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PROTEOME-METABOLITE INTERACTIONS IN WINE.

TRACKING DOWN THE X FACTOR.

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ABSTRACT

Protein haze formation is a recurrent problem in white and *rosé* wines. Formation of his type of haze is dependent on only for the protein content of the wine, but is strictly related to one or more non-proteinaceous wine components termed as X factor. A selected wine from the variety Moscatel of Alexandria was fractionated using a preparative RP-18 chromatography column in eight methanolic fractions that were subsequently subjected to heat stability test with wine isolated protein in model wine solution. The results obtained indicate that the X factor is not a single compound, but rather an interaction of several compounds with wine proteins. A heat stability test involving the combination of four of these fractions with an isomer of the major compound identified in the water soluble fraction, the erythritol, presented the higher haze formation. Nuclear magnetic resonance (NMR) and mass spectrometer (GCT) analysis allowed the identification of two compounds present in the methanolic fractions. As a whole, this work constitutes a step forward in the difficult process of purification and identification of the wine non-proteinaceous components that modulate the wine protein haze formation.

Keywords: protein haze, wine, X factor

RESUMO

A casse proteica é, ainda hoje, um problema recorrente em vinhos brancos e rosés. Esta casse é causada não só pelo teor em proteína existente nos vinhos mas está, também, estritamente relacionada com um ou vários compostos não-proteicos genericamente designadas por factor X. Foi seleccionado um vinho monovarietal da casta Moscatel de Alexandria, que foi posteriormente fraccionado numa coluna de cromatografia preparativa RP-18 em oito fracções metanólicas. Após testar a estabilidade ao calor destas fracções, conclui-se que o factor X não é um composto, mas sim uma interacção de vários compostos com a proteína existente no vinho. A fracção que apresentou maior turvação foi a combinação de cada uma das quatro fracções isoladas na fracção solúvel em metanol, com um isómero do composto maioritário isolado na fracção solúvel em água, o erythritol. Análises às fracções metanólicas que provocam turvação proteica por ressonância magnética nuclear (RMN) e espectrometria de massa (GCT) permitiram identificar dois compostos maioritários. Os resultados obtidos no presente trabalho representam um avanço na purificação e identificação dos compostos não-proteicos que modulam a formação de casse proteica nos vinhos.

Palavras-chave: casse proteica, factor X, vinho

RESUMO ALARGADO

A casse proteica é, ainda hoje, um problema recorrente em vinhos brancos e rosés. Esta casse é causada não só pelo teor em proteína existente nos vinhos mas está também estritamente relacionada com um ou vários compostos não-proteicos genericamente designados por factor X. Apesar das evidências do envolvimento estrito de um factor não-proteico na modelação desta casse, nenhum componente específico ou condição foi identificada como sendo a principal causa deste fenómeno.

Com o objectivo de isolar os compostos que interactuam na casse proteica, foi seleccionado um vinho monovarietal, da casta Moscatel de Alexandria, com elevado teor em proteína sendo posteriormente fraccionado. A fracção <3 kDa do vinho, reconhecida como tendo na sua constituição os compostos que interactuam na casse proteica (Batista *et al*., 2009), foi separada em dois extractos, um solúvel em metanol e outro em água. O extracto metanólico foi posteriormente fraccionado numa coluna de cromatografia RP-18 preparativa obtendo-se uma fracção solúvel em água e uma fracção solúvel em metanol. Após análise por NMR, identificou-se o composto maioritário da fracção aquosa como sendo o composto erythritol. Após novos testes de estabilidade ao calor, registou-se que, por si, o composto L-threitol, isómero do composto erythritol, em solução modelo (12% v/v etanol, 4 g/L ácido tartárico) com proteína isolada de vinho (250 mg/L proteína isolada de vinho branco) não provocava um aumento da turvação.

A segunda extracção de metanol foi então eluida uma vez mais numa coluna de cromatografia RP-18 preparativa desta vez com um gradiente de metanol entre 10 e 100% (v/v) de metanol, onde se obteve a separação de oito diferentes fracções. Após testar a estabilidade ao calor destas oito fracções em solução modelo com proteína isolada de vinho, concluiu-se que o factor X não é um composto, mas sim uma interacção de vários compostos com a proteína existente no vinho. O teste que apresentou maior turvação foi a combinação de cada uma das quatro fracções mais polares isoladas da fracção solúvel em metanol, com o isómero do composto maioritário isolado na fracção solúvel em água, o L-threitol. Análises de RMN e GTC permitiram a identificação de dois compostos maioritários presentes em duas das fracções do extracto metanólico que interage com a turvação proteica e que ainda não estão descritos na bibliografia.

Os resultados obtidos no presente trabalho representam um avanço na purificação e identificação dos compostos não-proteicos que modulam a formação de casse proteica nos vinhos.

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LITERATURE REVIEW

Introduction

Wine has a history of more than 7500 years of existence. The first evidence of wine production appears in the representations of wine presses from the reign of Udimo, 5000 years ago (Petrie, 1923). Nowadays, wine is a beverage with great importance in the human diet, with an annual consumption of around 240 ML of liters (OIV, 2006).

The wine, like all fermented beverages, has a significant nutritional value. This nutritional value is directly proportional to the content in carbohydrates and alcohol produced in the alcoholic fermentation of the fruit or cereal. In wines, carbohydrates and alcohol are the major components, followed by organic acids, mineral matter, phenolic compounds and nitrogen compounds (Ribéreau-Gayon *et al*., 2006a). The identification of wine complex matrix has only been possible thanks to the evolution of technologies but there are some molecules, mainly minority compounds, whose structure has not been elucidated.

The nitrogen compounds supply the required building blocks for yeast protein biosynthesis. In addition, they directly contribute to the flavour of food and are precursors for aroma and compounds formed during thermal or enzymatic reactions in production, processing and storage of food (Belitz *et al*., 2004).

The wine nitrogen compounds, mainly proteins, are not directly related to the nutritional value of the beverage because of its low content, about 15 to 300 mg/L (Ferreira *et al*., 2002; Waters *et al*., 2005). However, they assume great importance in terms of technology, economy and sensorial evaluation of the wine. The nitrogenous components play an important role in the vinification process, since nitrogen is an essential nutrient for yeast in winemaking. Lack of nitrogen is one of the main factors limiting yeast growth and sugar attenuation (Nernández-Orte *et al*., 2006). These compounds also influence clarification and microbial instability. They may affect the development of wine aroma, flavor (Bell *et al*., 2005) and foam characteristics in sparkling wines (Moreno-Arribas *et al*., 2000; Marchal *et al*., 2006). From the degradation of these nitrogen compounds, some metabolic byproducts may be produced which are considered detrimental to human health, e.g. ethyl carbamate, biogenic amines (Zoecklein *et al*., 1999; Bell *et al*., 2005).

The effect of a low level of nitrogen-containing compounds in grape juice on slow and stuck fermentation is perhaps the most widely studied (Siler & Morris, 1996; Mendes-Ferreira et al., 2007a, 2007b). Nitrogen-containing compounds in grape juice and wine are made up

of an ammonia component and a more complex amino-acid based component, e.g. free amino acids, oligopeptides, polypeptides, proteins, amide nitrogen, bioamines, nucleic acids, amino sugars, pyrazines, vitamins and nitrate (Henschke *et al*., 1991; Ough *et al*., 1991; Zoecklein *et al*., 1999)

Proteins in must and wine may have different origins, such as grape berry, yeasts, bacteria, and fungi (Dambrouck *et al*., 2003). Such proteins are of great interest, not only because they are the major nitrogen compounds in wines but also due to the fact that they are involved in a technological turbidity problem, usually called protein casse.

The protein casse is a problem that occurs in white wines, mainly due to temperature abuse, which consists in the flocculation of hydrophobic colloids. The denatured proteins can precipitate forming amorphous sediments, or flocculate producing foam and visible haze in bottled white wines (Waters *et al*., 1991; Ferreira *et al*., 2002). Red wines hardly contain any free proteins, as they are precipitated by tannins. White and rose wines, on the other hand, may have variable protein concentrations of up to a few hundred mg/L (Ribéreau-Gayon *et al*., 2006a).

Post-bottling haze formation in white wines is a prevailing problem that affects ,many wines from different regions. Some of these hazes, like protein haze, are not well understood and, in most cases, is associated with a decrease in the quality of wine or microbiological modification (Waters *et al*., 1999). According to the standard ISO 9000, the quality of a product is “the degree to which a set of inherent characteristics fulfills requirements”. Being the clarity of the product an inherent characteristic, protein haze in wine can decrease the quality of the product and consequently lead to a rejection of the product by the consumer. Consumers usually reject wines that are cloudy or show any type of material in suspension, regardless of the flavor that the wine can have. This happens because this haze gives the impression of microbiological problems in the wine (Waters *et al.*, 1999). As such, wines ready for dispatch, must remain clean and stable, irrespective of the storage conditions (Ferreira *et al*., 2002).

Being a natural component of wines, proteins have been known, for many years, to cause haze in wine. The first documented studies about protein haze in wines are dated back to 1896, by G. Colby (Colby, 1896). However, the first studies on the nature of the compounds that cause haze in wines appeared later (Moretti *et al*., 1965; Feuillat 1974), based on the quantification of the total nitrogen compounds found in the wine using the Kjeldahl method. The protein content was estimated using a conversion factor, to exclude the nitrogen present in other non-proteinaceous nitrogen-based compounds.

Nowadays, it is not yet fully established the protein limits to which wines are considered stable to protein haze formation. Moreover, the nature of haze-responsible proteins is still controversial. Increasing evidence suggests that non-proteinaceous factors play a fundamental interaction in the formation of protein haze in wines.

The protein content of a wine, in particular a sparkling wine, has a significant importance in the quality of the produced foam. Wine proteins are hydrophobic and, thanks to their isoelectric point and molecular weight, they have the potential to form foams (Brissonnet *et al*., 1993). This has a significant importance in the production of sparkling wines, e.g. Cava, Champagne, because of the positive relation between wine protein content and foam quality of the wine (Feuillat *et al*., 1988; Silva *et al*., 1990; Brissonnet *et al*., 1991; Malvy *et al*., 1994).

Grape proteins

Comparing to other fruits, the total soluble protein profile of grapes is relatively simple. After analysis by 1-D electrophoresis, a majority of proteins with low molecular weight is revealed (Yokotsuka *et al*., 1983; Hsu *et al*., 1987; Murphey *et al*., 1989; Pueyo *et al*., 1993). Great difficulty has been demonstrated for the extraction of grape proteins at several maturation stages. Nevertheless, a significant increase in protein content of grapes after veraison has been shown, due to a small synthesized in significant quantities (Tattershall *et al*., 1997). These proteins, synthesized during grape maturation, are mainly PR ("*Pathogenesis-Related*") proteins, including chitinases and thaumatine-like proteins (Tattershall *et al*., 1997), which have molecular weights of 32 and 24 kDa, respectively (Robinson & Davies, 2000). These are the major proteins synthesized during all maturation stages of the grape following veraison (Pocock *et al*., 2000) and act as a defense mechanism for the plant against pathogen agents (Van Loon, 1985; Somssich *et al*., 1998; Odjakova *et al*., 2001). However, other authors, using N-terminal sequencing analysis, identified the principal proteins present in ripen grapes from Moscatel of Alexandria (*Vitis vinifera*) as thaumatin-like and osmotin, failing in finding any chitinase in the samples analysed(Monteiro *et al*., 2003). The expression and accumulation of these protein families are determined by the environmental and pathological conditions at which the grapes are exposed during the maturation period (Monteiro *et al*., 2003).

The group of grape PR-like proteins is very similar to the classic PR proteins, but they include some homologue proteins that are synthesized constitutively in a specific plant tissue during its development (Van Loon, 1999). These proteins are mainly basic and are located

inside the vacuole of the cell (Linthorst, 1991). Their location, as well as their differential induction by both endogenous and exogenous compounds and other stimuli, suggests that this type of proteins can play an important role in the plant, besides the protection effect against pathogen attacks (Van Loon, 1999).

Some of the 17 groups of known PR proteins were found in grapevine, each one with different composition and function (Van Loon, 1999; Ferreira *et al*., 2007). The groups found in vines comprise PR-5 proteins, including thaumatine-like proteins and osmotines, PR-2 (β-1,3-glucanases), PR-3 and PR-4, that are the group of chitinases. The PR-5 group is known for creating transmembranar pores, the PR-2 degrade β-1,3-glucans and the PR-4 group degrade chitin (Ferreira *et al*., 2004). Such kind of activities is what gives these proteins their antifungal properties, since they act upon fungal structural features.

Several studies were made to elucidate the induction mechanisms of the PR proteins in grapevine and, consequently, their accumulation during the maturation process (Tattersall *et al*., 2001). PR proteins are the predominant proteins during all maturation stages after veraison (Pocock *et al*., 2000), with their concentration gradually increasing until harvest. This fact can indicate that the haze potential of the wine made with these grapes may increase along the maturation period.

PR proteins can be induced in grapes and leaves during their development, acting as part of the defense mechanism of the plant against factors that normally induce their synthesis: lesions, chemical elicitors, pathogen attacks or abiotic tensions (Jacobs *et al*., 1999; Robinson *et al*., 2000). One example of this is provided by the fungus *Eryshiphe necator*, the casual agent of powdery mildew, which induced an increase in thaumatin-like proteins in infected grape berries, when compared to healthy grapes (Monteiro *et al*., 2003). The work of Monteiro *et al.* (2003) also showed that PR proteins present in grape berries exhibit antifungal *in vitro* activity against some fungus that normally attack vines. These results corroborate the principal function of this type of proteins *in vivo*, i.e. the protection of the plant against fungal invaders.

The type of grape harvest (i.e. manual or mechanical) can influence the final content of protein that will be present in the must. The mechanical harvest originates typically musts that possess higher content of proteins compared to handpicked clusters (Paetzold *et al*., 1990). This happens due to the presence of grape stems during the pressing operation with the handpicked clusters, unlike the mechanical harvest, in which grape stems are not present. Grape stems contain a high content in phenolic compounds, which are going to enrich the must with this type of compounds (Ribéreau-Gayon *et al*., 2006a). The higher content of phenolic compounds in must leads to a higher complexation and precipitation of

the proteins present in the must (Paetzold *et al*., 1990). However, the mechanical harvest associated with delays in the transportation of grapes from the vine to the winery can lead to musts with even PR proteins content (Pocock *et al*., 1998). This phenomenon occurs thanks to high temperatures in the transportation bins, coupled with skin maceration from broken berries. This causes nitrogenated substances to dissolve more readily from the skins and seeds (Ribéreau-Gayon *et al*., 2006). These musts lead to wines that had to be fined with higher doses of bentonite (almost two times more) in order to assure protein stability, compared to wines made with grapes from the same vine but handpicked.

Wine proteins

Determination of total soluble protein composition of wines is an actual investigation area where the development of new analytic techniques is directly associated with its better characterization. Initially, denaturing electrophoresis was used to separate the proteins on basis of their molecular weight. Four distinct bands were initially observed indicating four different proteins, varying in concentration among them according to the different varieties studied (Moretti *et al*., 1965; Bayly *et al*., 1967). Newer techniques enabled a better characterization of proteins, like ion-exchange chromatography using FPLC (Waters *et al*., 1992; Waters *et al*., 1993; Dorrestein *et al*., 1995); FPLC with chromatofocusing (Dawes *et al*., 1994); molecular exclusion chromatography (Pellerin *et al*., 1993); affinity chromatography (Pellerin *et al*., 1993; Waters *et al*., 1993; Dizy *et al*., 1999); HPLC (Tyson *et al*., 1981; Santoro, 1995); and isoelectric focusing (Hsu, *et al*., 1987). The purification, separation and characterization of proteins present in musts and wines are a complex work, due to their low concentrations and their interaction with wine non-proteinous compounds like phenolic compounds. Hsu *et al*. (1987) used polyvinylpirrolydone (PVPP) and Amberlite XAD-4 to remove the phenolic compounds present in a sample wine in order to study the wine proteins. In this way, several fractions were found with molecular weighs between 11.2 ans 65 kDa. However, the predominant proteins in wine have low molecular weight (between 20 and 30 kDa), low isoelectric points (4,1< pI < 5,8) and are positively charged at the pH that normally occurs in this type of beverages (Hsu *et al*., 1987; Brissonnet *et al*., 1993; Ferreira *et al*., 2000). Other studies, involving Australian wines, revealed that the isoelectric point of the principal proteins present in wines had values between 4.8 and 5.7 (Lee, 1986).

Protein content of wines is usually lower than protein content present in musts. This difference is related to the proteolytic activity manifested during the vinification process,

precipitation due to polyphenols and to unfavorable wine conditions, such as the low pH and alcohol content (Sauvage *et al*., 2010). However, the major proteins remaining in wines are PR proteins thanks to their stability at low pH and high resistance to proteolysis (Linthorst 1991). During the vinification process, vacuolar acids and hydrolytic enzymes are released to the must precipitating and/or degrading many proteins present in grapes, thus favoring the predominance of PR proteins (Ferreira *et al*., 2002). The combination of all these factors ensures that only the resistant proteins, in this particular case PR proteins, resist to the vinification process, making them the principal precursory for protein haze formation in white wines (Waters *et al*., 1996; Waters *et al*., 1999; Ferreira *et al*., 2002).

The proteolytic activity during the vinification process is intensified during fermentation, thanks to secreted proteolytic enzymes from non-*Saccharomyces* yeasts (Lagace *et al*., 1990) present in the early stages of fermentation.

