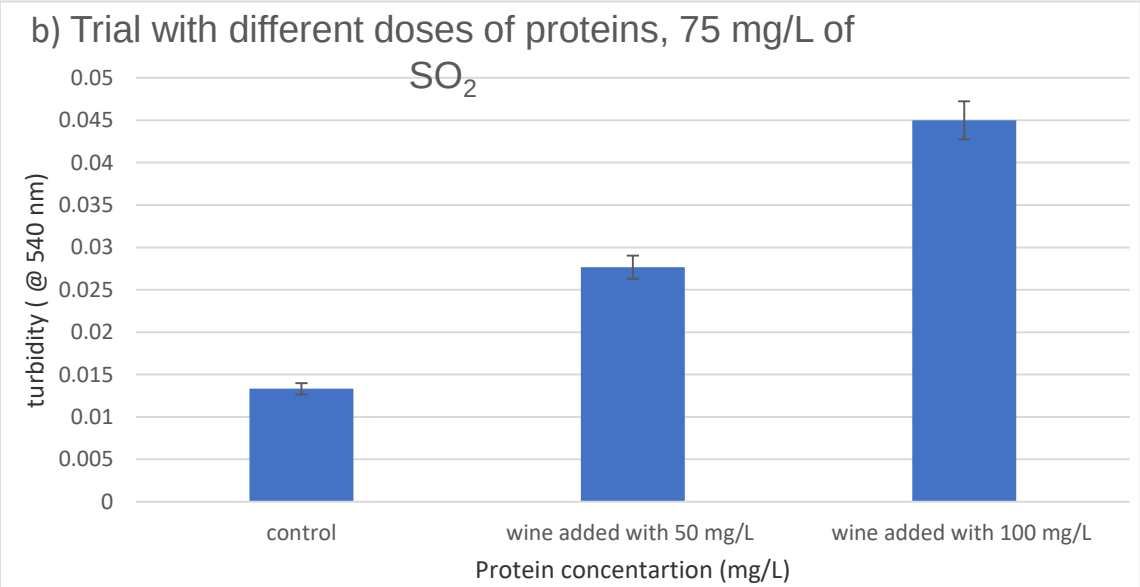
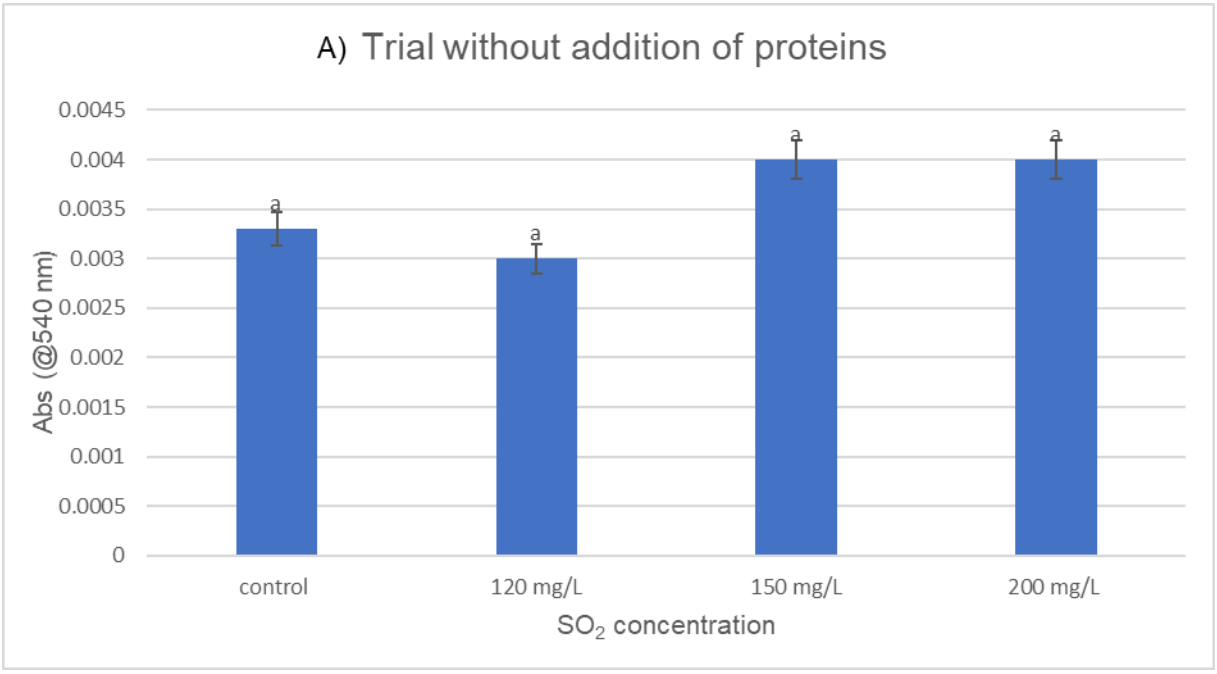
