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Estabilização proteica de vinhos: avaliação de alternativas para minimizar a aplicação de bentonite

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"Os nossos conhecimentos são a reunião do raciocínio e experiência de numerosas mentes."

Ralph Emerson

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Comunicação oral

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Resumo

As características sensoriais de um vinho, assim como a sua estabilidade, são fatores determinantes para a aceitabilidade do vinho no mercado, face à exigência dos consumidores atuais.

A desnaturação das proteínas do vinho é responsável pelo aparecimento de turvação nos vinhos brancos. Esta instabilidade proteica pode ser causada por fatores intrínsecos ou extrínsecos, como peso molecular e ponto isoelétrico das frações proteicas no vinho, força iónica, grau alcoólico e pH do vinho ou condições de armazenamento.

Para prevenir a instabilidade proteica são usados vários produtos enológicos com objetivo de remover as proteínas instáveis, evitando assim a sua desnaturação/precipitação. O agente de colagem mais usado para adsorção das proteínas do vinho é a bentonite sódica. Esta possui carga elétrica negativa, com capacidade de interagir eletroestaticamente com as proteínas do vinho de carga maioritariamente positiva, conduzindo à sua floculação e consequente precipitação. Apesar da colagem com bentonite ser o método mais utilizado, apresenta algumas limitações, particularmente quando aplicada em doses elevadas. Isto sucede, porque a bentonite para além de remover proteínas pode também interagir com outros compostos, nomeadamente com os que contribuem positivamente nas características sensoriais, tais como os compostos voláteis.

Assim, um dos objetivos do presente trabalho foi avaliar a aplicação de aditivos enológicos que permitam estabilizar as proteínas do vinho branco, em alternativa à bentonite. Numa fase preliminar, foram testados diferentes produtos nomeadamente bentonite, taninos enológicos, carboximetilcelusose (CMC), enzimas, gel de sílica, quitosana e manoproteínas em diferentes doses, com o intuito de avaliar o seu efeito na estabilização proteica do vinho branco. A bentonite e as manoproteínas foram os que apresentaram melhores resultados de estabilidade proteica.

Considerando os resultados obtidos, foram selecionadas cinco bentonites e onze manoproteínas comerciais, para ensaios de estabilização das proteínas dum vinho branco. Foi avaliada a influência destes produtos enológicos na composição fenólica, capacidade de acastanhamento, características cromáticas e sensoriais do vinho branco.

Os resultados obtidos confirmaram a eficiência da bentonite na estabilização proteica dos vinhos. Por outro lado, algumas manoproteínas estudadas também mostraram uma influência positiva na estabilização proteica do vinho. Inserido no presente estudo foi ainda efetuada uma caracterização das manoproteínas quanto à sua composição em açúcares (quantitativo e qualitativo) e o conteúdo em proteína. Com base na caracterização efetuada às manoproteínas foi possível estabelecer uma ligação entre a percentagem de manose e a sua eficiência na estabilização proteica do vinho, dependendo esta da percentagem de manose.

A bentonite não influenciou a composição fenólica, mas algumas manoproteínas diminuíram os compostos fenólicos totais. Relativamente às características cromáticas as manoproteínas de um modo geral conduziram a um aumento da luminosidade (L*) e a um aumento da coordenada da cromaticidade (b*), contudo apenas um dos vinhos tratados apresentou uma variação de cor detetável pelo olho humano.

Na análise sensorial, não foram detetadas diferenças significativas nos vinhos analisados, porém, após a análise de componentes principais, foi possível descriminar os vinhos em três grupos, sendo o grupo mais pontuado aquele que continha apenas vinhos tratados com manoproteínas. Estes resultados estão de acordo com a caracterização dos açúcares efetuada às manoproteínas, sugerindo que a elevada pontuação atribuída a este grupo, se encontra relacionada com a elevada percentagem em glucose.

Este trabalho pode fornecer informações importantes, conducentes a alternativas eficientes na estabilização proteica de vinhos brancos e que simultaneamente incrementa as características sensoriais do vinho.

Palavras-chave: vinho branco, proteínas, instabilidade proteica, testes de estabilidade proteica, turvação, bentonite, manoproteínas, características sensoriais.

Abstract

The sensory characteristics of a wine, as well the stability, are determinant factors for acceptability of wine in the market, face of current consumer exigency.

White wine proteins denaturation is responsible for the appearance of haze in white wine. This protein instability may be caused by intrinsic or extrinsic factors, such as molecular weight and isoelectric point of wine protein fraction, ionic strength, pH and alcohol content of wine or storage conditions.