Non-proteinaceous factors that interact with wine protein to form haze

As summarized in previous sections, the proteins responsible for wine protein haze are known as PR proteins. These proteins have their origin in grapes, have the ability to resist to all vinification process and, if not removed, can precipitate after bottling. Although PR proteins are the major compounds of the precipitate when haze occurs, some authors suggest that other non-proteic compounds may be involved in the wine protein haze mechanism (Pocock *et al*., 2007). This thesis corroborates the results obtained by some authors (Moretti *et al*., 1965; Bayly *et al*., 1967) which suggested that the total protein content, by itself, failed to correlate with wine heat instability.

Despite the present evidence for the absolutely required involvement of a non-proteic wine component in the protein haze formation, no particular component or condition has yet been identified. Nevertheless, the ability of such components and factors to modulate the intensity of heat-induced haze is widely described in the recent bibliography.

Phenolic compounds

Of all non-proteinaceous factors that may be involved in wine protein haze, phenolic compounds are the most studied and substantial evidence exists to propose that their interactions with proteins are significant (Waters *et al*., 2005). Koch & Sajak (1959) were among the first investigators to determine that isolated grape proteins were associated with

tannins. Sieber *et al.* (1998) showed that protein haze formation in beer and apple juice could decrease using PVPP (a fining agent used to remove polyphenols) to remove haze active polyphenol. Pocock *et al*. (2007) added PVPP to white wines and observed that the removal of phenolic compounds from the wine turned it more stable when submitted to heat stability test, confirming previous tests performed by other authors (Yokotsuka *et al*., 1983; Waters *et al*., 1995). In fact, Pocock *et al*. (2007) concluded that all the wines fined with PVPP presented lower haze formation after heat stability test, when compared to unfined wines.

Yokotsuka *et al.* (1983) tested high doses of isolated white wine tannins with isolated must proteins, showing that protein-polyphenol interaction can cause haze formation. The same authors also found that the monomeric phenolic fraction did not interfere in the haze formation. Despite these results, the tannin content used in the tests do not reflect the normal content of tannins present in white wines, an observation which questions some of the work conclusions.

Marangon *et al*. (2010) showed that haze formation in wines seems to be related to hydrophobic interactions occurring between proteins and tannins. These interactions should occur on hydrophobic tannin-binding sites, whose exposition on the proteins can depend on both protein heating and reduction. They also hypothesized that, during the time after bottling, the decrease of the wine redox potential together with temperature fluctuations during storage, could cause the exposition of hydrophobic binding sites on wine proteins available for tannin complexation, resulting in haze formation during storage of white wines.

Polysaccharides

Polysaccharides are polymers containing more than ten monosaccharide units linked by glycosidic bonds, which may be linear or branched structures (Murray *et al*., 2003). Pellerin *et al*. (1994) tested 15 different polysaccharides of different origins, concluding that they either did not affect or increased haze during heat test. Mesquita *et al*. (2001) showed that a particular fraction containing polysaccharides as major compounds increased the protein instability in wine, in particular, at a range of temperatures between 40 and 50 ºC. A multifactorial study revealed a particular kind of polysaccharide, pectin, to be important in haze formation (Fenchak *et al*., 2002). Pectin represents from 0.02 to 0.6% of the fresh grape weight but, due to the recurrent use of commercial enzymes, a large number of these polymers are hydrolyzed. These enzymes, with principal origin on grape or those added by the winemaker, are mainly pectinases, polygalacturonases, cellulases and hemicellulases, which main function is on the colloidal structure of the juice, facilitating natural settling (Ribéreau-Gayon *et al*., 2006). Despite its importance in haze formation in musts, pectin

also is active as a protective colloid, inhibiting the growth of nuclei and crystallization of potassium bitartrate (Ribéreau-Gayon *et al*., 2006).

Yeast-derived mannoproteins are other polysaccharides that have an effect in protein haze formation. Waters *et al.* (1993) described the effect of this "haze protective factor" as an exciting prospect for preventing protein haze formation in white wines. Quirós *et al*. (2010) showed that wines produced with three transgenic wine yeast strains, deleted for genes involved in cell-wall biogenesis causing them to release increased amounts of mannoproteins, required 20 to 40% less bentonite to assure protein stabilitazion than those made with their wild-type counterparts.

Metal ions

Metal ions, in particular copper and iron, are known to be a non-proteic haze origin. However, their role in protein haze formation is poorly understood. Besse *et al.* (2000) reported that the concentration of copper in wine decreased after heat treatment and protein haze removal, suggesting that copper is part of the protein precipitate. In view of the involvement of a protein support in the colloid flocculation occurring in copper casse in white wine, bentonite may be used to treat this problem. However, ferric casse has no proteins involved, so bentonite is ineffective (Ribéreau-Gayon *et al*., 2006).

pH

The effect of pH on protein haze formation is incompletely studied and the existing published work focuses mainly in other beverages like beer. Siebert *et al*. (1996) studied the effect of pH on the formation of protein-polyphenol complexes in wine, but the use of white wines non-characteristic proteins and polyphenols (i.e. gelatin and catechin respectively), turned out questionable the significance of these results. Mesquita *et al*. (2001), using wine samples instead of wine model solutions, showed that white wine became increasingly heat stable as the pH rose from 2.5 to 7.5. This demonstrated that wine proteins gradually became more stable to heat with increasing pH, suggesting that pH does play an important role in protein haze formation. However, Batista *et al.* (2009) indicated the existence of at least two different mechanisms responsible for the heat-induced precipitation of the white wine proteins: one occurring at higher pH values, that appears to result mainly from the isoelectric precipitation of the wine proteins and the other, at lower pH values (but possibly operating also at other pH values), that depends on the presence of a non-proteinaceous factor, known as the X factor.

Ethanol

The alcohol content of white wines can present a significant variability. This is an important factor since, due to climate change, the alcohol levels in wines from regions like

Alsace, Australia or Napa are increasing (Jones, 2007). Mesquita *et al*. (2001) studied the interaction between alcohol content and protein haze formation, demonstrating that the addition of extra alcohol to white wine samples (0.5, 1 and 2% v/v), had no influence on it. These results corroborate the data presented by Siebert & Lynn (2003) which showed, in the particular case of beer, that alcohol concentration had little influence in haze formation at the normal pH of the beverage.

Organic acids

Organic acids constitute another factor with great interest in what concerns protein instability. Batista *et al*. (2010) tested five different organic acids (L(+)-tartaric, L(-)-malic, citric, succinic and gluconic acids) and analyzed their effect on wine protein haze potential. The results indicate that these acids induce a stabilizing effect upon the haze potential of wine proteins at all pH values tested from 2.8 to 3.8. The same work raised the hypothesis that organic acids, carrying a net negative electric charge at wine pH, interact electrostatically with the wine proteins, positively charged at the wine pH, from pH 2.8 through to pH 3.8, preventing the interaction of the X factor with the wine protein. The tartaric acid is also capable of interacting directly with the X factor, either in the presence or absence of protein, removing it from solution in the form of a tartrate crystal precipitate and, consequently, stabilizing the wine.

Based on these results, organic acids are another factor that needs to be considered as involved in wine protein haze formation, supporting at the same time the existence of the X factor.

Oenological practices to prevent turbidity due to protein precipitation in white wines

Clarity is an important aspect of a consumer first contact with a wine. If the wine presents some kind of haze, probably, that bottle will be rejected, leading to prejudice to the winery that produced that beverage. To avoid turbidity in white wines due to protein precipitation, the oenologist needs to test the wine haze potential and treat the wine if necessary.

Testing the wine for protein haze

To understand the haze potential of a wine there are some tests and commercial kits available. Among all the kits, the most used one is Bentotest, followed by Proteotest, Prostab and, recently, Immuno Test π.

Bentotest is a commercial test kit developed by Jakob (1962), which uses a solution of phosphomolybdic acid prepared in hydrochloric acid to denature and precipitate wine proteins. The precipitation obtained with this test is proportional to the protein content of the wine, and it can be used to determine the bentonite addition levels required for protein stabilization (Zoecklein, 1991).

Proteotest is a test that involves the addition of highly reactive tannins to the wine. The over excess tannin content forces the protein in the wine to precipitate, ensuring the formation of haze. The propensity of the wine to produce haze is measured in a nephelometer, comparing the initial haze and the haze after addition of the tannins (Lankhorst *et al*., 2009).

Prostab (Martin Vialatte OEnologie, Epernay, France) is other commercial kit available, but there is no information about its mechanism of action.

The Immuno Test π is the most recent commercial kit, developed in 2005. This kit differs from the previous thanks to its selectiveness to the haze responsible proteins using an immunological assay (Sofralab, 2008).

Under laboratory conditions, there are some possible assays to foresee the protein haze potential in wine with chemicals. These include precipitation of protein using ethanol, ammonium sulfate, trichloroacetic acid, phosphomolybdic acid, phosphotungstic acid and tannic acid (Zoecklein, 1991). Most of these precipitation tests are much more severe than heat tests, causing a denaturation and precipitation of all protein fractions (Zoecklein, 1991).

Esteruelas *et al*. (2009) made some research comparing several of these methods (including Bentotest, Prostab, slow heat test, fast heat test, ammonium sulfate, TCA, tannins and ethanol) and concleded that the generated precipitates obtained with the kits are very different from the natural precipitate of the wine.

The fast heat test (40 mL wine subjected to a temperature of 90 °C for 1 h in a thermostatic water bath, followed by a incubation of 4 °C for 6 h in a refrigerator (Sarmento et *al.*, 2000)) appears to be the most similar to the natural precipitate in terms of chemical composition, indicating that this test may be the most appropriate stability test (Esteruelas *et al*., 2009).

Treating the wine to avoid protein haze

One of the most effective ways to treat a wine in order to avoid protein haze formation is fining. Fining consists in the addition of a substance to the wine that captures the particles responsible for turbidity or instability in the wine, thus clarifying and stabilizing it (Ribéreau-Gayon *et al*., 2006a). The principal way to eliminate protein from white wines is to perform a fining with bentonite, along with immobilized tannic acid, cross-flow membrane ultrafiltration and ion-exchange resin adsoption (Sun *et al*., 2007).

Bentonite is mainly composed of montemorillonite and is negatively charged thanks to some of the Al^{3+} ions present in the octahedral positions being displaced by Mg^{2+}, Fe^{2+} and Fe^{3+}, leading to charge imbalances (Brindley, 1984). This clay interacts electrostatically with positive charged wine proteins because of its net negative charge at wine pH, which produces flocculation (Hsu *et al*., 1987). Due to its non-selectiveness for proteins, fining with bentonite interferes negatively with the organoleptic characteristics of the wine since it adsorves other molecules with positive electric charge (Miller *et al*., 1985; Rankine 1989; Voilley *et al*., 1990). Besides its effectiveness to remove protein from wine, it has been demonstrated that bentonite treatment negatively affects the flavor (Lubbers *et al*. 1996) and texture (Guillou *et al*. 1998) of wine. However, recent studies indicate that the effect of bentonite treatments on aroma substances in white wine depends on the chemical nature and initial concentration of the volatile compounds and on the abundance and nature of proteins in the wine (Lambri *et al*., 2010).

Hydrating the bentonite before fining the wine increases its efficiency due to the separation of its layers and, consequently, increments its specific area (Weiss *et al*., 2001), but it may be that the polypeptides are primarily absorbed near the edges of the bentonite sheets rather than within the interlayer spaces between the sheets (Gougeon *et al*., 2003).

Another problem arising from the application of bentonite is the volume of formed lees and, consequently, the volume of wine that is lost after fining. Between 5 and 20% of the wine may stay occluded in the lees after a fining treatment (Lagace *et al*., 1990; Tattersall *et al*., 2001) and, after a possible rotary vacuum drying systems (RDV) filtration, this occluded wine may be downgraded in quality comparing to the fined wine mainly thanks to oxidation phenomena (Waters *et al*., 2005). The handling and disposal of used bentonite continues to be a great problem due to high labour input, associated costs, safety issues and ambient impact, given the high content of ethanol and phenolic compounds present in the lees (Waters *et al*., 2005; Musee *et al*., 2006).

There has been several attempts to stabilize white wine using continuous processes. Studies using immobilized tannic acid were effective in removing proteins and tannins

without affecting the level of peptides and the acidity of the wine. On the other hand the high cost of the method could make it not viable (Weetall *et al.,* 1984).

Other options for continuous process stabilization were tested, like packed columns or percolated beds (Sarmento *et al*., 2000). Studies of packed columns with ion-exchange resins (Sarmento *et al*., 2001) showed that the level of polyphenols and proteins decreased, but the color and aroma of the wine were affected.

Metal-oxides (Fukuzaki *et al*., 1996; Pachova *et al*., 2002) and zirconia (Pashova *et al*., 2004) were tested as well with model wine solutions. There was some organoleptic impact on the wine treated with the metal-oxides, and no information about the zirconia treated wine. After analysis, neither treatment significantly affected the physicochemical properties of the final product.

The removal of specific proteins from the wine may be one of the ways of stabilizing it, but several factors in its matrix may derail some technologies. One example is immobilized antibodies. They can be specific to remove certain proteins from the wine, but due to the low wine pH, the interaction antibody-antigen is compromised (Ferreira *et al*., 2002). The immune test π described in the previous section uses antibodies for specific proteins but the test solution raises the pH of the wine to usable values where the antibody-antigen interaction is assured.

Using molecular biology techniques it may be possible to silence the protein PR expression genes in the vines, leading to wines without protein but turning the vine susceptible to fungus and other tensions (Ferreira *et al*., 2004).

Pre-fermentative clarification is other technique used in winemaking that promotes the production of higher quality wine. The most used clarification method is natural settling that consists in the natural settling of the suspended solids followed by careful racking (Ribéreau-Gayon *et al*., 2006b). Clarification has been known to improve the fermentation aromas of white wines (Crowell *et al*., 1963; Bertrand,1968; Ribéreau-Gayon *et al*., 1975), removing suspended solids that have heavy, green aromas and biter tastes (Ribéreau-Gayon *et al*., 2006b). About 2.6% of the lees removed in the clarification operation consists of nitrogen compounds (Alexandre *et al*., 1994), indicating that some proteins can be removed from must in this operation. To increase the yield in the clarification, some adjuvants may be added sush as bentonite, enzymatic preparations, or activated charcoal. The most effective of these adjuvants for removing protein in the clarification is bentonite, but it can damage the organoleptic quality of the wine if the wine is maintained in contact with its lees for several months (Ribéreau-Gayon *et al*., 2006a).

Enzymatic preparations, mainly pectinases, can be added to the must during the clarification operation. The objective is to reduce the content in pectic substances of the must that difficult the settling of the suspended solids. These pectic substances are complex heteropolysaccharides with origin in the plant cell walls (O'Neill, 1990), which represent from 0.02 to 0.6% of fresh grape weight (Ribéreau-Gayon *et al*., 2006a). These substances are present in colloidal form, protecting the must proteins and difficulting their removal. In general, pectic substances increase the haziness and viscosity of the must (Grassin, 1992), causing clarification and stabilization problems (Feuillat, 1987). The commercial pectolytic enzyme preparations may contain some proteases in their composition, but they are not the majority and their action is not selective. The musts treated with this type of commercial enzymes show greater clarity (Lao, et al., 1996) and in some barrel fermented wines the addiction of these enzymes lead to the production of superior wines with higher levels of alcohols, esters and terpenic compounds (Aleixandre, et al., 2003). Regarding the eventual degradation of wine proteins that interact in the protein haze formation by these commercial enzymes, there are no positive effects described in the literature.

Protease activity has been reported in grape berries (Cordonnier & Dugal, 1968), wine yeast (Charoenchai *et al*., 1997; Dizy *et al*., 2000) and in malolactic transformation bacteria *Oenococcus oeni* (Leitão *et al*., 2000), but because of their low specifity, they have low activity towards haze-forming proteins. A heat treatment combined with the addition of proteases can reduce the incidence of haze formation but, once more, because of the low specifity of the commercially available proteases, the possibilities offered by this method are compromised (Pocock *et al*., 2003).

A number of different proteolytic enzymes are produced by yeasts (Klar *et al*., 1975; Barrett *et al*., 2004). *Saccharomyces cerevisiae* is the principal yeast responsible for the alcoholic fermentation of grape must (Ribéreau-Gayon *et al*., 2006a), and its secreted proteolytic system is quite complex, consisting of carboxypeptidases, aminopeptidases, proteinases and several specific inhibitors (Béhalová *et al*., 1979). Some studies revealed the presence of extracellular acid protease production among various species of *Saccharomyces* (Bilinski *et al*., 1987; Rosi *et al*., 1987; Conterno *et al*., 1994; Moreno-Arribas *et al*., 1996; Iranzo *et al*., 1998), but the main part of the yeast proteases, in particular of *S. cerevisiae*, are intracellular and located in various compartments (cytosol, vacuole, mitochondria, endoplasmic reticulum, and Golgi complex) and cellular membranes of the cell (Klar *et al*., 1975; Barrett *et al*., 2004). After cell lysis and death, these proteases can be released to the surrounding medium where they may retain activity. Of the many cellular proteases present in yeasts, the vacuolar acid protease (endoproteinase A) was studied widely since it has been considered to play a significant role in enology (Moreno-Arribas *et*

al., 1996; Alexandre *et al*., 2001). This protease A is classified to be an aspartic protease and as endoproteinase; pepstatin is its inhibitor (Beynon *et al*., 1990). This vacuolar acid protease appears to be very active in degradation of grape proteins once released from the cells and its activity is detected for long periods of time during aging on the yeast lees (Carnevillier *et al*., 2000). However, PR proteins, the principal precursors for protein haze formation in white wines (Waters *et al*., 1996; Waters *et al*., 1999; Ferreira *et al*., 2002) have high resistance to proteolysis (Linthorst 1991; Waters *et al*., 1996; Pocock *et al*., 2003), remaining in the wine even after the vinification process.