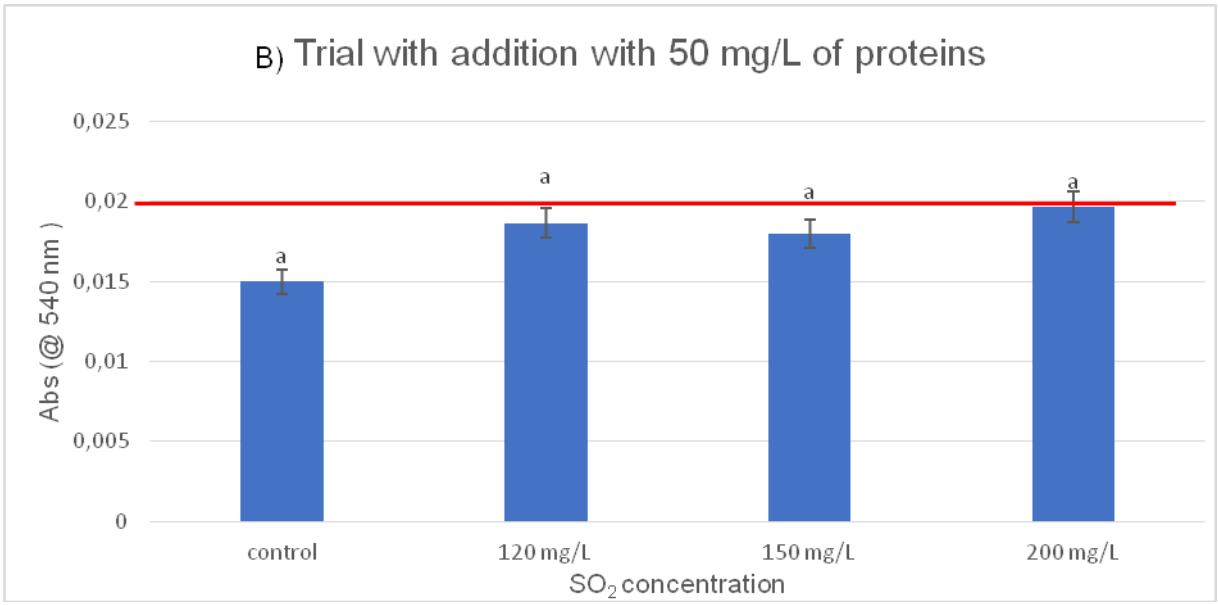