To prevent protein instability, several oenological products are used, with the aimed to remove instable proteins, preventing their denaturation/precipitation. Sodium bentonite is the most commonly fining agent used to adsorption of wine proteins. This compound has a negative electrical charge, with capacity to interact electrostatically with wine proteins charged mostly positively, leading to flocculation and consequently precipitation. Although fining with bentonite being the most commonly used method, present some limitations, particularly when applied in high doses. This occurs, because addition of bentonite remove proteins and may interact with other compounds, namely with compounds that contribute positively in sensorial characteristics, such as volatile compounds.

Thus, the propose of this work was to evaluate the application of oenological additives, that enable stabilize white wine proteins, as an alternative to bentonite. In a preliminary trial, were tested different types of products, namely bentonite, oenological tannins, carboxylmethylcellulose (CMC) enzymes, silica gel, chitosan and mannoproteins with different dosage, in order to evaluate their effect in white wine proteins stabilization. Bentonite and mannoproteins presented the best results. Based on these results we select five bentonites and eleven commercial mannoproteins. In this trials, it was evaluated the influence of this oenological products in phenolic composition, browning potential, chromatic and sensory characteristics of a white wine.

The results obtained confirm the efficiency of bentonite in wine protein stabilization. Moreover, some mannoproteins studied also showed a positive influence in wine protein stabilization. Inserted into this study, it was also performed a mannoprotein characterization on sugars composition (quantitative and qualitative) and protein content. Based on mannoprotein characterization, it was possible to establish a relation

between percentage of mannose and its efficiency in wine protein stabilization, depending on mannose percentage.

Bentonite did not influence phenolic composition, but some mannoproteins decreased the total phenolic compounds. Regarding chromatic characteristics mannoproteins generally lead to an increase in lightness (L*) and an increase in yellowness (b*), however just one treated wine showed a variation in colour detected by human eye.

In sensory analyses, no significant differences were detected among the analyzed wines, however, after principal components analyses; it was possible to discriminate wines into three groups, being the high scored group, which contains just wine treated with mannoproteins. This results are in accordance with sugars characterization performed at mannoproteins, which may suggest that high score attributed at this group is related with high percentage of glucose.

This work may provide important information, leading to efficient alternatives in white wine proteins stabilization, and simultaneously, increase sensory characteristics.

Keywords: white wine, proteins, unstable protein, protein stability tests, protein precipitation, haze, bentonite, mannoproteins, sensory characteristics.

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Abreviaturas e símbolos

Ag – Silver

AgCl – Silver Chloride

$Ba(OH)_2$ – Barium Hydroxide

Ca – Calcium

CMC – Carboxylmethylcellulose

Cu – Copper

Fe – Iron

g – Grams

g- gravity force

HCl – Cloridric acid

HPLC – High performance liquid chromatography

H_2SO_4 – Sulphuric acid

IEF – Isoelectric focusing

K – Potassium

kDa – Kilo Dalton

L – Litre

MALDI-TOF – Matrix Assisted Laser Desorption Ionization – Time of Flight

Mg – Magnesium

mL – Millilitre

Mm – Molecular mass

MW – Molecular weight

Na – Sodium

Na_2CO_3 – Sodium Carbonate

NaOH – Sodium Hydroxide

Nm –Nanometres

NTU – Nephelometric turbidity unit

PAGE – Polyacrylamide gel electrophoresis

pI – Isoelectric point

PR – Pathogeneses-Related

SDS – Sodium dodecyl sulfate

TCA – Trichloroacetic acid

% – Percentage

% v/v – Percentage on volume

μL – Microlitre

1. Introdução

1.1 Introdução geral

O vinho é um produto com elevado interesse económico e cultural, sendo crescente a exigência por parte dos consumidores a nível da qualidade e estabilidade do produto.

A instabilidade proteica do vinho branco não é uma problemática recente, porém é um problema que ainda não foi possível colmatar eficazmente, sem modificar significativamente as características físico-químicas e sensoriais dos vinhos.

As proteínas, apesar de existirem em baixa concentração no vinho (15 a 300 mg/L), são dos constituintes mais importantes na estabilidade coloidal, podendo afetar a sua limpidez e consequente estabilidade. Estas podem originar turvação e/ou formação de depósitos amorfos no vinho engarrafado, resultante da desnaturação de proteínas instáveis que posteriormente podem precipitar (Waters *et al.*, 2005). Esta instabilidade é considerada um defeito do vinho branco, podendo conduzir à sua rejeição, uma vez, que a limpidez do vinho é uma qualidade exigida pelo consumidor (Ribéreau-Gaynon *et al.*, 2006; Sauvage *et al*., 2010). A concentração de proteínas, bem como a composição das frações proteicas presentes no vinho, estão relacionadas com fatores como casta, condições climáticas, estado de maturação das uvas e processo de vinificação (Pashova *et al.*, 2004; Sauvage *et al*., 2010), podendo a sua precipitação ser induzida por condições de armazenamento desfavoráveis (Ferreira *et al.*, 2002).