A number of studies allowed the identification of some components described to be haze protective factors, in the wine colloidal fraction. As presented above, some polysaccharides apparently protect the wine proteins against heat-induced haze formation (Waters *et al*., 1991). Mannoproteins are considered to be a haze-protective factor, and represent the majority (80%) of all exocellular polysaccharides released by yeast during fermentation and aging on the lees, containing 90% of mannose and 10% of protein. However, the purified heat-stabilizing product is a 31.8 kDa mannoprotein (known as MP32), consisting of 27% protein and 62% mannose (Ribéreau-Gayon *et al*., 2006a). This glycoprotein was isolated from a wine of the variety Moscatel and showed protective effect on wine proteins, even if the wine suffers heat test (Waters *et al*., 1999).

The active component of the mannoproteins was identified as being an invertase fragment from the yeast, with a molecular mass of around 32 kDa (Moine-Ledoux & Dubourdieu, 1999). This invertase fragment, identified as compound MP32, is not the same active component of the mannoproteins that inhibit tartrate crystallization (Ribéreau-Gayon *et al*., 2006a). Another mannoprotein, analogue to the Moscatel isolated one, was purified from 600 L of a wine from the variety Carignan Noir, in mg amounts and is referred to as Hpf1p. A second haze protective mannoprotein (Hpf2p) has also been isolated by ethanol precipitation of a chemically defined grape juice medium fermented by the winemaking strain of *S. cerevisiae*, Maurivin PDM (Dupin *et al.*, 2000b). The haze-protective effect of mannoproteins was independently confirmed by Ledoux *et al.* (1992) but the exact mechanism by which mannoproteins afford haze protection remains unclear (Waters *et al*., 2005). Nevertheless, it was described that these compounds do not inhibit proteins from precipitate but decrease the size of the suspended particles, making them invisible (or hard to detect) to the naked eye (Waters *et al*., 1993).

Dupin *et al.* (2000a) showed that the extraction methods for mannoproteins from *S. cerevisiae* are not very efficient and estimated that in order to decrease 20% of the haze, 500 mg of mannoproteins need to be applied to one litter of wine. However, Dubourdieu *et al.*

(1994) after applying 250 mg/L of mannoproteins extracted by enzymes (MPEE) from yeast cell walls (purified by ultrafiltration and dried), demonstrated that they were capable of halving the dose of bentonite required for protein stabilization of extremely heat-sensitive wines.

Dupin *et al*. (2000a) also showed that the invertase fragment from *S. cerevisiae* was present in the wine after heating and removal of the haze. Since the majority of the haze-protective factor was in the supernatant, it was suggested that these factors act by competing with other wine proteins for other non-proteinaceous wine components (X Factor), required for the formation of large insoluble aggregations of protein.

Trade names are used in this review for information purposes only. Neither the Instituto Superior de Agronomia nor the Universidade Técnica de Lisboa warrant those mentioned nor do they intend or imply discrimination against those not mentioned.

As previously stated, despite the present evidence for the required involvement of a non-proteic wine component in the protein haze formation, no particular component or condition has yet been identified. In this work it was sought to purify, analyze and characterize different fractions in the <3 kDa wine fraction and study their role in the protein haze formation in wines.

MATERIALS AND METHODS

Preparation of wine

Seven wines from the varieties Viosinho, Alvarinho, Arinto, Moscatel of Alexandria, Macabeu and Encruzado were tested. All wines were from the 2009 vintage, one from the Terras do Sado region (of the variety Moscatel of Alexandria) and six from the Lisbon region. The wines from Lisbon where produced at Instituto Superior de Agronomia, as a result of vinification trials. The winery Bacalhôa kindly offered a sample of its Moscatel of Alexandria varietal wine, from the Terras do Sado region, Portugal.

The traditional white wine vinification method was used for all grape varieties. The musts were fermented without skin maceration under controlled temperature. Bentonite was not added during clarification or fermentation processes.

After protein quantification, the selected wines were divided in 80 mL aliquots and stored at -20 ºC until used. To avoid repeated thawing and freezing, a different aliquot was used for each experiment.

Protein quantification

There are several methods for protein quantification but, some of them, are imprecise. This lack of precision is mainly due to interference caused by the presence of non-proteic factors, e.g. the content of phenolic compounds in the sample.

The chosen procedure for this work was a modification of the Lowry method, as proposed by Bensadoun and Weinstein (1976). This method is widely used in the literature, easy to apply and provide high sensitivity results. In this method, the protein reacts with cupric sulphate and tartrate in alkaline solution, which results in formation of a tetradentate copper-protein complex, reducing the Folin-Ciocalteu reagent. The blue colored, water-soluble product can be quantified at 750 nm.

Procedure

The calibration curve was constructed with bovine serum albumin (BSA), with a protein gradient between 1 and 25 µg, in a final volume of 250 µL (0.004 – 0.1 g/L). Five

different concentrations of BSA were prepared, in triplicate, starting from a BSA 0.5 g/L solution (stored at -20 ºC). To this preparation, 50 µL of a 1% (w/v) solution of sodium desoxycholate and 1 mL of trichloroacetic acid 10% (w/v) were added, leaving to incubate for 10 min. After incubation, the samples were centrifuged at 10000 *g* for 5 min, rejecting the supernatant and dissolving the pellet in 1 mL of solution C.

Solution C has to be freshly prepared, combining 1 part of solution B to 100 parts of solution A. The solutions A and B are composed by:

Solution A: sodium carbonate deca-hydrate 2% (w/v), sodium hydroxide 0.4% (w/v), sodium tartrate dihydrate 0.1% (w/v) and Sodium Dodecyl Sulphate (SDS) 1% (w/v).

Solution B: copper sulphate pentahydrate 4% (w/v)

After preparation, solution C was be agitated and incubated at 25 ºC, since the SDS can precipitate at low temperatures. Finally, 100 µL of Folin-Ciocalteau reagent (Sigma), diluted in a ratio of 1:1 with MilliQ water, was added. The final mixture was agitated in a vortex and placed in the dark, at room temperature, during 45 min.

The absorbance values were measured at 750 nm in a spectrophotometer (Shimadzu UV-2100) in plastic cuvettes of 1 cm path length, against a blank where the volume of the protein solution is substituted by MilliQ water.

Determination of the protein content of the samples was performed analogously, diluting the sample with Milli Q water until a final volume of 250 µL. This dilution is critical since the absorbance values measured have to be within the calibration curve limit values.

The absorbance is a linear function of the protein concentration between 5 and 50 µg of BSA. Under these conditions, the extrapolation of the protein content was performed based on the tendency line, acquired by the least squares method, from the average value of absorbance relative to the each BSA concentration (Bensadoun, 1976).

Isolation of the total protein wine fraction using FPLC

To isolate the total protein of the wine, the wine sample (Moscatel Bacalhôa 09) has to be previously desalted. Desalinization was performed by gel filtration using PD-10 prepacked Sephadex G-25M columns (GE Helthcare), previously equilibrated with 25 mL of Milli Q water. The eluate was homogenized and lyophilized. This procedure ensures the removal of about 90% of the <5 kDa molecules.

To isolate the total protein from the desalted wine, ion-exchange chromatography technique using a “Fast Protein, Peptid and Polynucleotid Liquid Chromatography” (FPLC) system was applied. A Resource S column (Pharmacia) was used. This column has an hydrophilic resin constituted by particles of 10 µm with great physical and chemical resistance. It can operate under several different conditions, with a pH range from 2 to 12, temperature range from 4 to 40 ºC and it is stable to pressures as high as 10 MPa. The charged group on the gel is –CH2–SO3, or sulphonyl group.

A sample (2 mL) containing the desalted wine was subjected to FPLC cation exchange chromatography on the Resource S column, previously equilibrated in 20 mM citrate-NaOH buffer, pH 2.5. The flow rate was 1.5 mL/min and the bound proteins were eluted with 1 M of NaCl in 20 mM citrate-NaOH buffer, pH 2.5. The fraction containing the wine total soluble protein was collected, subsequently desalted into water in PD-10 columns and lyophilized. The protein content of the sample was quantified using the modified Lowry method (Bensadoun, 1976).

Heat stability test

The wine protein haze can be induced in order to evaluate the susceptibility of a wine to form protein haze. There are several haze potential assessing tests described in the literature, differing in the presence/absence of tannins or in the time/temperature ratio. In this work the procedure described by Pocock and Rankine (1973) was selected.

Wine aliquots were thawed and centrifuged at 10 ºC and 10000 *g* for 15 min. Heat stability of the wines was subsequently determined. All measurements were made in triplicate. Five mL samples of the wines in study were saturated with nitrogen and sealed in test tubes with screw caps. The tubes were posteriorly heated at 80 ºC for 6 h in a water bath, held at 4 ºC for 16 h in a refrigerator and then, allowed to warm to room temperature. The increase in turbidity was detected spectrophotometrically at 540 nm and 25 ºC in 1 mL plastic cuvettes.

Wine fractionation

Fractionation of the wine in <3 kDa and >3 kDa compounds

The wine was fractionated in <3 kDa and >3 kDa compounds using ultrafiltration. Ultrafiltration filters Centriplus (YM-3, Millipore) with a molecular exclusion limit of 3 kDa were used. To the separation, the samples were centrifuged at 2500 *g* during 1 h. In all experiments only the <3 kDa fraction was used, corresponding to the fraction that pass through the 3 kDa cut-off membrane.

Thin layer chromatography (TLC)

Thin layer chromatography is a technique used to separate compounds present in a mixture. This separation is possible due to the differential adhesion strengths of the molecules present in the mixture to a mobile phase and to a stationary phase. The mobile phase is normally a solvent, and the stationary phase, in this case, is the thin layer sheet. This difference in the adhesion force translates into more or less movement along the sheet of each component, allowing its separation.

The stationary phases used were: alumina, silica gel and silica gel RP-18 TLC sheets (EMD Merck Precoated Aluminum Back TLC Sheets). The samples were applied in the TLC plate using a Pasteur pipette. Chromatography took place after inserting the plate inside a glass container with the respective eluent.

For the alumina and silica gel sheets, a solution of diclorometh ane with 10% (v/v) of methanol was used as mobile phase. The silica gel RP-18 sheet was activated with methanol, and the elution was made with a solution of water and 70% (v/v) methanol.

Reverse phase chromatography

In thin layer chromatography, the stationary phase is a thin layer of silica gel or alumina on a glass, metal or plastic plate. Column chromatography works on a much larger scale by packing the same materials into vertical glass columns. In this work, two glass columns with different sizes were used. The size of the column was chosen based on the mass of the sample to fractionate.

Analogously to the TLC experiments, the column was packed with silica gel RP-18. In this work, methanol and water were used as eluents, with different concentrations depending on the experiment.

High-performance liquid chromatography (HPLC)

Some fractions were analyzed by HPLC after fractionation by reverse phase chromatograhy. The HPLC system used included an L-7100 pump, a Rheodyne type injector, a D-7000 interface and a L7450A diode array spectrometric detector. The column was a reverse-phase C18 Lichrospere 100 (250 mm x 4.6 mm, 5 µm) (Merck, Darmstadt, Germany), and the separation was performed at room temperature. The elution conditions were as follows: 1.0 mL/min flow rate; solvent A: water (100%); solvent B: methanol (100%), 0-100% B linear from 0 to 25 min, 100-0% B linear from 25 to 40 min, followed by washing (100% solvent B) and reconditioning of the column in methanol.

NMR spectroscopic analysis of wine fractions

The sample fractions were dried in a Buchi Rotavapor inside appropriate vials. The samples were then dissolved with 0.5 mL of methanol D4 99.95% (v/v) (CD_3OD) and transferred to 5 mm NMR tubes. The deuterated methanol provided a chemical shift reference (^1H, δ 3.31 ppm). Proton and carbon nuclear magnetic resonance spectra (^1H and ^{13}C NMR) were recorded on a Bruker ARX (400 MHz) spectrometer. Chemical shifts are expressed in ppm, downfield from TMS; J-Values are given in Hz. The exact attribution of NMR signals was performed using two dimensional NMR experiments. Mass spectra were taken in a Micromass GC-TOF (GCT) mass spectrometer.

Compound 1 - ^1H NMR (400MHz, MeOD, δ): 7.01 (d, 2H, J=8.2, H2/6), 6.68 (d, 2H, J=8.3, H3/5), 3.67 (t, 2H, J=7.2 H2'), 2.70 (t, 2H, J=7.2, H1'); ^{13}C NMR (90MHz, MeOH, δ): 156.8 (Ar4), 130.9 (Ar2/6), 116.1 (Ar3/5), 64.6 (C2'), 39.4 (C1'); EIMS m/z 138 (M+).

Compound 2 - ^1H NMR (400MHz, MeOD, δ): 7.52 (d, 1H, J=7.8, H1), 7.31 (d, 1H, J=8.0, H6), 7.06 (m, 2H, H2/4), 6.98 (t, 1H, J=7.4, H5), 3.80 (t, 2H, J=7.3 H2'), 2.96 (t, 2H, J=7.2, H1'); ^{13}C NMR (90MHz, MeOH, δ): 138.1 (Ar6a), 128.9(Ar2a), 123.5 (Ar4), 122.2, 119.5, 119.2, (Ar1/2/5), 112.7 (Ar3), 112.2 (Ar6), 63.7 (C2'), 29.8 (C1'); EIMS m/z m/z 162 (M+).

Statistical analysis

The data is presented as the mean ± standard deviation. The comparison of treated means (Tukey test, 5% level) was performed using ANOVA analysis using Statistica Software 7.0 (StarSoft, Inc).

RESULTS AND DISCUSSION

Wine selection

To be able to induce protein haze in a white wine, it is desirable a non-fined wine with high content in protein. To the assays to be undertaken in the present work, nine wines from Lisboa and Terras do Sado regions, Portugal, all from the 2009 vintage, were chosen. Eight wines from vinification assays of Instituto Superior de Agronomia (ISA), from the varieties Viosinho, Alvarinho, Arinto, Moscatel de Alexandria, Macabeu and Encruzado were selected. The 9th wine was from the company Bacalhôa, of the variety Moscatel of Alexandria.

From the ISA wines, six where produced using musts obtained with low-pressure extraction, and two using high-pressure extraction. The wine from Bacalhôa was a mixture of low and high-pressure extraction must, in an unknown ratio.

The difference between low-pressure extraction and high-pressure extraction focuses on the equipment used to press, and the extraction pressure. In the Bacalhôa wine was used a pneumatic press was used, but there is no information about the working pressure. On the other hand, the ISA wines where elaborated using an immediate continuous extraction procedure.

Immediate continuous extraction is a process that has speed and volume of work as its mains advantages. In this process (Fig. 1), the grape clusters are crushed in a roller crusher and fall by gravity into a continuous inclined dejuicer, also called dynamic drainer.

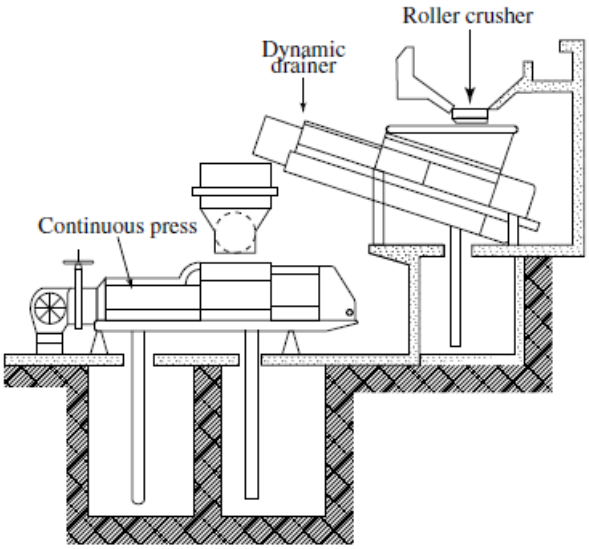


Fig. 1- Continuous juice extraction process (Ribéreau-Gayon *et al*., 2006)

The dejuicer contains a helicoidal screw that transfers the crushed grapes into a continuous press placed below. Continuous-type presses have the advantage of running uninterruptedly but the resulting juice or wine have a poorer quality when compared to the result of a pneumatic or vertical press (Jackson, 2008).

For this work, the must obtained in the dejuicer was considered the low-pressure must, also called free-run juice. This free-run fraction, when compared to the press-run juice, is clearer and possess lower levels of suspended solids, phenolic compounds and flavorants derived from skin maceration. On the other hand, the press-run juice contains increasing amounts of suspended solids, tannins and skin flavorants. Press-run fractions are also more likely to suffer oxidation (containing a higher concentration of polyphenol oxidase), posssess lower acidity (higher potassium contents), and higher concentration of polysaccharides, gums and soluble proteins (Jackson, 2008).

After protein quantification in all studied wines, using the modified Lawry method (Fig. 2), the variety with higher protein content, is the Moscatel of Alexandria. Similar results were described by Mesquita *et al*. (2002).