Figure 24. *Protein turbidity trend in a) Moscatel de Setúbal 2018 with increasing concentration of proteins at 80 mg/L of total SO_2 and b) Macabeu 2017 with increasing concentration of protein* at 75 mg/L of total SO_2

The results of the synergistic effect of incrementing doses of both proteins and sulfur dioxide for the Moscatel de Setúbal 2018 and for tre Macabeu 2017 are shown in figures 25 and 26, respectively.


A) Trial without addition of proteins
0.0045
0.004
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
Abs (@540 nm)
a
a
a
a
control
120 mg/L
150 mg/L
200 mg/L
SO2 concentration


B) Trial with addition with 50 mg/L of proteins
0,025
0,02
0,015
0,01
0,005
0
Abs (@ 540 nm)
a
a
a
control
120 mg/L
150 mg/L
200 mg/L
SO2 concentration

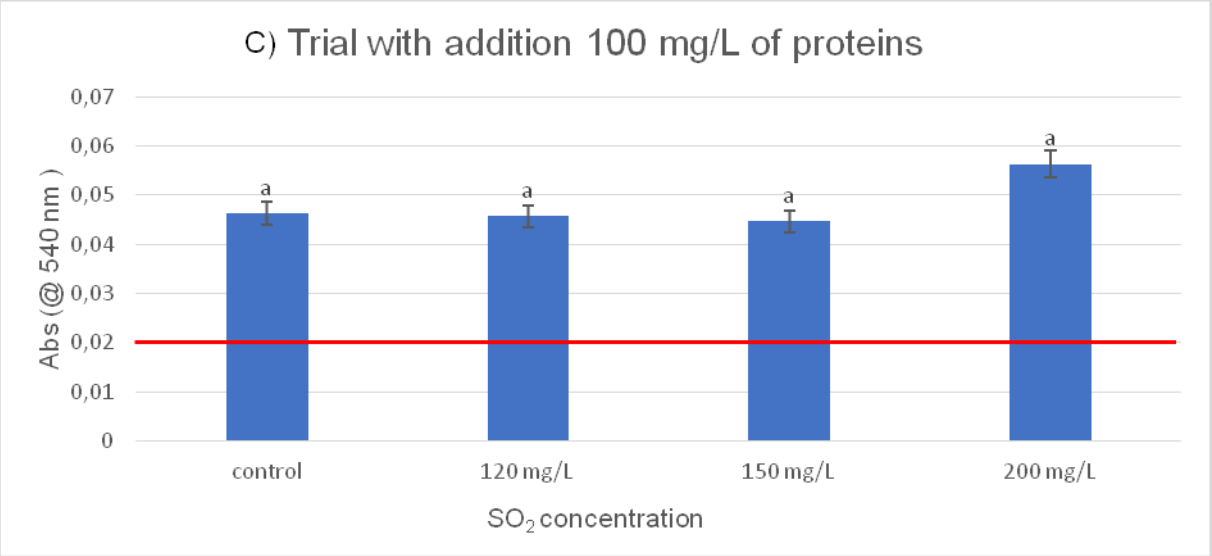
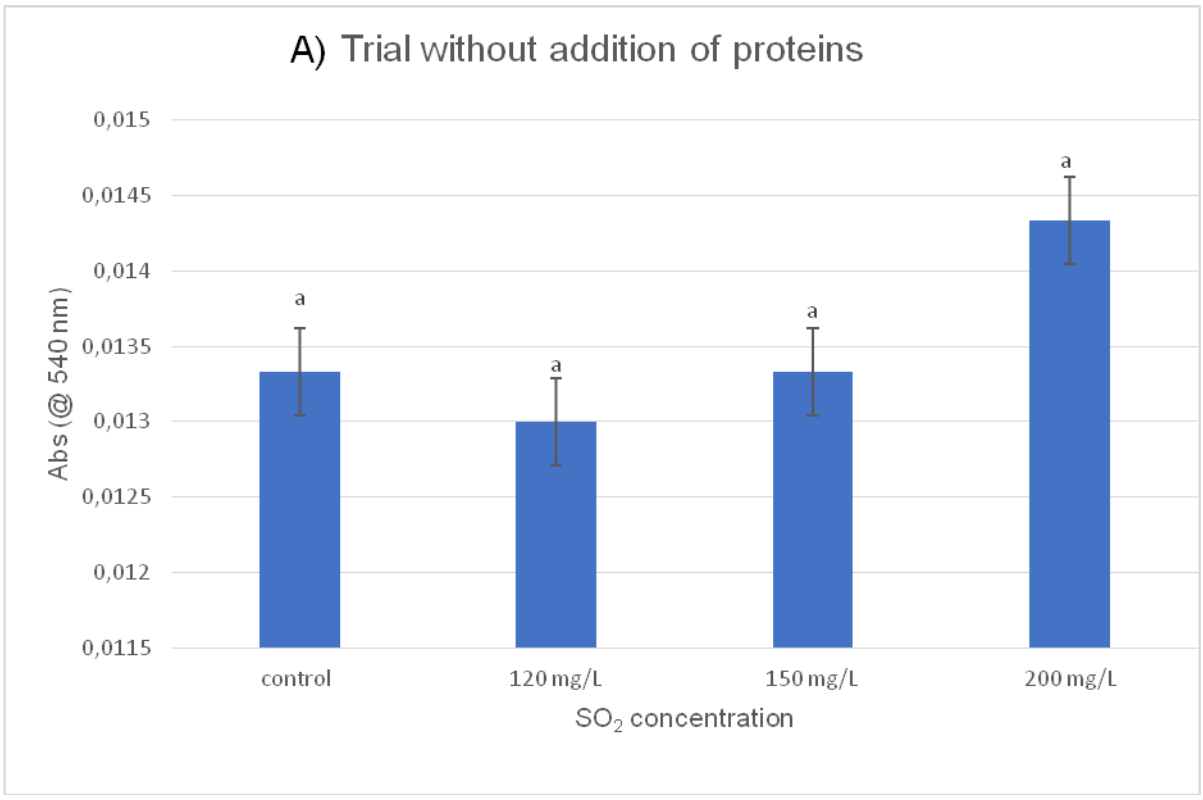
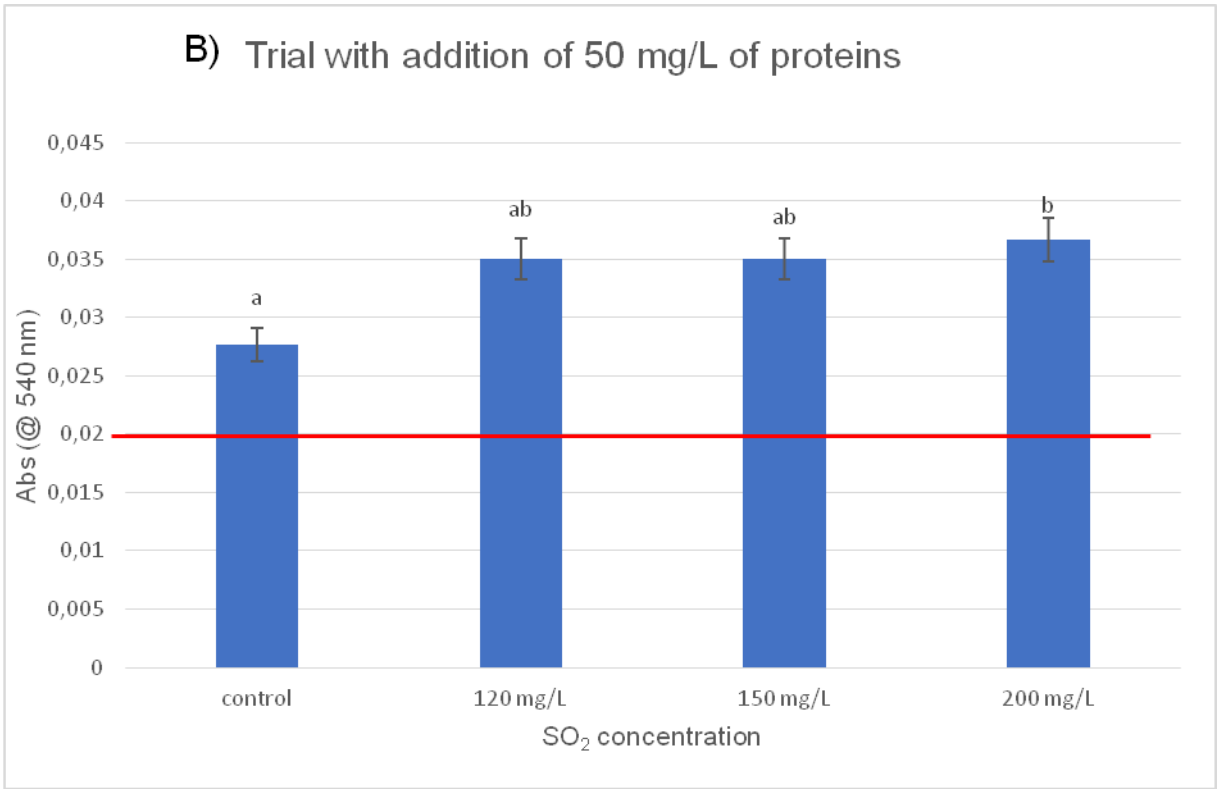