As proteínas do vinho provêm da uva, de *Vitis vinifera*, e da autólise das leveduras, *Sccharomyces cerevisiae* (Ferreira *et al*., 2002; Zoecklein, 1988), porém a maior fonte são as uvas (Waters *et al*., 2005), tal é demonstrado recorrendo a testes imunológicos (Ferreira *et al*., 2000) e testes electroforéticos (Esteruelas *et al*., 2009b). Alguns autores afirmam que a instabilidade está relacionada com a concentração de proteína total presente no vinho (Mesquita *et al.*, 2001; Ferreira *et al*., 2002). Contudo, uma vez que as diferentes frações proteicas se comportam de maneira distinta e possuem uma sensibilidade diferente à desnaturação (Bayly e Berg, 1967; Hsu e Heatherbell, 1987b; Esteruelas *et al*., 2009a,b), a instabilidade está dependente de frações proteicas específicas (Fusi *et al.*, 2010). A ocorrência de precipitação proteica pode estar na causa de alterações intrínsecas ou extrínsecas, como o valor de pH, o teor de etanol, o teor de compostos fenólicos e a temperatura, sendo que estas alterações podem ocorrer durante o loteamento dos vinhos (Boulton, 1980; Sarmento *et al*., 2000a).

As principais proteínas do vinho responsáveis pela turvação, são as quitinases e as proteínas do tipo taumatina (Falconer *et al*., 2010), estas pertencem ao grupo das proteínas relacionadas com a patogenicidade que são sintetizadas durante a maturação das uvas, como mecanismo de defesa de infeções por agentes patogénicos (Waters *et al*., 2005). Devido ao seu mecanismo de resistência à proteólise, bem como à sua estabilidade ao pH ácido do vinho, as proteínas relacionadas com a patogenicidade têm capacidade de persistir após o processo de vinificação (Linthorst, 1991; Vincenzi *et al*., 2005; Waters *et al*., 2005). Sauvage *et al*. (2010) afirma que outras famílias do grupo das proteínas relacionadas com a patogenicidade podem estar na causa da turvação do vinho branco, como por exemplo as glucanases. Também está descrito que o peso molecular e o ponto isoeléctrico das frações proteicas são relevantes na estabilidade, visto que as frações com menor peso molecular (12.6 kDa – 30 kDa) e menor ponto isoeléctrico (4.1 – 5.8) são as mais instáveis ao pH do vinho (Hsu e Heatherbell, 1987a; Waters *et al*., 1991).

A limpidez deve ser uma característica permanente do vinho, daí a importância da estabilização proteica dos vinhos. Para determinar a instabilidade proteica de um vinho, bem como o aditivo enológico a adicionar, assim como a dose correta para prevenir a instabilidade, é necessário recorrer a ensaios laboratoriais e a testes de estabilidade (Sarmento *et al*., 2000a). Os testes de estabilidade proteica podem ser classificados de acordo com os seus mecanismos de ação em testes de desnaturação térmica, ensaios de proteína total, desnaturação química e diminuição de solubilidade (Boulton, 1980; Mesrob *et al*., 1983; Dawes *et al*., 1994; Sarmento *et al*., 2000a, Esteruelas *et al*., 2009a). Os testes mais utilizados são, o teste do calor (Berg e Akiyoshi, 1961; Pocock e Rankine, 1973), o teste do ácido tricloroacético (Berg e Akiyoshi, 1961, Boulton, 1980), o bentotest (Rankine e Pocock, 1971), o teste do etanol (Boulton, 1980) e o teste do tanino (Mesrob *et al*., 1983). Porém, todos os testes referidos produzem precipitados muito diferentes, quando comparados com o precipitado natural, não sendo considerada uma reprodução perfeita do fenómeno, já que o precipitado geralmente contém um teor de proteína, compostos fenólicos e polissacarídos mais elevados (Esteruelas *et al*., 2009a). Também a dose de agente de colagem a adicionar no vinho, para prevenir a instabilidade, depende do teste de estabilidade efetuado (Esteruelas *et al*., 2009a). Apesar disso, o teste do calor, também conhecido como teste de transporte, não só é o mais usado, como é o que apresenta resultados mais próximos do comportamento

normal do vinho, sendo um indicador adequado para determinar a dose correta de produto enológico para estabilizar o vinho quanto à instabilidade proteica (Sarmento *et al*., 2000a). Também o teste do ácido tricloroacético apresenta resultados favoráveis aproximando-se do teor de proteína total (Boulton, 1980).