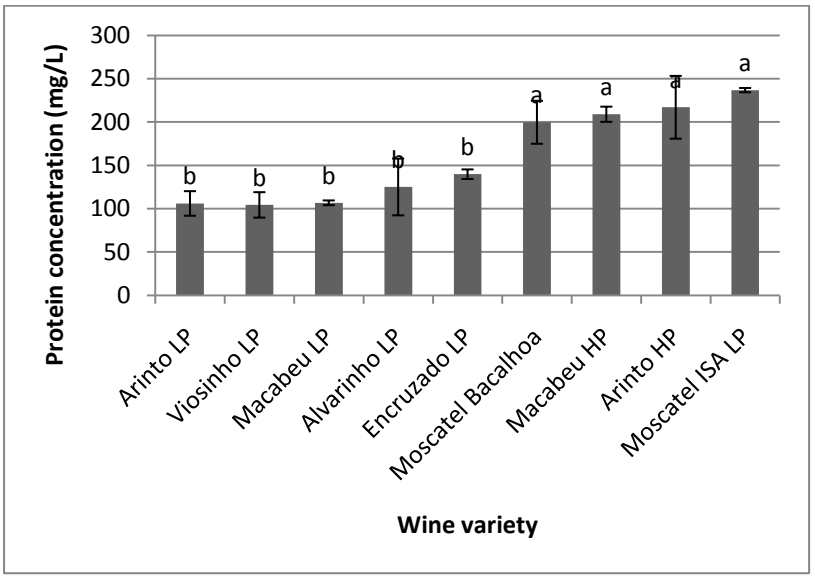


Fig. 2 - Protein concentration of the studied wines from Instituto Superior de Agronomia and Bacalhôa, 2009 vintage. LP for low-pressure extraction, HP for high-pressure extraction

Other predictable result is that high-pressure extraction wines contain more protein than low-pressure extraction wines, a phenomenon also described by other authors (Ribéreau-Gayon *et al*., 2006; Jackson, 2008).

The four wines that contained higher content in protein, were submitted to a haze potential test (Fig. 3). The tested wines were from the varieties Moscatel de Alexandria (LP), Macabeu (HP), Arinto (HP) and also Moscatel from Bacalhôa. The method used for measuring the haze potential was the classic heat test (Pocock & Rankine, 1973) described in the materials and methods section.

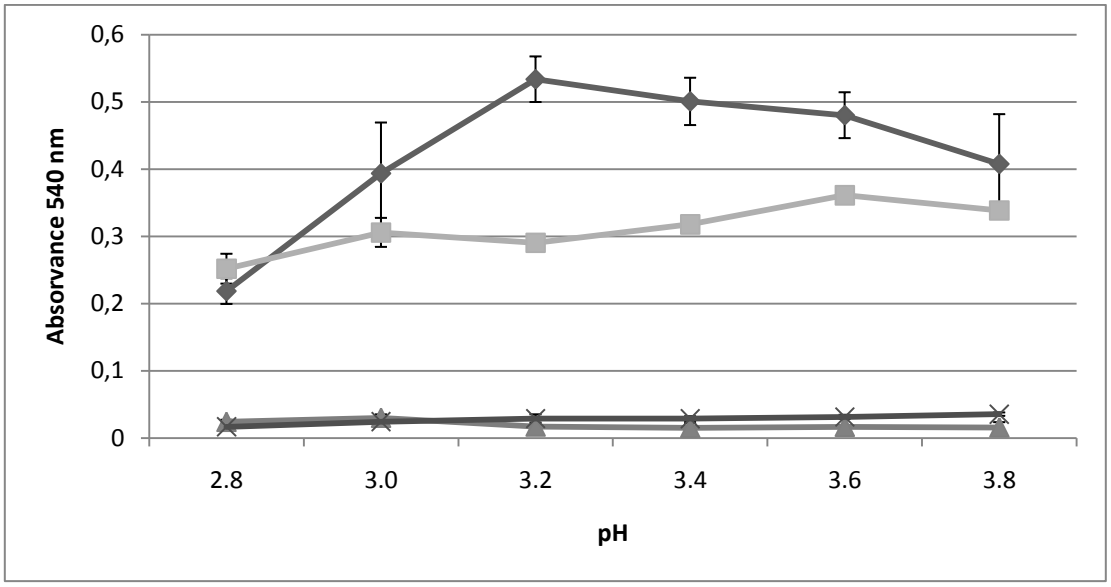


Fig. 3 - Changes in turbidity (detecting by measuring the absorvance at 540nm) observed after Heat Test of the wines from Moscatel Bacalhôa ( ), Moscatel ISA ( ), Arinto ISA HP ( ), Macabeu ISA HP ( )

The wine that expressed higher haze potential was the Moscatel de Alexandria from Bacalhôa, mainly at pH 3.2 (Fig. 3). This wine was not the richest in protein (Fig. 2); however, it showed the higher haze potential after the heat test. These results corroborate some studies which indicate that the haze formed in white wine is not proportional to its total protein content (Moretti *et al*., 1965; Bayky *et al*., 1967; Dawes *et al*., 1994).

The haze potential of the high-pressure extraction and low-pressure extraction wines showed a significant difference. High-pressure wines had no significant difference in the protein content relatively to the low-pressure extraction ones, but demonstrated a significant difference in the haze potential test at all pH values analysed. Can this result be related to the quantity of the factor X present in the press-run wines? High-pressure extraction wines, or press-run wines, have higher concentration of many compounds, including phenolic compounds (Ribéreau-Gayon *et al*., 2006a) which are known to be one of the strongest candidates as responsible in the protein haze (Waters *et al*., 2005).However, these results indicate the opposite. Indeed it may be that lower molecular weight tannins play an inducible role in protein haze formation, unlike higher molecular weight tannins which may remove wine proteins from solution by precipitation. Therefore, it would be important to determine if high-pressure extraction wines contain higher levels of lower or higher molecular weight

tannins than lower-pressure extraction wines. The phenolic compounds present in these wines could have precipitated some of the responsible proteins for protein haze, but that should have been reflected in the overall protein content of the wine. In the other hand, press-wines have higher content in polysaccharides that can act as protective colloids. Protein haze in white wines can occur because of tannins and proteins form aggregates, but the formation of these aggregates may be inhibited by the presence of polysaccharides (De Freitas *et al*., 2003; Riou *et al*., 2003).

The diagram illustrated in Fig. 4 represents a proposed mechanism for the formation of tannin-protein aggregates and the putative role of polysaccharides as haze protective factors.

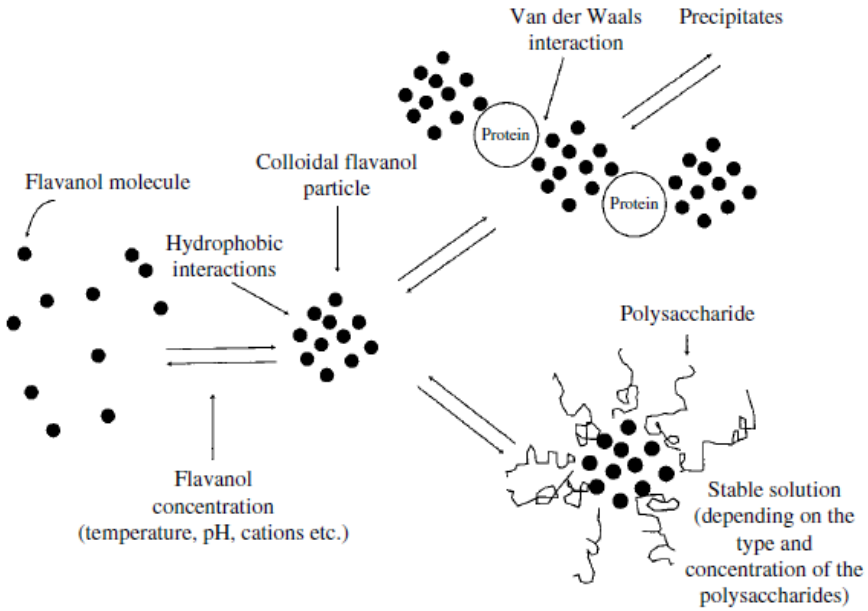


Fig. 4- Model of the colloidal properties of flavanols (tannins) (Saucier, 1997)

However, another study showed that polysaccharides might increase protein instability, particularly at moderate to high temperatures (Mesquita *et al*., 2001). The type of polysaccharides considered may explain the apparent discrepant observations.

These results may have been punctual, an observation that indicates that more assays are needed to validate this observation.

For the subsequent tests, a single pH value, the pH of 3.2, was selected. The main reasons for the choice of this pH are the normal occurrence of this pH value in bottled white wines and the significant results in the haze potential tests using sample wines at this pH. Moreover, at higher pH, the haze formation tends to increase thanks to the proximity to the isoelectric point of the proteins present in wine. The protein fractions that contribute to

protein instability in white wines are known to have molecular weights from 10 to 32 kDa, and isoelectric points of 4.1 to 5.8 (Hsu *et al*., 1987).

Fining assay

Fining consists in the addition of a substance to the wine, producing flocculation and precipitation of some compounds that cause turbidity or colloidal instability (Ribéreau-Gayon, 2006). The fining agents may have several objectives, depending of the constitution of the agent. There are fining agents to help decanting suspended solids, to remove protein, to remove phenolic compounds or to stabilize some components of the wine like the action of arabic gum on iron. To better understand the roll of phenolic compounds on protein haze formation, a wine sample was fined and the difference of haze potential between the fined and the non-fined wine measured.

Phenolic compounds are non-proteinous factors that are potentially involved in white wine protein haze formation, with substantial evidence that their interactions with proteins are significant (Waters *et al*., 2005). In order to remove phenolic compounds from the wine sample, a fining agent specific to these compounds was selected. The chosen fining agent was casein. Casein is a protein that is extracted from cow milk. Addition of sodium or potassium bicarbonate increases its solubility. This protein is used as a fining agent, reducing preferentially the wine content in highly polymerized proanthocyanidins and also those esterified with gallic acid (Ricardo-Da-Silva *et al*., 1991).

Some authors using different finig agents performed similar assays. Waters *et al*. (2007) reported that wines fined with PVPP formed less protein haze after heat test, when compared to unfined controls.

Using inappropriate amounts of fining agents, in this case casein, can lead to an over-fining. This phenomenon rarely occurs in red wine but, in white wines, it may cause the suspension of residual protein and therefore haze formation. A concentration of 50 g casein/hL wine was used in this assay, described as the maximum recommended dose of casein (Cardoso, 2007).

The protein quantification was performed on both fined and non-fined wine using the modified Lowry method (Bensadoun, 1976). There was no significant difference on the protein content of the fined wine, indicating that there was no over-fining with the concentration of 50 g/hL of casein (Fig. 4).

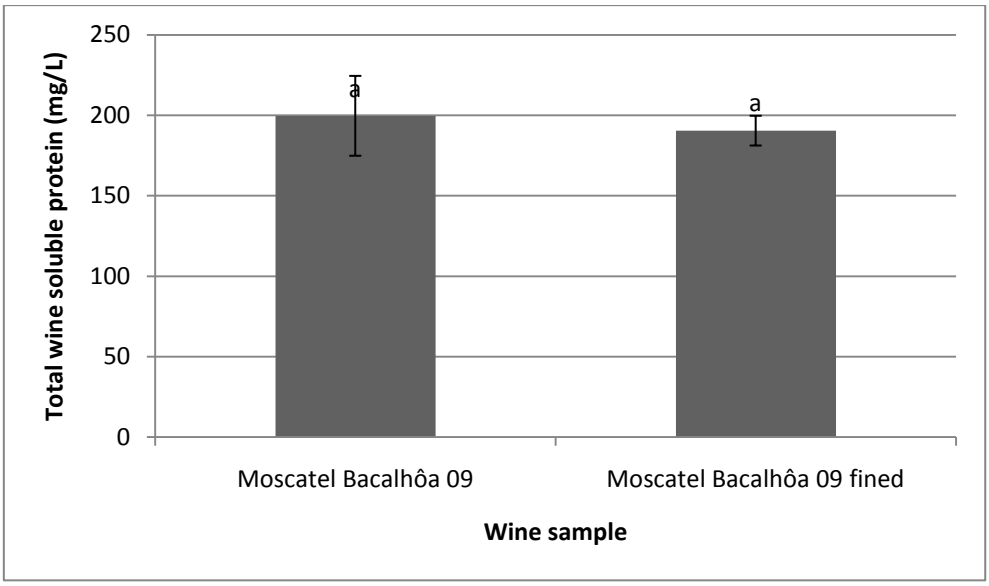


Fig. 5 - Protein content of the wines Moscatel Bacalhôa 09 and Moscatel Bacalhôa 09 fined with 50 g casein /hL wine.

After protein quantification, both wines were subjected to a heat test to evaluate their haze potential. The difference between the fined wine and the non-fined wine is significant (Fig. 5). The fined wine presents much less haziness, showing that some of the phenolic compounds that were removed by the casein treatment interact with the protein forming haze in white wines, corroborating the results of Waters *et al.* (2005) with PVPP. The Casein fining experiment confirmed previous results (Yokotsuka *et al*., 1983; Waters *et al*., 2005) which suggested that phenolic compounds are involved in protein haze formation.

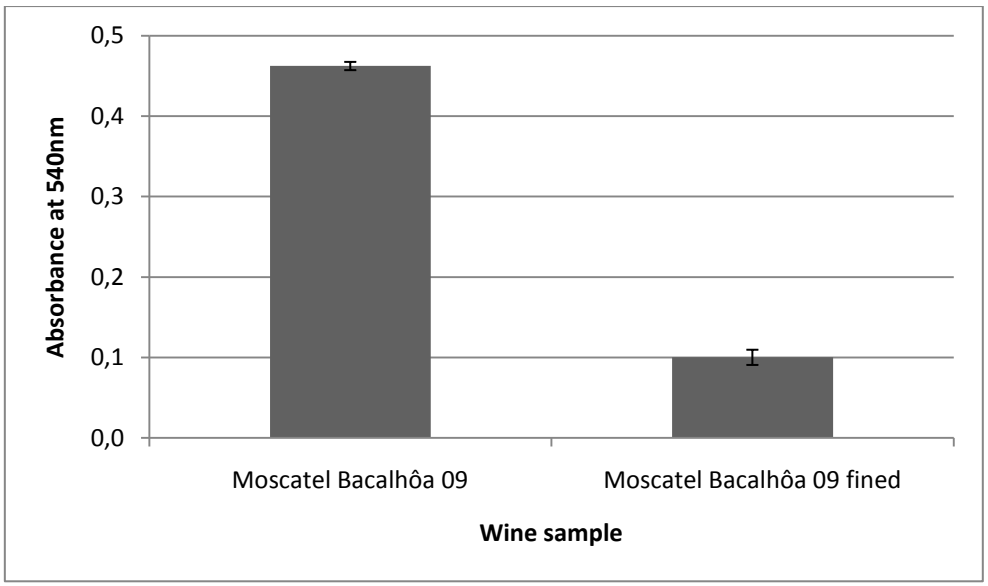


Fig. 6 - Changes in turbidity (detecting by measuring the absorbance at 540 nm) observed after heat stability test of the wine Moscatel Bacalhôa 09 and the wine Moscatel Bacalhôa 09 fined with 50 g/hL of casein.

Therefore, the effect of casein in the treatment of haze formation can result from the removal phenolic compounds, mainly nonflavonoid compounds has suggested by Cosme *et al.*(2007). Hereupon, it would be of great interest to understand what compounds were removed and to what fraction do they belong.

Wine fractionation

Baptista *et al*. (2009) reported that at low pH, protein haze formation in white wine exhibits an absolute requirement for a low molecular mass (<3 kDa) wine component, that sensitize proteins for heat-induced denaturation at low wine pH. With the aim of identifying this or these compounds, the wine was subjected to ultrafiltration using a 3 kDa cut-off Amicon Ultrafilter. A heat stability test was performed to the <3 kDa wine fraction, and compared to the pattern of turbidity of the Moscatel Bacalhôa 09 wine (Fig. 7).

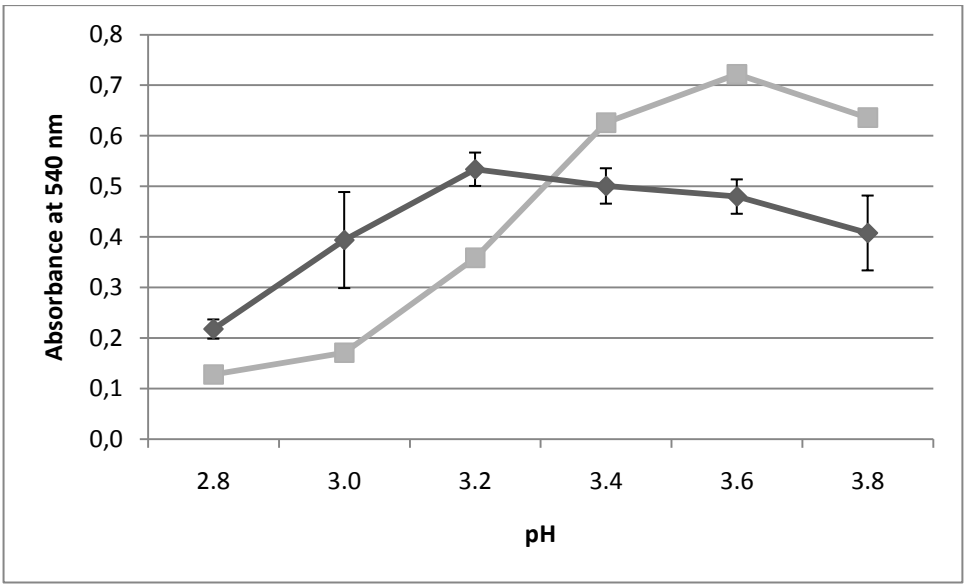


Fig. 7- Changes in turbidity (detecting by measuring the absorbance at 540 nm) as a function of pH observed after heat test of the wine Moscatel Bacalhôa 09 ( ) and the <3 kDa fraction of the wine Moscatel Bacalhôa 09 back-added with isolated wine protein ( ).