Figure 25. *Effect on protein turbidity, assessed with the absorbance at 540 nm, at increasing protein and sulfur dioxide doses on the Moscatel de Setúbal 2018 wine; a) trial without the addition of proteins, b) with the addition of 50 mg/L of proteins, and c) with the addition of 100 mg/L of proteins.*

A shown in figure 25, the Moscatel de Setúbal 2018 is stable without the addition of proteins. Increasing the amount of sulfur dioxide does not correlate with an increment of the turbidity. When 50 mg/L of proteins are added the wine turbidity increases, and at a concentration of 200 mg/L of SO_2 the wine reaches the upper limit of the stability, namely 0.0195 units of absorbance after the HST (the threshold to be considered unstable is 0.02 units of absorbance) (de Bruijn *et al.*, 2009). Instead, with the addition of 100 mg/L protein, the wine is always unstable, but raising from 80 mg/L of SO_2 to 200 mg/L leads to a higher increment of turbidity than in the trial with 50 mg/L protein. Even after statistical analysis, these differences cannot be considered significant, according to the ANOVA test, performed to assess the statistically significant differences.


A) Trial without addition of proteins
0,015
0,0145
0,014
0,0135
0,013
0,0125
0,012
0,0115
Abs (@ 540 nm)
a
a
a
a
control
120 mg/L
150 mg/L
200 mg/L
SO2 concentration


B) Trial with addition of 50 mg/L of proteins
0,045
0,04
0,035
0,03
0,025
0,02
0,015
0,01
0,005
0
Abs (@ 540 nm)
a
ab
ab
b
control
120 mg/L
150 mg/L
200 mg/L
SO2 concentration

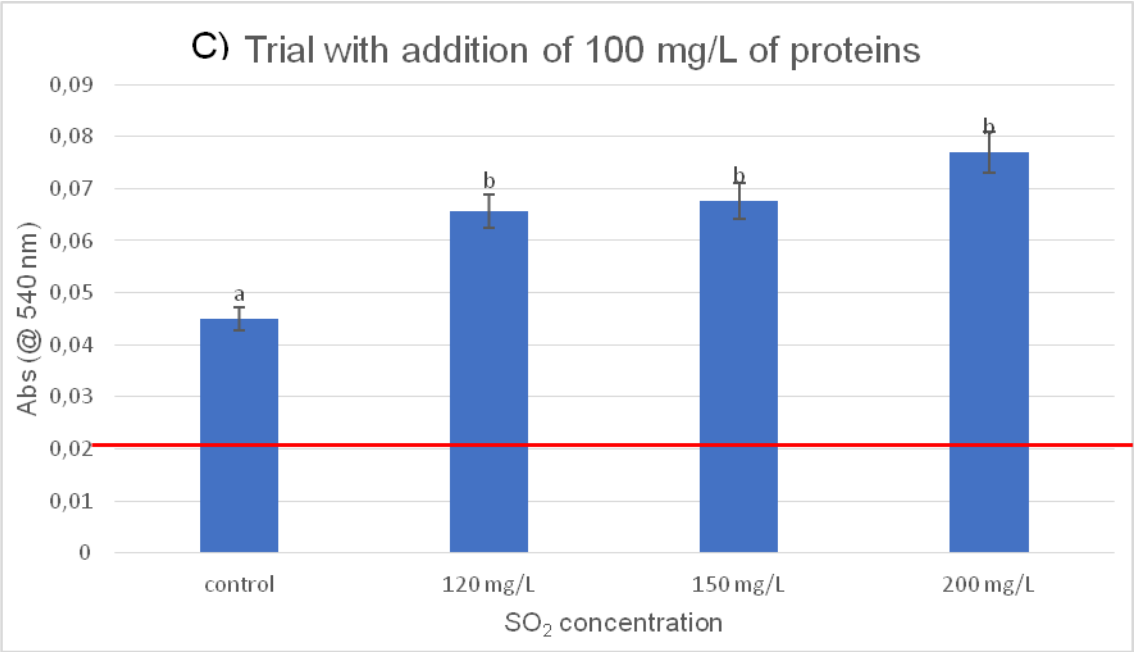


Figure 26. *Effect on protein turbidity, assessed with the absorbance at 540 nm, at increasing protein and sulfur dioxide doses on the Macabeu 2017 wine; a) trial without the addition of proteins, b) with the addition of 50 mg/L of proteins, and c) with the addition of 100 mg/L of proteins.*

The Macabeu 2017 wine samples were not treated with bentonite, and at the end in the various tests carried out, it presented a protein concentration slightly higher than the Moscatel de Setúbal 2018 wine (20 mg/L higher). This could explain why it reaches slightly higher turbidity values in all three tests when compared to the Moscatel. Moreover the treatment with bentonite can affect the composition of wine, such as the decrement of phenolic compounds and this can explain the higher stability of Moscatel. Also, it must be emphasized that to the Macabeu wine, Viosinho wine proteins were added, an unstable wine with a protein profile similar to Moscatel, in terms of proportion of TLPs present, as shown below.

The trial without the addition of proteins, for the Macabeu 2017 wine, going from 75 mg/L to 200 mg/L of sulfur dioxide gave an increment of turbidity more visible than in the Moscatel's trial, even if in this case there are no statistically significant differences in relation to the control. In contrast, in the trials with the addition of proteins and according to the analysis of variance, there are results significant and highly significant, both for the trial of 50 mg/L and for 100 mg/L of proteins added. Respectively, in the first case the absorbance at 540 nm was 0.027 in the control and reaches 0.036 absorbance units at 200 mg/L of sulfur dioxide. For these values in fact, the control, without the addition of sulfur dioxide, is different from the

trial with 200 mg/L SO_2. Also, the two tests with 120 and 150 mg/L of SO_2 are different from the control but lower when compared to the 200 mg/L SO_2 test.