The <3 kDa fraction back-added with isolated wine protein (250 mg/L) presented a gradual increase in turbidity from pH 2.8 to 3.6 (Fig. 7). The wine Moscatel Bacalhôa 09 had the maximum turbidity at pH 3.2, and did not follow the same pattern of turbidity for higher pH values than the <3 kDa fraction with the isolated protein. This difference may be tentatively explained by the presence of large molecular weight haze-protective factors (e.g.

polysaccharides) which are present in the wine but not in the back-added isolated protein (Waters *et al*., 1993; 1994).

The different turbidity values observed at pH 3.2 (selected value for this work; Fig. 7) can be also explained by the tartaric stabilization of the MBA09 wine that was ultrafiltered. In order to protect the sample wine Moscatel Bacalhôa 09, it was store frozen at -20 ºC, temperature which leads to tartrate precipitation. Using the slow cold stabilization treatment equation (Ribéreau-Gayon *et al*., 2006b; Fig. 8), the temperature at which tartrate precipitation occurs for the Moscatel Bacalhôa 09 wine is -5º C, confirming that the storage temperature used may have contributed to wine stabilization.

$$Temperature\ treatment = -\frac{\text{Alcohol content}}{2} - 1$$

Fig. 8 - Slow cold stabilization treatment equation (Ribéreau-Gayon *et al*., 2006)

A new heat stability test was performed with the Moscatel Bacalhôa 09 wine after tartrate precipitation. The results presented a significant difference with or without tartaric stabilization (Fig. 9).

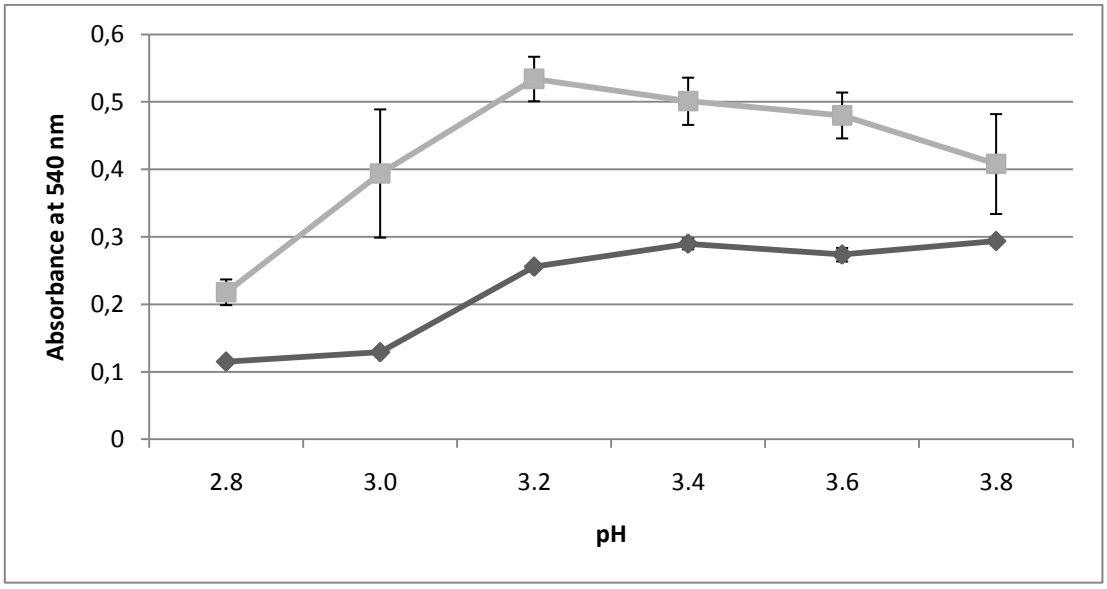


Fig. 9 - Changes in turbidity (detecting by measuring the absorbance at 540 nm) as a function of pH observed after heat test of the wine Moscatel Bacalhôa 09 without tartaric stabilization at 5 October 2009 ( ) and the wine Moscatel Bacalhôa 09 with tartaric stabilization at 20 October 2009 ( )

There was a decrease in the absorbance values for the Moscatel Bacalhôa 09 wine after 15 days at -20 ºC. This difference can be explained by the precipitation of some wine components with potassium hydrogen tartrate crystals. Vernhet *et al.* (1999) reported that there were tartrate esters of phenolic acids and polysaccharides on potassium hydrogen tartrate. Other molecules like tannins, proteins and pectic polysaccharides are also known to bind with organic acids present in wine (Correa-Gospore *et al*., 1991; Vernhet *et al*., 1999).

Batista *et al.* (2010) suggested the hypothesis that some organic acids, in particular tartaric acid, can bind with other molecules preventing them to bind with proteins. The same authors measured the absorption spectral curve of the tartrate precipitate of white wine and compared to the absorption spectral curve of the precipitate resulting from the addition of tartaric acid to the <3 kDa fraction of white wine. In the same work it was proposed that tartaric acid reacted with the same type of molecules in both fractions tested, corroborating the presence of a protein instabilization factor present in the <3 kDa fraction.

To evaluate what compounds interact with the protein to form haze, fractionation of the wine was performed. The goal was to isolate a compound, or family of compounds, that are considered the X factor, acting as a precursor for protein haze formation.

1st experiment

The Moscatel Bacalhôa 09 wine was prepared for fractionation, following the methodology described in the Materials and Methods section. The dried wine sample was dissolved in 10 mL of methanol (100% v/v), decanted and filtered. The precipitate, formed after dissolving in methanol, is the water-soluble fraction (WF1), whereas the supernatant contains the methanol-soluble fraction (MF1).

To understand the structural differences between fractions, NMR analysis was employed, as described in the Methods section. After confirming the differences between the fractions (NMR spectra), a heat stability test was performed.

The haze potential of the methanolic extract is significantly higher than the water fraction (Fig. 10). These results indicate that the methanolic extract contain compounds that increase the protein haze potential. The methanol-soluble fraction was compared to the wine sample in what concerns heat stability test, to evaluate the difference in haze formation (Fig. 11).

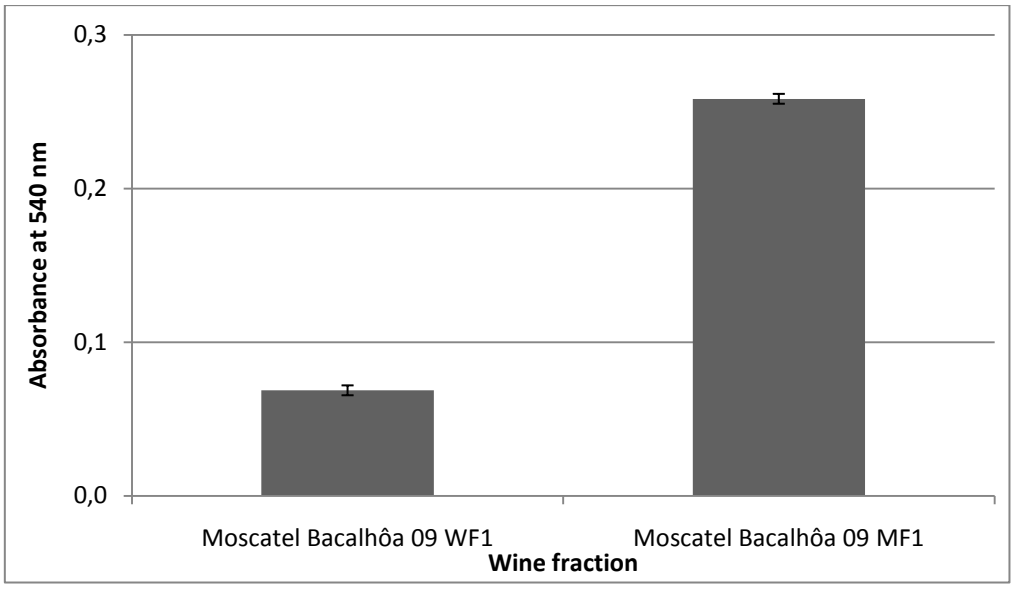


Fig. 10 - Changes in turbidity (detecting by measuring the absorbance at 540 nm) observed after heat test of the wine Moscatel Bacalhôa water-soluble fraction (WF) and methanol-soluble fraction (MF) in a wine model solution (WMS; 12% ethanol 4 g/L tartaric acid) with isolated wine protein (IWP; 250 mg/L)

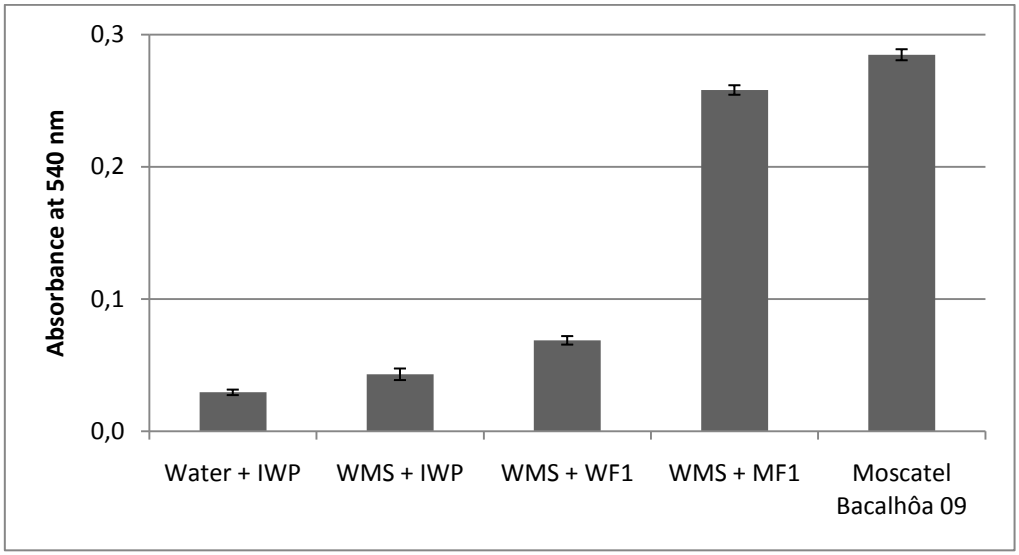


Fig. 11 - Changes in turbidity (detecting by measuring the absorbance at 540 nm) observed after heat stability test performed in the following samples: water with wine isolated protein (water + WIP), wine model solution with isolated wine protein (WMS + IWP), wine model solution with water-soluble fraction of the Moscatel Bacalhôa 09 wine (MWS + WF1), wine model solution with methanol-soluble fraction of the Moscatel Bacalhôa 09 wine (MWS + MF1) and the Moscatel Bacalhôa 09 wine.

The haze formed in the methanol-soluble fraction was only slightly lower than that obtained for the sample wine, suggesting that the major compounds involved in the protein haze formation are present in that fraction.

Previous work developed in our lab identified a major component of the methanolic extract as a *meso*-erythritol, or one of its two stereoisomeric tetritols, D-threitol and L-threitol. These compounds were individually subjected to heat stability test, revealing no direct involvement in the protein haze formation (unpublished data).

The methanolic extract (MF1) was separated in two fractions by reverse phase chromatography using a RP18 column and, as solvents, water (100% v/v) for the first elution and methanol (100% v/v) for the second elution. Both methanolic (MF2) and water (WF2) fractions were subjected to heat stability test and NMR analysis (Fig. 12).

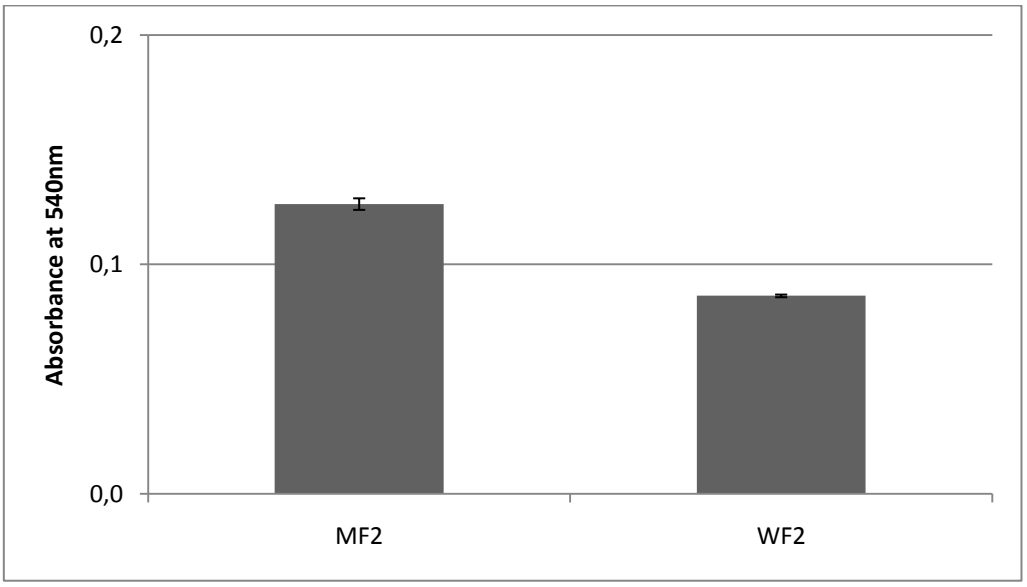


Fig. 12 - Changes in turbidity (detecting by measuring the absorbance at 540 nm) observed after heat stability test performed to the methanol fraction (MF2) and water fraction (WF2) obtained after the fractionation of the methanolic extract in a RP18 chromatography column. Both fractions were back-added with isolated wine protein (250 mg/L).

The MFM fraction had a significantly higher haze potential, indicating that the factor that contributes more intensely to the haze formation is present in that fraction.

After the analysis of the NMR spectrum obtained for both fractions, it was concluded that the major compound of the methanolic extract (MF), the meso-erythritol, was completely washed-out to the WF2 fraction.

As a resume, the 1st experiment is schematically represented in Fig.13.

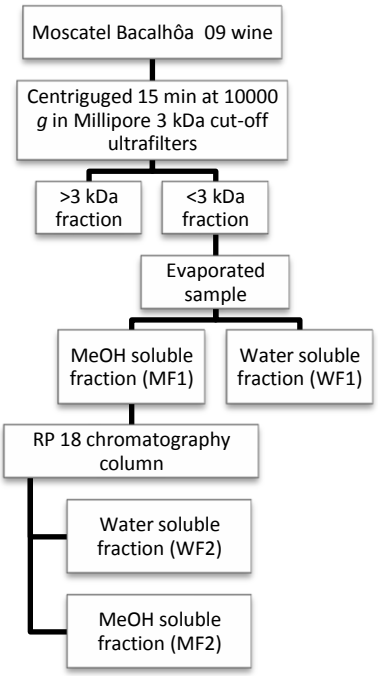


Fig. 13 - 1st experiment scheme

2nd experiment

To better understand the complexity of each experiment, the following experiment schemes will be presented at the beginning of the chapters.

As a resume, the 2nd experiment is schematically represented in Fig.14.

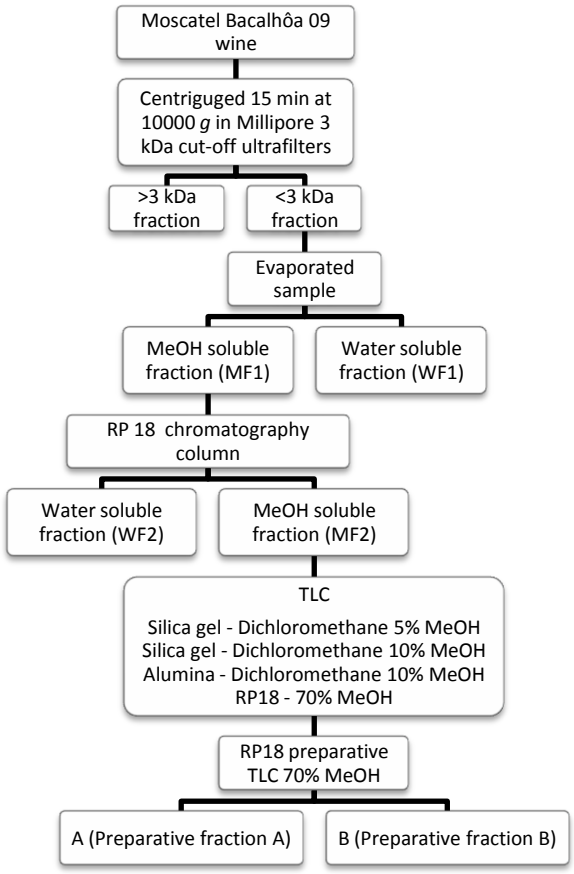


Fig. 14 - 2nd experiment scheme

One hundred mL of MBA09 wine were performed for fractionation by the method described in the Materials and Methods section. Analogously to the 1st experiment, the dried wine sample was diluted in 10 mL methanol and decanted, separating the wine in to a methanolic-soluble fraction (MF1) and a water-soluble fraction (WF1).