In the trial with the addition of 100 mg/L of proteins, the absorbance goes from 0.045 to 0.077 of absorbance units when passing from the control to the 200 mg/L SO_2 test. In this latter case, the wine became highly unstable, so much that the increase in turbidity, if compared with the control, was visible and appreciable with the naked eye. In this case, according to the analysis of variance, the control is different from all the other tests; in contrast, between the tests at 120 mg/L, 150 mg/L and 200 mg/L of SO_2 there aren't statistically significant differences.

The results show that the increment of turbidity is not so noticeable, like in the work of Chagas *et al*. (2016): in the latter, the turbidity increased twice in the sample with sulfur dioxide compared to the one without. Indeed unlike the of by Chagas et al. (2016) work carried out on a model wine solution, in this work was not possible to evaluate the turbidity after the heat stability test in the complete absence of sulfur dioxide, since was used real wine. Anyway, the increment is statistically significant in respect to the control in the Macabeu's trial with the addition of 50 and 100 mg/L of proteins.

Moreover, in comparison with the work of Chagas *et al*. (2016) there are differences in the effect of the increment doses of the sulfur dioxide on the turbidity: in the quoted work the higher effect of the SO_2 is observed between 0 and 100 mg/L of added SO_2, but further increases in SO_2 lead to successively lower increments in protein turbidity until a saturation point is reached. As stated before in this work, was used real wine, as consequence the minimal level of sulfur dioxide at which was performed the HST was determinated by the sulfur dioxide content in the wines.

In the opposite way, in this work, the higher increment of protein turbidity is observed within the range 120-200 mg/L of sulfur dioxide. This could be explained, by the fact that there are additional factors implicated in the pathway of instabilization of protein (Marangon *et al*., 2011). Therefore, a different wine matrix could give different results.

After fractionation, it is possible to state that in all wine samples used in this work, a specific fraction is present at higher concentrations, which could potentially be identified with the TLPs. Accordingly, on the description of their structure (Marangon *et al*., 2014), this class of proteins, due to the presence of different regions in which different disulfide bridges are present, would be the most sensitive to interact with the sulfur present in wine. Therefore, during heating, but even more during cooling of the wine during the HST, the denaturation of the native structure of the protein due to the attack of SO_2, would lead to the aggregation of the proteins and to protein haze formation.

This test aims to support the protein haze model, already studied and validated by another study (Chagas *et al*., 2016), putting in this case the attention on the role and interaction of TLPs and sulfur dioxide.

These results are in agreement with the results of Chagas *et al*. (2016) regarding the effect of sulfur dioxide in the destabilization of heat-unstable wine proteins. This additive, present in the majority of commercial wines, induces protein thiosulfonation or *S*-sulfonation, acting not only as a reducing agent but also a sulfhydryl-blocking reagent, hindering thiol-disulfide exchange during protein interactions (Zhang & Sun, 2008).

3.5. The sulfur dioxide and TLPs interaction on HST

In this work the fractionation of total proteins by HIC was done for each sample of wine, to assess the protein profile. In this case each sample presented from 2 to 4 peaks: the biggest was probably identified as TLPs which, as demonstrated by Chagas *et al*. (2016), is the class of proteins that induce a high increment in turbidity, after a heat test, in the presence of sulfur dioxide. In the previous trials the relationship between sulfur dioxide and total proteins was studied. Now, in this work, an attempt was made to evaluate in a more specific way the interaction of sulfur dioxide and TLPs. In table 10 the integrals for each HIC fractionated protein peak are shown. It is is also reported a comparison between the proportion of the TLPs, termed P1, and the sum of all the other peaks, collectively called P2.

Table 10. *Values of the areas corresponding to protein peaks obtained by HIC. P1 represents the* proportion *of TLPs, whereas P2 is the sum of the proportions of all the other peaks.*

Wine	Integral peak 1	Integral peak 2	Integral peak 3	integral peak 4	P1	P2
Encruzado 17	0.41	70.09	28.13		71	29
Alvarinho 17	28.05	54.68			66	34
Viosihno 17	1.48	137.11	34.84		79.05	20.95
Arinto 17	34.21	90.69			72.6	27.4
Moscatel Galego 17	2.49	1.61	179.37	21.24	87.6	12.4
Macabeu 17	21.29				not detectable	
Moscatel of Setubal 17	20.54	641.29	76.04		86.90	13.10
Encruzado 18	11.46	5.40	149.93	16.33	82.00	18.00
Alvarinho 18	0.83	94.18	13.10		87.00	13.00
Viosihno 18	0.29	116.19	16.49		87.30	12.70
Arinto 18	1.69	230.37	92.82		71.0	29.00
Moscatel Galego 18	11.84	6.87	554.73	64.87	87.00	13.00
Macabeu 18	1.11	43.90	17.88		70.00	30.00
Moscatel of Setubal 18	4.95	1.11	187.29		96.90	3.10