The methanolic-soluble fraction (MF1) was further separated in two fractions by reverse phase chromatography using a RP18 Column and, as solvents, 50 mL of water (100% v/v) for the first elution and 50 mL of methanol (100% v/v) for the second elution.

Once more, NMR analysis was performed to both fractions to guarantee that all the meso-erythritol was washed-out into the water-soluble fraction (WF2).

To understand the complexity of the methanolic fraction (MF2) composition, thin-layer chromatography (TLC) of the sample was performed, testing several TLC sheets and concentrations of eluents.

Initially a silica sheet was tested with dichloromethane (5% v/v) and methanol (95% v/v) as eluents (Fig.15A). The sample stayed in the application point, meaning that the polarity was not enough to fractionate the sample components. The same result occurred with the silica sheet with dichloromethane (10% v/v) in methanol (90% v/v) (Fig. 15B), and to the alumina sheet with dichloromethane (10% v/v) in methanol (90% v/v) (Fig. 15C).

Fig. 15 - TLC of the methanolic extract (MF2) using different sheets. A - Precoated silica TLC sheet using dichloromethane (5% v/v) and methanol (95% v/v) as eluent B - Precoated silica TLC sheet, using dichloromethane (10% v/v) and methanol (90% v/v) as eluent. C - Precoated alumina TLC sheet, using dichloromethane (10% v/v) and methanol (90% v/v) as eluent.

A RP18 TLC sheet was tested with methanol (70% v/v) has the eluent. After elution, the methanolic fraction (MF2) was separated in two bands, differing in their polarity (Fig. 16).

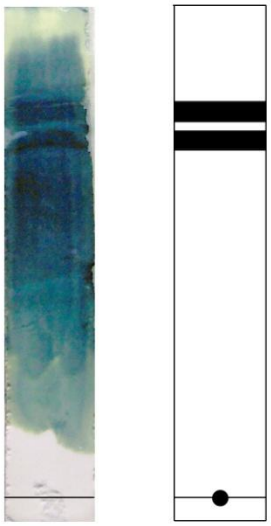


Fig. 16 - Thin-layer chromatography of the methanolic fraction (MF2) from the Moscatel Bacalhôa 09 wine using a RP18 TLC sheet with methanol (70% v/v) as the eluent.

This type of chromatography sheets allow the separation of the sample by using hydrophobic interactions of the stationary phase with wine compounds of appropriate molecular structure. In this particular case, it was used a RP18 medium was used, which means reversed phase with an organofunctional octadecyl group. Reversed phase means that the relative polarities of the stationary and mobile phases are reversed, compared with unmodified sorbents like silica gel or alumina (Fredric, 2003).

This result prompted the use of preparative layer chromatography. PLC, or preparative layer chromatography, is used to isolate 10 to 500 mg or more of material on layers thicker than those used for analytical TLC (Fredric, 2003). With the purpose of separating both fractions obtained previously in the analytical TLC, a preparative RP18 layer (Fig. 17) was pre-activated with methanol and methanol (70% v/v) in water was used as the eluent. Thirty mg of sample were applied, containing the methanolic fraction (MF2) from the methanolic extract (MF1).

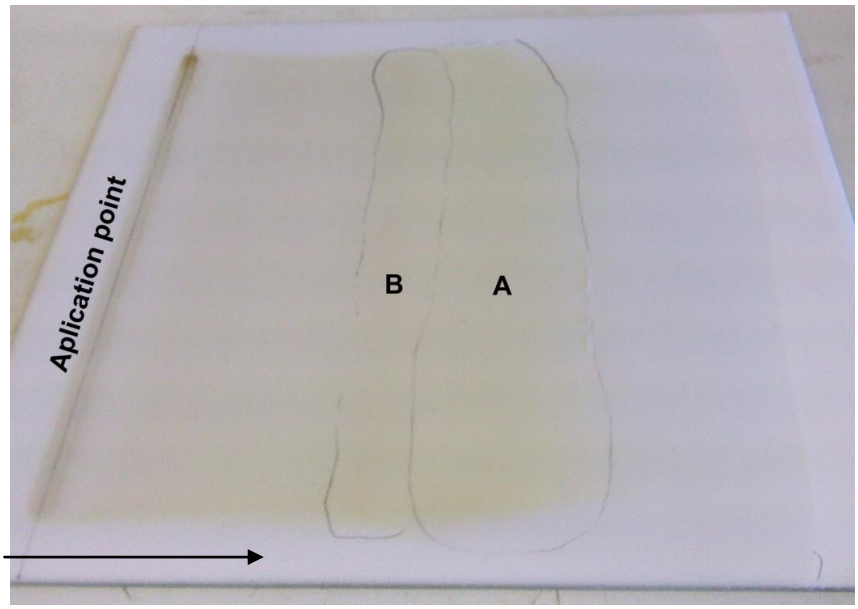


Fig. 17 - Preparative layer chromatography of the MF2 fraction from the Moscatel Bacalhôa 09 methanolic extract (MF1).

The preparative chromatography step allowed the separation of fractions A and B (Fig. 17), previously identified in the analytical TLC (Fig. 16). Being a reversed phase layer, the A fraction is the most polar and the B fraction the less polar.

Both bands A and B, present in the silica, were individually scraped from the glass, dissolved in methanol (100% v/v) and decanted. After isolating both fractions, a haze stability test was performed (Fig. 18).

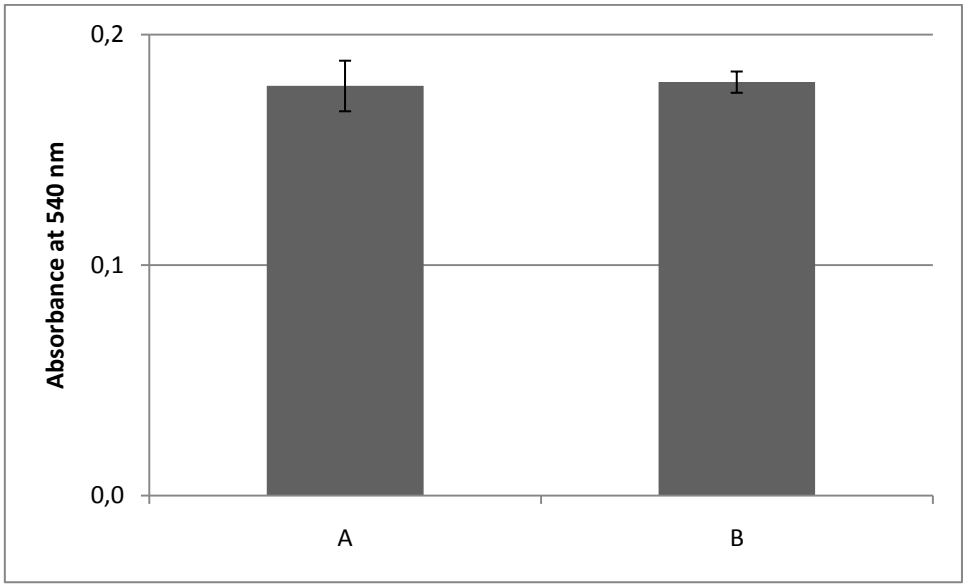


Fig. 18 - Changes in turbidity (detecting by measuring the absorbance at 540 nm) observed after heat stability test performed to fractions A and B obtained by the fractionation of the MF2 fraction using preparative RP18 chromatography.

Considering the standard deviations, there is no significant difference between both samples in the haze potential test. This indicates that the compounds involved in the protein haze formation are present in both fractions.

3rd experiment

As a resume, the 3rd experiment is schematically represented in Fig.19.

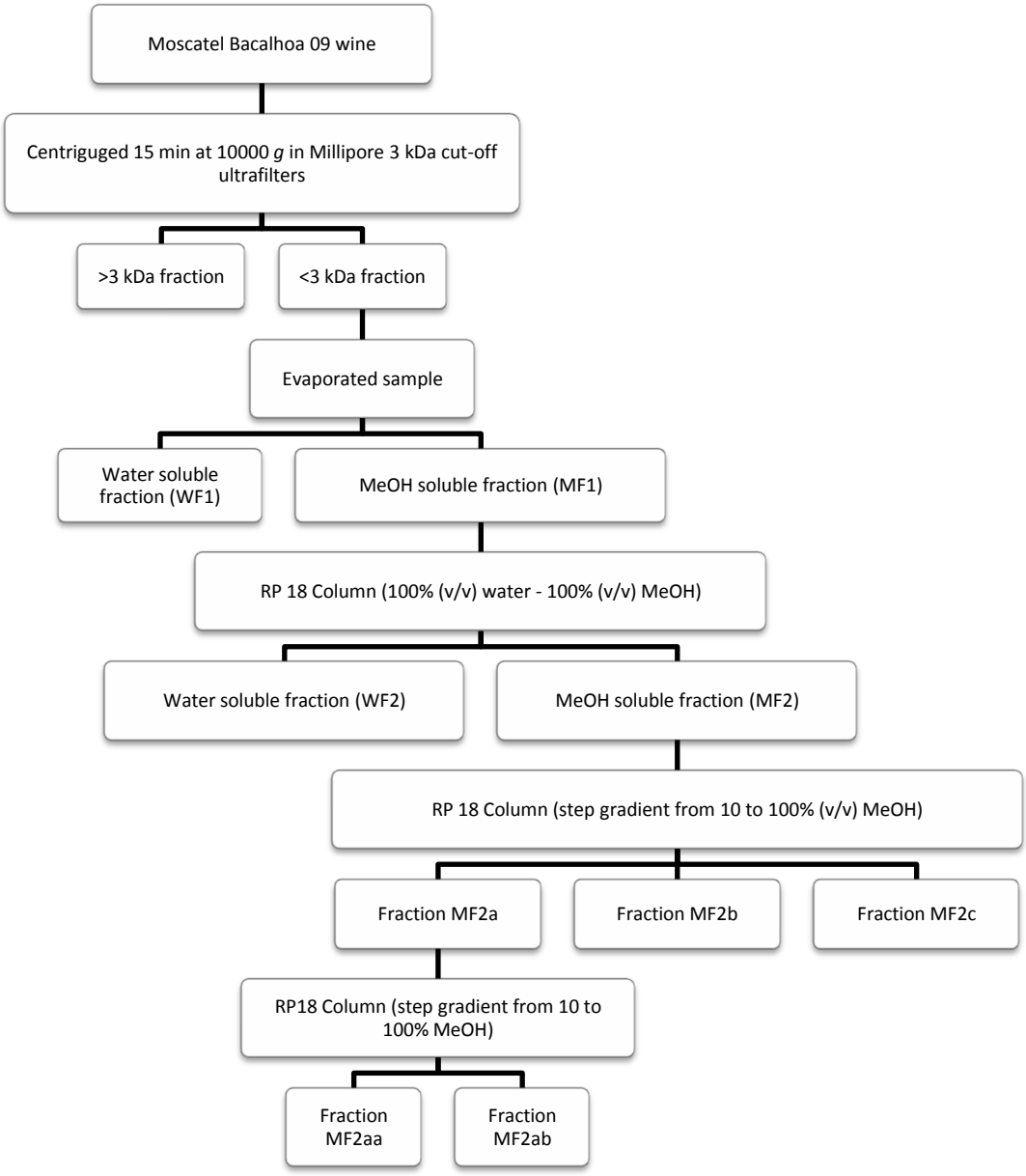


Fig. 19 – 3rd experiment scheme

To isolate the compounds involved in haze formation present in both fractions obtained in the 2nd experiment TLC (Fig. 17), another experiment was attempted with the objective of achieving a finer fractionation of the MF2 fraction. With a greater number of fractions, there is a higher probability of separating the reactive compounds from the non-reactive.

The fractionation procedure of the Moscatel Bacalhôa 09 wine, as described in the Materials and Methods section, was repeated. The methanolic extract (MF2) was fractionated, once more, in the same chromatography RP18 Column but with a gradient of methanol from 10 to 100% (v/v). Ten sub-fractions were collected into test tubes, which were subsequently subjected to TLC analysis in an analytical RP18 chromatography sheet with methanol (70% v/v) as the eluent (Fig. 20).

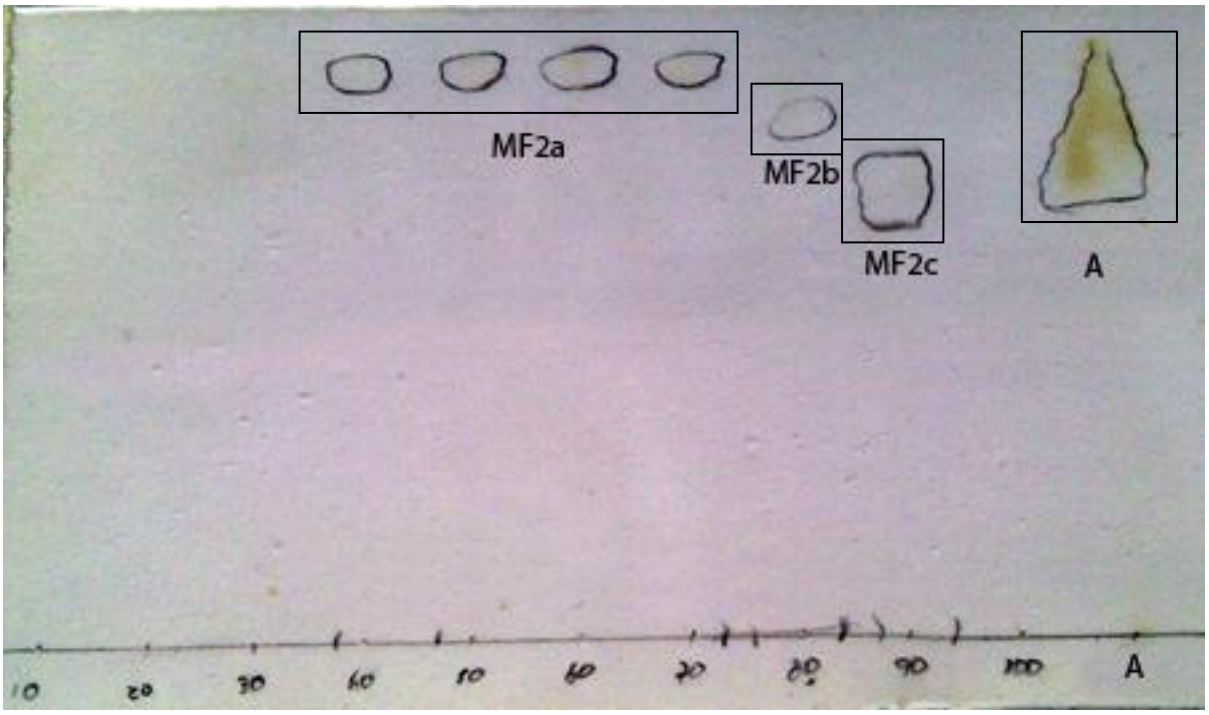


Fig. 20 - TLC analysis using an analytical RP18 sheet with methanol (70% v/v) as eluent of the ten sub-fractions from the fractionation of the methanolic extract (MF1) of the Moscatel Bacalhôa 09 wine with step gradient from 10 to 100% (v/v) methanol. At the right, the A band from the PLC of the 2nd trial.

TLC analysis using UV light, allowed the selection of three different fractions taking their differential retention factor (Rf) as reference (Figs. 20 and 21). The first fraction selected results from the combination of the fractions 40, 50, 60 and 70 (MF2a), with an Rf of 0.93. The fractions 80 (MF2b) and 90 (MF2c) had a different Rf, 0.82 and 0.72 respectively.

$$Rf = \frac{\text{distance moved by the solute}}{\text{distance moved by the mobile phase front}}$$

Fig. 21- Retention factor equation (Fredric, 2003)

At the same time, a sample of the A band from the 2nd experiment was applied to the TLC sheet. Because of the excessive concentration of the applied sample, the revealed TLC was blurry, with no focused spots. Nevertheless, the A band can be considered the combination of the different fractions that composed the methanolic extract (MF2), as evidenced by comparing the Rf values of the individual fractions with the Rf values of band A components.

NMR analysis was applied to the fraction MF2a. Among other compounds, it stood out a probable phenolic acid ester bound to a sugar, presumably a rhamnose.

Fraction MF2a was passed through a chromatography RP18 column; elution took place with a gradient of methanol between 10 and 100% (v/v). These fractions were subjected to analytical TLC analysis, revealing different bands in the 50 and 60% (v/v) methanol fractions (Fig. 22).

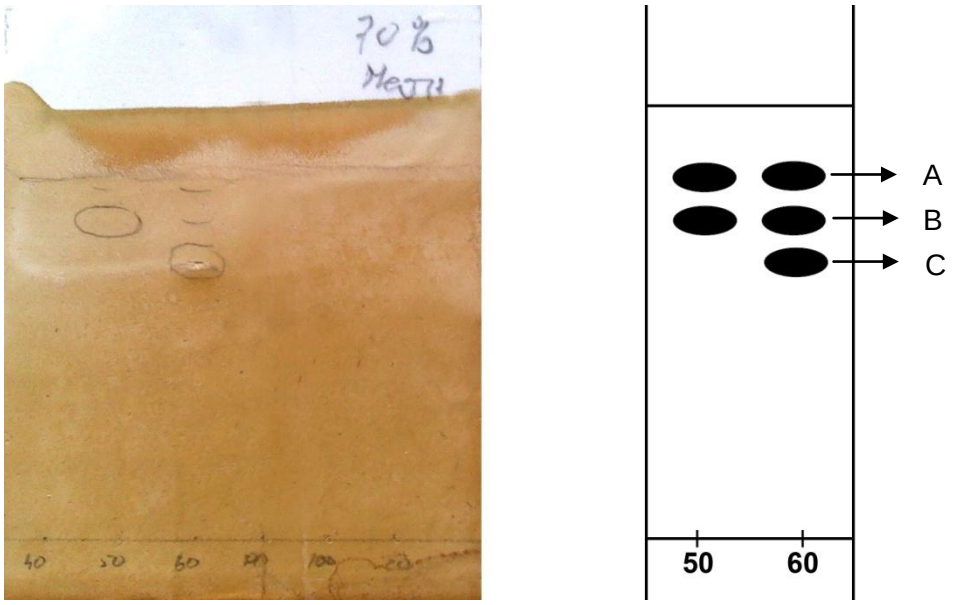


Fig. 22 – Analysis of the MF2a fraction using an analytical RP18 TLC sheet with methanol 70% (v/v) as the eluent. The scale represents the methanol concentration used in the RP18 chromatography column during the fractionation of the MF2a fraction. TLC revealed with potassium permanganate.

The TLC was revealed with potassium permanganate, oxidizing the compounds present in the bands. The bands were marked, appearing one more band with a different R_f in the 50 fraction (MF2aa) comparing to the 60 fraction (MF2ab).

Table 1 - Rf values of the different compounds present in the MF2aa and MF2ab sub-fractions.

Band	R_f
A	0.93
B	0.84
C	0.73

The TLC shown in Fig. 22 turned light brown with time, hiding the bands but, at the moment of application, it was purple and the stains appeared yellow. This visualization reagent reacts mainly with alkenes and alkynes, but alcohols, amines, sulfides and mercaptans can be also oxidized. The remaining fractions did not present any bands or stains.

NMR analysis revealed that the sugar present in the MF2a fraction was washed-out in the MF2aa fraction and the aromatic compound went to the MF2ab fraction.

Both samples MF2aa and MF2ab (Fig.22), along with the fractions MF2b and MF2c (Fig.20), were subjected to heat stability test (Fig. 23).

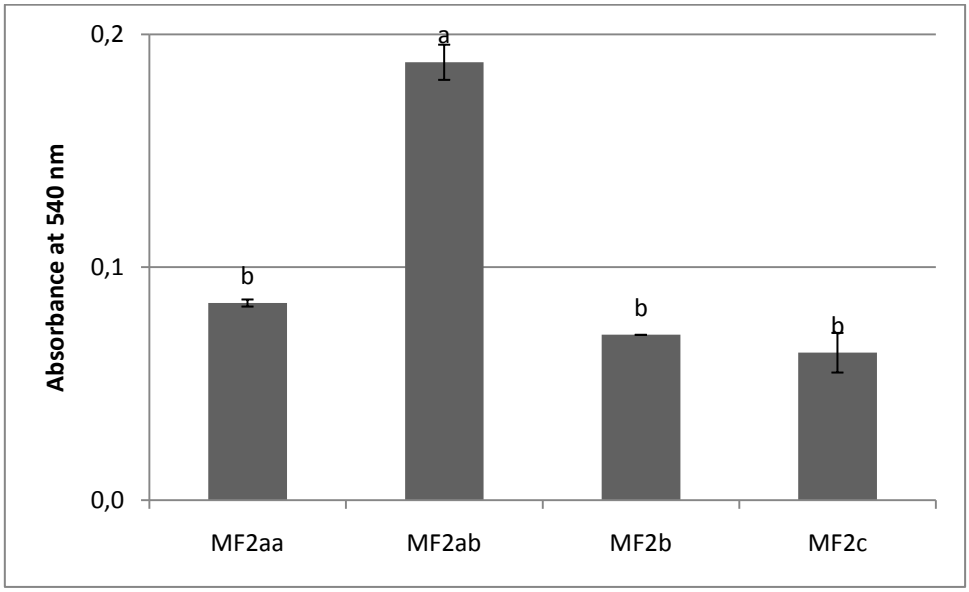


Fig. 23 - Heat Changes in turbidity (detecting by measuring the absorbance at 540 nm) observed after heat stability test performed to fractions MF2aa, MF2ab, MF2b and MF2c. All fractions were back-added with isolated wine protein (250 mg/L).

Fraction MF2ab proved to be the fraction with higher haze potential (Fig. 23). The NMR analysis of fraction MF2ab revealed mainly a compound containing aromatic protons, together with other minor compounds.

4th experiment

As a resume, the 4th experiment is schematically represented in Fig.24.

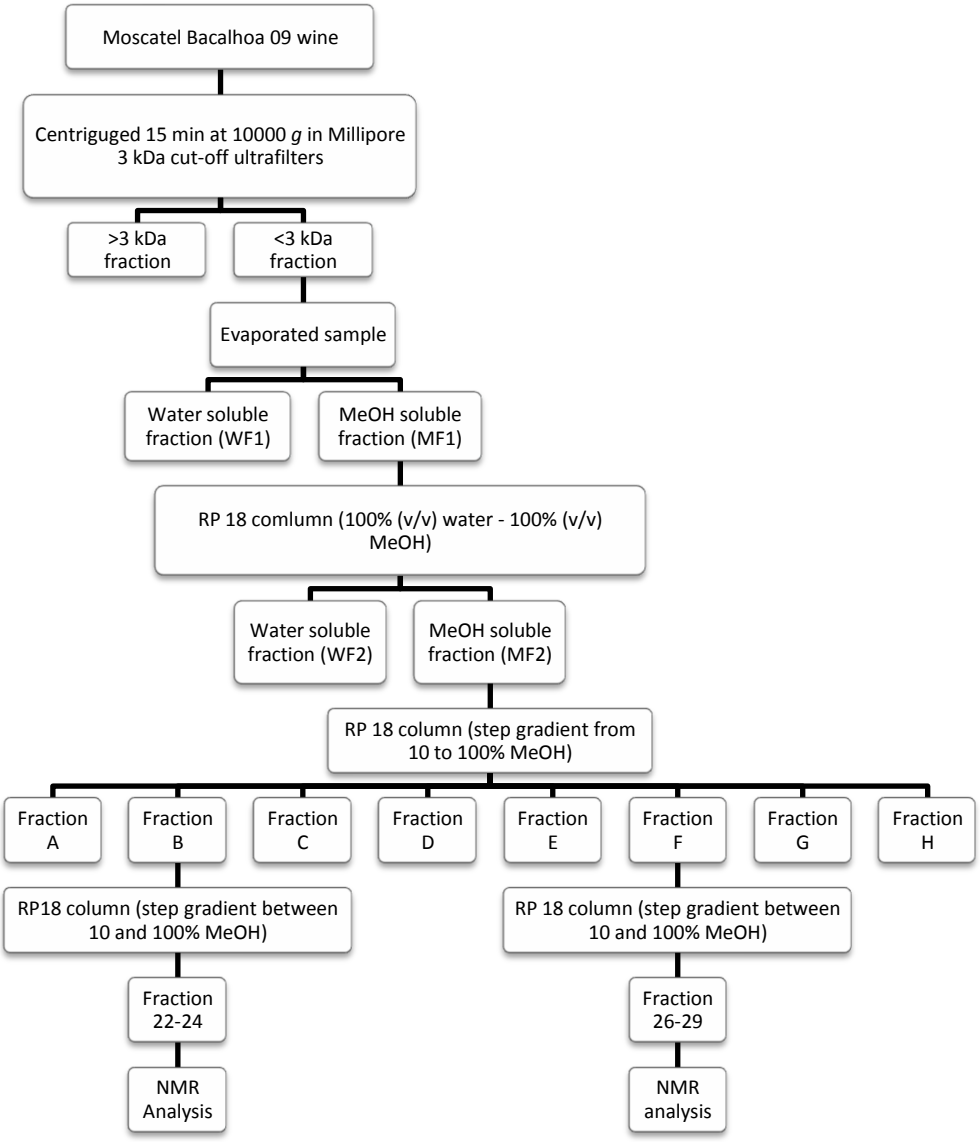


Fig. 24 - 4th experiment scheme

Due to the impurities present in the MF2ab fraction, observed in the NMR spectra, and due to the small amount of the sample, another experiment was performed to isolate these compounds to a higher purity level.

Analogously to the other experiments, the wine was fractionated until the separation of the WF2 fraction (containing the erythritol) from the MF1 fraction (containing the factor X, among other compounds).

The WF1 fraction was passed through a RP18 chromatography column with a gradient of methanol from 10 to 100% (v/v), collecting every 5 mL of eluent. After fractionation, 40 fractions of 5 mL were obtained. A sample of each collected aliquot was subsequently applied in a RP18 TLC chromatography sheet, using methanol (70% v/v) as eluent, in order to understand the complexity of each aliquot (Fig. 25).

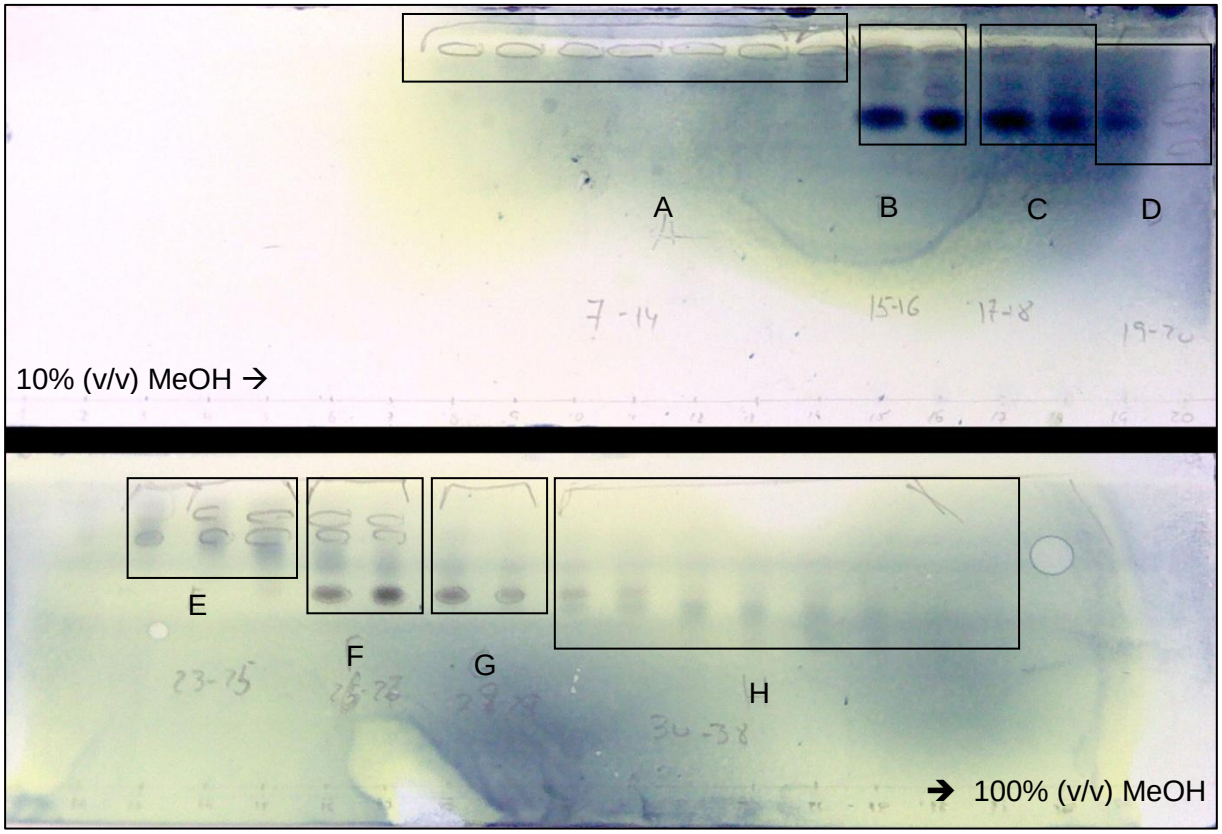


Fig. 25 – Analysis of the 40 aliquots collected in the RP18 column, using a RP18 TLC sheet with methanol (10 to 100% v/v) as the eluent. The TLC sheet was revealed with phosphomolybdic acid.

The 5 mL fractions pooled according to band similarity and, at the end, eight distinct fractions were obtained, designated A to H. All eight fractions were dried and weighed.

The eight fractions were subsequently injected in the HPLC using the methodology described in the Materials and Methods section. To prepare the samples, the fractions were dissolved in 0.5 mL of gradient-grade methanol for HPLC (CHROMASOLV - Sigma). An analytical C18 HPLC column was used and a volume of 10 µL of each sample was injected. The analysis revealed inconclusive.

After TLC analysis of the eight fractions, the two fractions that presented higher concentration of compounds were fraction B and fraction F (Fig. 25). The bands that correspond to their main compounds are the dark green bands clearly visible in the TLC.

Both fractions, B and F, were once more passed through a RP18 chromatography column with a gradient of methanol between 10 and 100% (v/v). Five mL fractions were collected. After fractionation, TLC analysis was applied to each aliquot from both fractions using TLC RP18 chromatography, with methanol 70% (v/v) as eluent (Fig. 26).

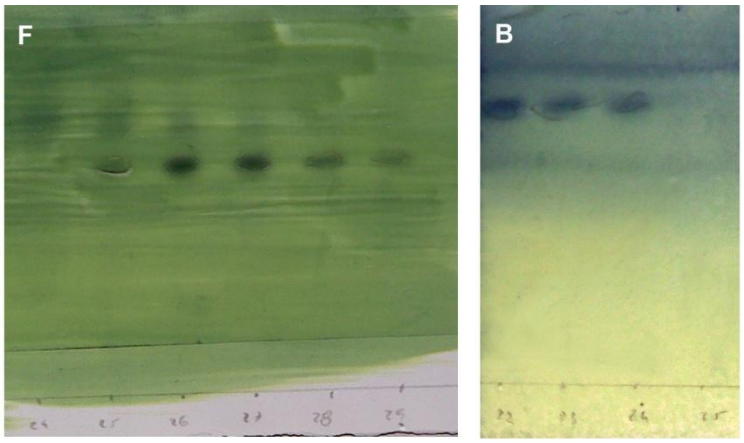
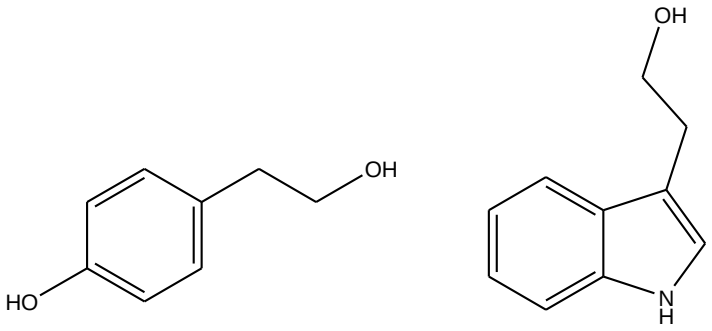


Fig. 26 – Analysis of the fractions B and F by TLC after fractionation using a RP18 chromatography column with a step gradient between 10 and 100% (v/v) methanol. The scale in the TLC sheets represent the methanol concentration used in the RP18 column during the fractionation.

After TLC analysis, the aliquots 26 to 29 from the F fraction, and aliquots 22 to 24 from the B fraction were pooled (Fig. 26). This selection was made based on band similarity and Rf values, which were respectively 0.57 for the F fraction compounds and 0.67 for the B fraction compounds. Both mixtures were analyzed by NMR in order to identify the major compounds present.

Structural determination on these two fractions was based on the two dimensional NMR experiments that allowed the clear attribution of all protons. The ^{1}H-NMR aromatic pattern for the aliquots 22 to 24 from B fraction and 26 to 29 from F fraction (respectively compounds 1 and 2) shows aromatic patterns with an 1,4 substitution (two doublets with the coupling constants near 8Hz) for compound 1 and a more complicated one (with 5 aromatic hydrogen atoms) for compound 2. Both compounds show two triplets for two hydrogen atoms each compatible with an ethanolic side chain. Mass spectrometry analysis agrees in both cases with the proposed structures (Fig. 27).



1 **2**

Fig. 27 – Proposed structure of compounds 1 and 2.

The eight fractions identified in the TLC sheet presented in Fig. 25, including the purified F and B fractions, were subjected to heat stability test (Fig. 28).