TLPs are always the most abundant class of proteins present in all wine samples, achieving in some cases 96.9 % of the total wine proteins, such as the case of Moscatel de Setúbal 2018. The wines with the highest proportion of TLPs, after Moscatel de Setúbal 2018, are Viosinho 2018, Moscatel de Setúbal 2017, and the Moscatel Galego 2018, which represent the most unstable wines in descending order. The Encruzado, despite having a proportion of TLPs lower than other wines, is unstable - In fact is in the same positive part of the plot with the other unstable wines, as shown below in the figure 26.

Other wines, like Moscatel Galego 2017 and Alvarinho 2018, even though containing a high proportion of TLPs, are stable. This is probably due to their low protein content, which makes them stable. This point has a relationship with the test done in this work, for the Moscatel de

Setúbal 2018 and the Macabeu 2017: in the test without the addition of proteins both wines, at low concentration of proteins, turned out to be stable even at high levels of sulfur dioxide.

Moreover, this relationship between the TLPs and wine instability is supported by another PCA analysis, that shown in figure 26. In this latter, the number of components extracted taken in consideration are fifteen.

The distribution of the four wines, delimitated in the pink area, with a high percentage of TLPs and highly unstable, close to the HST variable, is clearly visible, once again demonstrating the influence of this protein class in inducing protein instability.

All the stable wines are located in the green area, and therefore clearly separated from the unstable ones. Is important to underline that the Macabeu 2017 is the wine with the lowest values at the HST and at the same time the lowest concentration of proteins. Being in contraposition at the variable “TLPs proportion” should be the wine with the lower proportion of TLPs, but is not possible to state it, because as previously explained, it was not possible fractionate the total proteins for the low protein content wines.

PCA analysis shows that the proportion of TLPs in wines strongly influences protein stability, especially if present at high concentrations. Therefore, the protein quantity is a fundamental requirement in protein haze. The four wines in the red area have in fact the highest percentage of TLPs, and in addition also the highest protein content.

Regarding the total SO_2 and SO_2 free variables, it seems that in this case, it does not affect the distribution of wines in this PCA analysis. The cause of this is to be found in the fact that all the wines present more or less the same concentration of total sulfur dioxide, and from the fact that, according to the results obtained in this work, the sulfur dioxide has a role in inducing substantial differences in protein instability for higher values such as 150-200 mg/ L of total sulfur dioxide. The latter are values not present in the analyzed samples, but often can be found in commercial wines, especially in young wines after bottling.