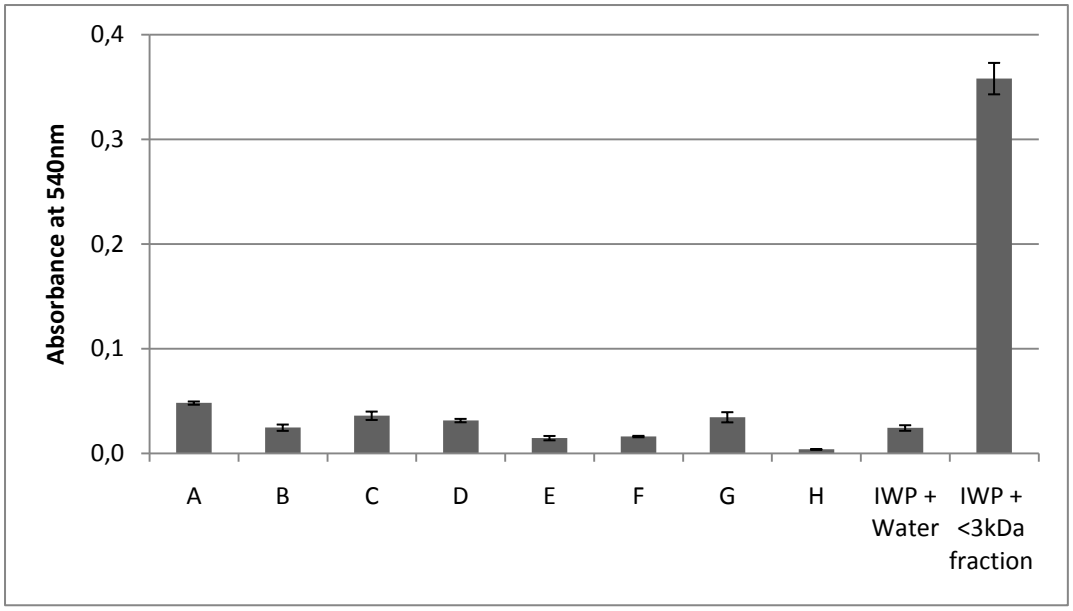


Fig. 28 - Heat Changes in turbidity (detecting by measuring the absorbance at 540 nm) observed after heat stability test performed to the fractions A to H back-added with isolated wine protein, isolated wine protein in water (IWP + water) and isolated wine protein with the <3 kDa fraction of the Moscatel Bacalhoa 09 wine (IWP + <3 kDa fraction).

After heat stability test, none of the fractions analyzed presented a similar haze formation potential compared to the wine isolated protein with the <3 kDa Moscatel Bacalhôa wine fraction.

These results demonstrate that neither fraction have, by itself, a significant role in the wine protein haze formation when isolated from each other. However, the first four fractions (fractions A, B, C and D) and fraction G showed higher formation after heat stability test (Fig. 28).

5th experiment

The fractions individually obtained in the 4th experiment had to be tested using combinations of the different fractions, in order to understand if the interaction of the distinct compounds present increases the protein haze formation potential. To perform the heat stability test with such fraction combinations, the same protocol used in the 4th experiment (Fig. 24) was repeated.

Analogously to the 4th experiment, the MF2 fraction obtained, which contained the compounds that interact in the wine protein haze, was passed through an RP18 chromatography column with a gradient of methanol between 10 and 100% (v/v) as eluent. Five mL fractions were collected. Sixty individual aliquots were obtained, and tested in a RP18 TLC sheet with methanol 70% (v/v) as eluent (Fig. 26).

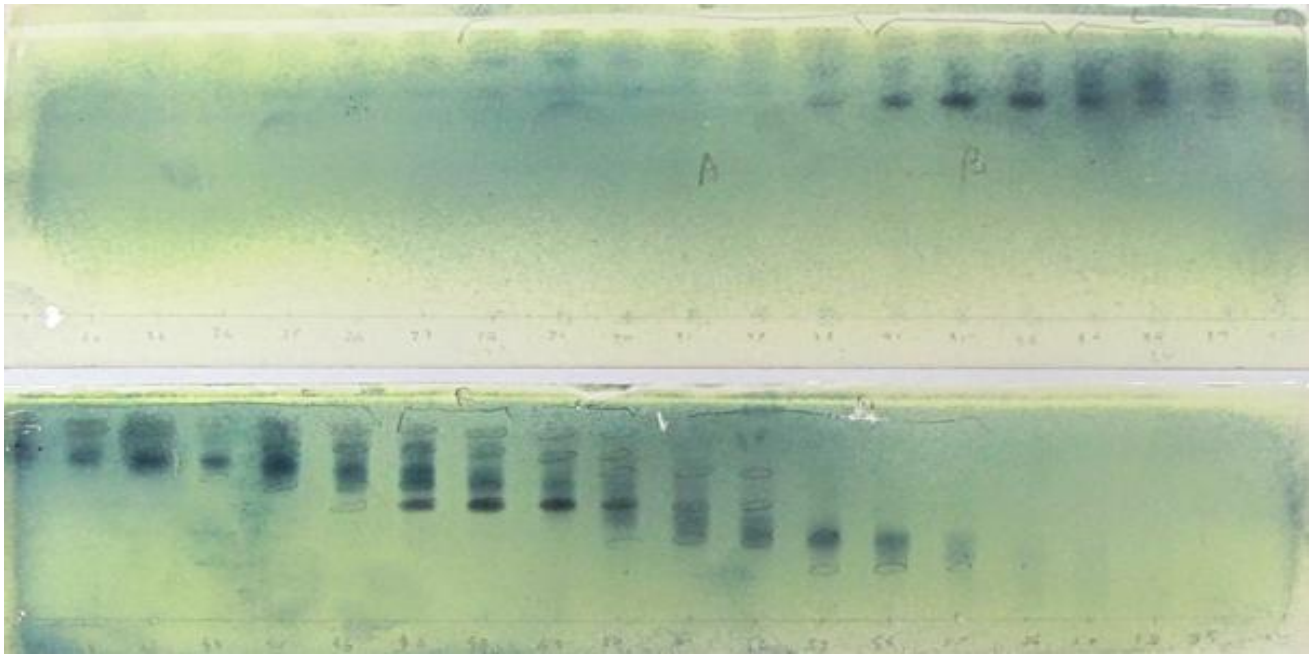


Fig. 29 - Analysis of the 40 aliquots collected in the RP18 column, using a RP18 TLC sheet with methanol (10 to 100% v/v) as the eluent. The TLC sheet was revealed with phosphomolybdic acid.

The obtained TLC (Fig. 29) has a very similar pattern when compared to the TLC in the 4th experiment (Fig. 25), confirming that the same compounds are present in these fractions. The fractions were pooled to obtain fractions similar to those obtained in the 4th experiment. Two pooled fractions were prepared, one resulting from the combination of fractions A, B, C and D, the other from the combination of fractions E, F, G and H.

Due to lack of wine isolated protein, two heat stability tests were chosen: one involving the fraction ABCD with isolated wine protein in model solution and other involving the fraction ABCD with L-threitol (Sigma-Aldrich, 99%), isolated wine protein and wine model solution (Fig. 30). Based on the weight of the water-soluble fraction (WF2) from the 1st experiment, it was considered that, being the erythritol the main compound of the WF2 fraction, it would be added the same mass of L-threitol to fraction ABCD, i.e. 10 g/L.

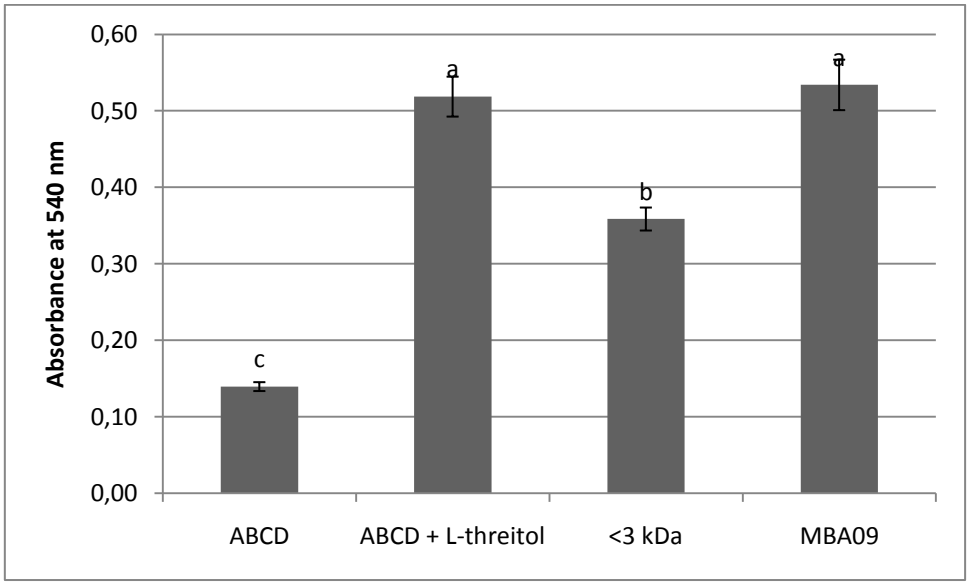


Fig. 30 - Heat Changes in turbidity (detecting by measuring the absorbance at 540 nm) observed after heat stability test performed to fractions ABCD, ABCD + L-threitol, <3 kDa Moscatel Bacalhôa 09 wine compounds and Moscatel Bacalhôa 09 wine. All fractions were back-added with isolated wine protein (250 mg/L) apart from the Moscatel Bacalhôa 09 wine.

Analyzing the data there is no significant difference between the fraction ABCD + L-threitol and the Moscatel Bacalhôa 09 wine. The fraction ABCD without the L-threitol showed much less haze formation when compared to the other fractions. These results indicate that the L-threitol may be involved in protein haze formation when added to the fraction ABCD.

There is a significant difference between the ABCD + L-threitol fraction and the <3 kDa Moscatel Bacalhôa 09 wine compounds. The two main reasons for this phenomenon are the possible excess of L-threitol in the ABCD + L-threitol fraction when compared to the wine and the concentration of extract used in the experiment. Due to sample loss during the fractionation process, the final extracts are five times concentrated, which can influence the haze formation after heat stability test.

FINAL CONSIDERATIONS

The presence of proteins in wine, in particular PR proteins, is a requirement for protein haze formation (Ferreira *et al*., 2002). As observed in the first experiments of this work, wines prepared from different grape varieties with different protein contents, exhibited distinct patterns of haze formation. The varieties with higher protein content presented higher haze formation potential, in particular the variety Moscatel of Alexandria. However, two Moscatel of Alexandria wines with no significant differences in protein content presented differential protein haze formation potentials, corroborating the results previously obtained by some authors (Moretti *et al*., 1965; Bayly *et al*., 1967; Pocock *et al*., 2007), suggesting that the total protein content, by itself, failed to correlate with wine heat stability. These results support the hypothesis that other non-proteic compounds may be involved in the wine protein haze mechanism besides the protein content of the wine. Among the reported factors that can interact with protein haze formation are the pH, the ethanol, polysaccharides, phenolic compounds, metal ions and organic acids (Waters *et al*., 2005; Batista *et al*., 2010), but none was sufficiently studied yet to understand its precise role in protein haze formation.

In the wine selection chapter several varietal wines were tested including two wines prepared using high-pressure extraction musts. These wines were designated by high-pressure extraction wines and showed a very different protein haze formation behavior. After heat stability test, these wines showed almost no turbidity, revealing to be more stable to temperature than the corresponding low-pressure wines. It was hypothesized that this phenomenon occurs because of the higher content in polysaccharides present in the high-pressure extraction wines, which may act as protective colloids. High-pressure extraction wines, or press-run wines, contain higher concentration of many compounds, including phenolic compounds and polysaccharides (Ribéreau-Gayon *et al*., 2006b) that can interact with protein haze formation. However, more studies have to be performed in order to evaluate if these results are reproducible and what was the reason for the higher stability of such wines.

At the fining assay experiment, a selected wine was fined with casein to evaluate the role of phenolic compounds in protein haze formation. A similar experiment was performed by Pocock *et al*. (2007) with PVPP, revealing an increase in the wine stability after fining the wine. Unlike the same authors, in the experiment conducted in this work the protein content of the wine was evaluated after casein fining to confirm that over-fining did not occur. The heat stability test revealed that the fined wine was more stable to protein haze formation when compared to the non-fined wine, corroborating previous results from other authors

(Yokotsuka *et al*., 1983; Water *et al*., 1995; Pocock *et al*., 2007). These results suggest that phenolic compounds, or any other compound that is also affected by the casein treatment, may modulate protein haze formation. It would be of great interest to understand which family of compounds were removed with the casein fining and to which wine fraction do they belong to.

In the first experiments of wine fractionation a heat stability test was performed to compare the haze formation pH-dependent pattern of the selected wine MBA09 to the <3 kDa fraction of the same wine. Both samples exhibited similar patterns of haze formation at pH 3.2, showing significant difference at higher pH values. Comparing the absorbance values of both samples at pH 3.2, there is a significant difference that can be explained by the tartaric precipitation occurred during the storage of the <3 kDa fraction at -20 ºC. With tartaric stabilization, some wine components that may interfere in wine protein haze can precipitate with potassium hydrogen tartrate crystals, explaining the difference witnessed in Fig. 9. However, Batista *et al*. (2009) suggested the hypothesis that some organic acids, particularly tartaric acid, can have some protective effect in protein haze formation, binding to other molecules so as to prevent their interaction with proteins.

Batista *et al*. (2009) provided evidence that at low pH, protein haze formation in white wine exhibits an absolute requirement for a low molecular mass (<3 kDa) wine component, that sensitize proteins for heat-induced denaturation. On this basis, the sample wine was fractionated in order to isolate the compounds that interact with protein haze formation. The fraction that demonstrated to be the most reactive with protein was the methanol soluble fraction from the <3 kDa compounds of the wine. After NMR analysis of the methanol soluble fraction, it was concluded that the major compound of this fraction is erythritol. In a previous work developed in our lab was concluded that this compound, by itself, revealed no direct participation in protein haze formation.

To isolate the different wine fractions, several solvents and chromatography techniques were tested. The first step was to isolate the erythritol from the rest of the wine components. The adopted procedure was to submit the sample (methanol soluble fraction from the <3 kDa wine compounds) to a silica gel RP-18 chromatography column with water (100% v/v) and methanol (100% v/v) as solvents. All L-threitol was washed-out in the water soluble fraction. The methanol soluble fraction presented a higher haze formation when the isolated wine protein was back-added and subjected to the heat stability test. Nevertheless, the haze obtained with the methanol soluble fraction was always lower than that obtained with the <3 kDa wine fraction, demonstrating that some compounds present in the water

soluble fraction interact with compounds present in the methanol fraction to modulate protein haze formation.

Different fractionations to obtain increasingly pure wine fractions were performed. Such fractionations allowed the separation of eight major fractions (designated by A to H) from the methanolic extract previously isolated from the silica gel RP-18 chromatography column, as shown in Fig. 25. Two of these fractions showed major compounds that were subsequently analyzed using NMR and GCT techniques. The results revealed two different compounds showing aromatic patterns with an 1,4 substitution (two doublets with the coupling constants near 8 Hz) for the compound identified as compound 1 and a more complicated one (with 5 aromatic hydrogen atoms) for the compound identified as compound 2 (Fig. 27). Both compounds show two triplets for two hydrogen atoms each compatible with an ethanol side chain. After structure identification it was concluded that compound 1 is the tyrosol and compound 2 the tryptophol. Individually, these compounds did not interact with wine proteins to form haze but they are part of the fraction that modulates that same haze formation.

After submitting, individually, all eight isolated fractions to a heat stability test, it was concluded that none of them protrudes. These results may indicate that the isolated compounds do not have a strict interaction with wine proteins, raising the hypothesis of an interaction of several compounds present in the methanol soluble fraction to modulate the haze formation. To test this hypothesis, a new heat stability test with a mixture of fractions A, B, C and D was performed. The difference was significantly higher for the mixture of the fractions ABCD.

As previously mentioned, the major compound isolated from the <3 kDa water soluble fraction, identified as L-threitol, did not present any detectable interaction with isolated wine protein in a heat stability test. However, in this work, after combining L-threitol with fractions A+B+C+D, the haze formation assumed a completely different behavior, showing significant higher haze when compared to the fraction ABCD by itself. As observed in Fig. 30, the haze formation revealed to be higher than the <3 kDa sample itself. These results can be explained by the concentration of the extracts and the presence of L-threitol. To compensate for sample losses during the fractionation process, the fractions were concentrated five times before heat stability test. The quantity of L-threitol used in the heat stability test was calculated based on the quantity present in the water-soluble fraction eluted from the silica gel RP-18 chromatography column during the experiments. In conclusion, it can be hypothesized that L-threitol has a significant effect in modulating wine protein haze when in the presence of some wine <3 kDa methanol soluble compounds.

Further work needs to be developed in order to qualify/quantify the exact role of each fraction/compound in the wine matrix, along with the precise quantification of L-threitol, which can lead to the identification of the X factor. Crossing this information with other factors previously identified, like the wine pH, may contribute to the development of an indispensable tool for winemakers to assess more precisely the amounts of bentonite that they have to apply to wines in order to achieve their protein stabilization. After validating the effect of the X factor in several wines, the next step will be the development of a kit, suitable to be used under cellar conditions, capable of accurately predict the risk of protein haze formation, identifying the situations in which bentonite fining is mandatory.

As previously described, the addition of bentonite to wine, removing the protein present, is currently the most effective way to stabilize it for protein haze formation. However, due to its non-selectiveness for proteins, fining with bentonite interferes negatively with the organoleptic characteristics of the wine since it adsorves other molecules with positive electric charge (Miller *et al*., 1985; Rankine 1989; Voilley *et al*., 1990). Consequently, it is necessary to develop a new methodology, suitable to use under cellar conditions, capable of removing, in a way as specific as possible, the X factor from wines, thus avoiding the use of bentonite.

The identification of the X factor may also open new investigation lines such as the characterization of the factors leading the presence / accumulation of the X factor in wines, including factors of biochemical (e.g. gene expression), physiological / pathological (e.g. weather conditions and dominant pathogenic agents during the growing season), genetic (e.g. grape variety) and technological nature (e.g. technology used in winemaking). After characterization of these factors, it will be of great interest to describe the conditions that will minimize the X factor content in wines.

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