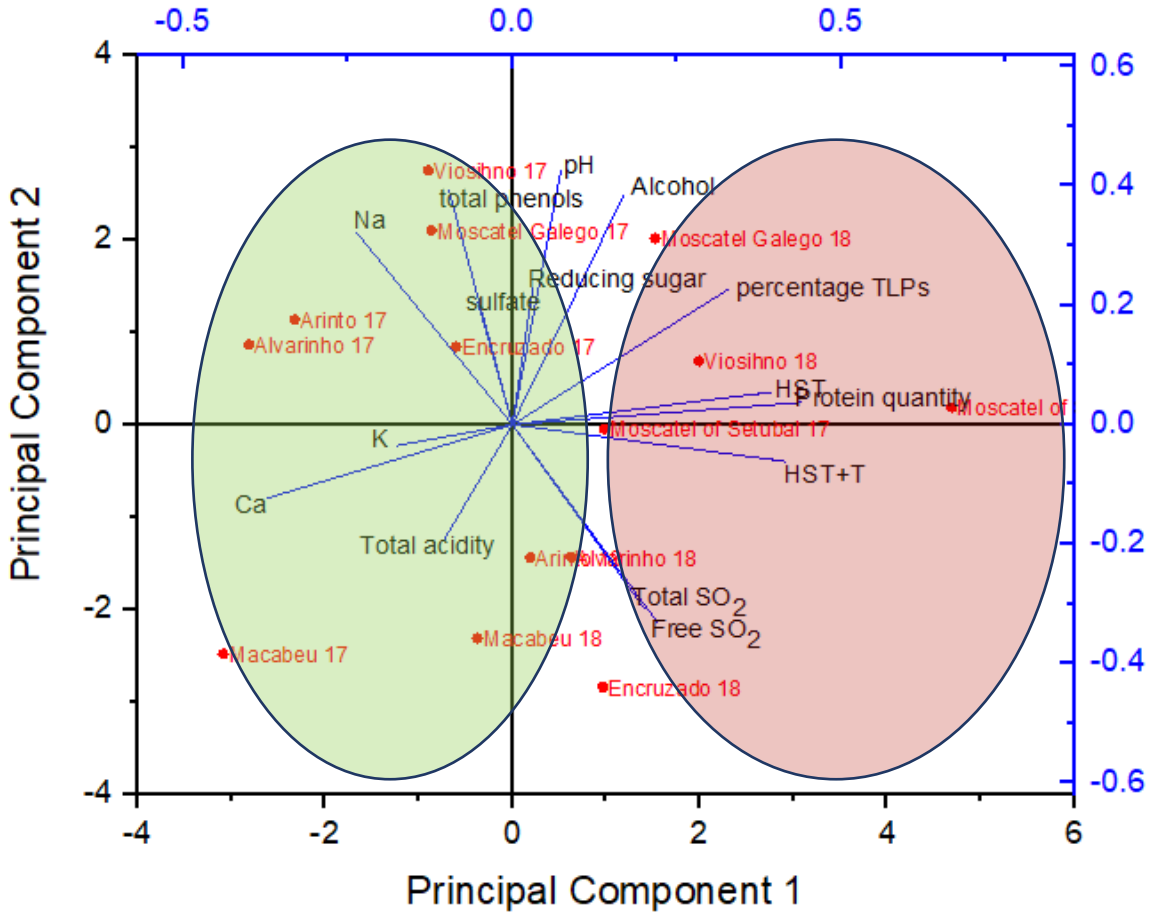


Figure 26. *PCA of all wines analyzed with some selected variables. The variables are the ones that affect the most protein instability. The cumulative percentage of the variance of PC1 and PC2, in this case, is 89.67%.*

4. Conclusions

Protein haze formation in white wines has been studied for many decades and many types of research have been carried out in this regard, in an attempt to define the bases of this defect and to prevent it. Although as previously mentioned, is a multifactorial process, which has not yet been completely clarified.

In several studies, many authors have tried to identify potential factors involved in this process, often using extreme concentrations of such factors in their tests, so as not to make it clear whether the effect on the protein instability, if produced, resulted from the “factor x” studied or from the extreme concentration of it.

Starting from the work of Chagas *et al*. (2016), in which it was shown how the presence of sulfur in musts and wines could be implicated in the hazing process, inducing protein aggregation once the wine is exposed to a thermal stress, an attempt was made in this work to study the synergistic effect on the protein turbidity of the co-presence of sulfur dioxide and of particular proteins such as TLPs.

From the results obtained in this work, it is possible to state that by increasing the concentration of proteins in wines, stable wines become highly unstable. This result agrees with the fact that the presence and above all the concentration of proteins is the first requisite for haze formation in white wines. Furthermore, from the analysis of the HST, protein content, and the protein profile of wines obtained by HIC it is possible to say that wines with a high proportion of TLPs but low concentration of proteins were more stable when compared to wines that had about the same proportion of TLPs but a higher protein content – Meaning a higher TLP wine concentration.

The combined effect of protein and sulfur dioxide increment was also evaluated in two wines that were initially stable. From this test, significant differences in the increase in turbidity were evaluated when sulfur dioxide was increased for the same amount of protein added in the wine.

From this we can state that sulfur dioxide not only has a reducing action in wine but probably also, as already claimed by Marangon *et al*. (2014) and by Chagas *et al*. (2016), induces the aggregation of proteins under some conditions. From the results of this work is possible to state that the ulfur dioxide has an action in the protein haze when present in the wine at high concentrations (e.g. 120-200 mg/L), a range commonly found in commercial wines.

Furthermore, since the added proteins comprised 87-96% TLPs, this could explain that the major increases in protein turbidity are due to this protein class. Like Chagas *et al.* (2018) have demonstrated, the presence of sulfur dioxide induces in the TLPs a modification of the

torsion angle of the same proteins, making them more susceptible to aggregation. This would explain the difference between the test in which there is an increase in protein, keeping the concentration of SO_2 constant, and the one where instead an increase in sulfur dioxide has produced an appreciable increase in turbidity visible to the naked eye, as confirmed by the results of the HST.

Protein haze formation is a complex process, deriving from multiple factors and therefore from the different composition of the wine matrix. In summary we can say that sulfur dioxide is certainly one of the main causes of this instability, especially when in the presence of a high concentration of TLPs.

5. References

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