A aplicação de bentonite é dos processos mais utilizados e mais efetivos para remover as proteínas do vinho, estando a sua eficiência dependente do tipo de bentonite, dose adicionada, temperatura, pH e composição do vinho (Ribéreau-Gayon *et al*., 2006). A bentonite é uma argila natural, formada sobretudo por montemorilonite, composta quimicamente por silicato hidratado de alumínio, sódio, cálcio e magnésio, e impurezas. As bentonites sódicas e cálcicas são as formas predominantes, contudo é a bentonite sódica a mais utilizada, devido ao seu elevado poder de hidratação e adsorção, (Catarino *et al*., 2004) em particular das proteínas estáveis e instáveis (Blade e Boulton, 1988; Zoecklein, 1988; Sarmento *et al*., 2000b). Após hidratação da bentonite, o líquido de intumescimento (água ou vinho), proporciona espaços vazios, sendo maior a eficiência em água, levando-a a expandir a sua área superficial, assim como, formar um gel com forte carga negativa ao pH normal do vinho. É esta carga negativa que interage eletoestaticamente com os colóides presentes no vinho, carregados positivamente, como as proteínas, e conduz à floculação (Lambri *et al*., 2010; Sauvage *et al*., 2010), sendo possível remover os agregados formados através de uma posterior filtração. Contudo, a utilização de bentonite também pode trazer consequências indesejáveis, uma vez que este agente de colagem não é específico apenas para proteínas, podendo remover também outras moléculas do vinho carregadas positivamente (Ferreira *et al*., 2002; Lambri *et al*., 2010) como por exemplo, compostos aromáticos e fenólicos, e consequentemente conduzir a alterações sensoriais dos vinhos.

Atendendo às especificidades descritas da bentonite para as proteínas instáveis do vinho, têm sido estudadas alternativas ao seu uso, como por exemplo a aplicação de colóides protetores como é o caso das manoproteínas (Waters *et al*., 1993; Waters *et al*., 1994a; Gonzales-Ramos *et al*., 2008), utilização de adsorventes como, por exemplo, óxidos de zircónio (Pashova *et al*., 2004; Salazar *et al*., 2006; Salazar *et al*., 2010), resinas de troca iónia, gel de sílica, hidroxipatite ($3Ca_3(PO_4)_2.Ca(OH)_2$), alumínio (Sarmento *et al*. 2000b), quitina (Vincenzi *et al*., 2005) e zeólitos naturais (Mercurio, *et al*., 2010), uso de ultrafiltração (Hsu *et al*., 1987; Flores, *et al*., 1990), adição de enzimas proteolíticas (Feuillat e Ferrari et al., 1982; Waters *et al*., 1992; Dizy e Bisson,

1999) e pasteurização (Francis *et al*., 1994; Pocock *et al*., 2003). Contudo estes métodos continuam a ser pouco satisfatórios, como por exemplo no caso da adição de enzimas proteolíticas já que ainda não foi encontrada uma protease capaz de hidrolisar as proteínas responsáveis pela turvação nas condições existentes nos vinhos.

A utilização de manoproteínas para estabilização proteica dos vinhos pode, no entanto, proporcionar um efeito positivo na qualidade dos mesmos (Gonzales-Ramos *et al*., 2008; Waters *et al*., 1993; Waters *et al*., 1994a), nomeadamente, prevenir a formação de turvação (Gonzales-Ramos *et al.*, 2006) e melhorar as características sensoriais (Escot *et al*., 2001). As manoproteínas são extraídas das células purificadas de paredes celulares de levedura, via enzimática usando β-glucosidase exo-1,3, para digestão dos glucanos (Klis *et al.*, 2002), ou via física ou química. Além das manoproteínas da parede celular das leveduras outras glicoproteínas têm demonstrado efeito protetor, incluindo as invertases (Moine-Ledoux e Dubourdieu 1999; Dupin *et al*. 2000), glicoproteínas da uva ou da maçã contendo arabinogalactanas (Waters *et al*., 1994b; Pellerin *et al.*; 1994) e goma-arábica (Pellerin *et al*., 1994). Porém, Waters *et al*. (1993) afirma que estes colóides protetores nos vinhos não previnem a desnaturação térmica das proteínas, mas diminuem a dimensão das partículas, adquirindo o vinho uma aparência mais límpida, explicando assim o seu efeito no controlo da estabilidade do vinho.

1.2 Objetivos e metodologia geral

A utilização de manoproteínas como agente estabilizante a adicionar ao vinho, carece ainda de estudos que envolvam a sua caracterização, dosagem e seus efeitos, quer a nível da estabilização proteica, quer a nível da qualidade sensorial dos vinhos. Assim neste contexto, o presente trabalho representa um contributo para o conhecimento dos efeitos do uso de manoproteínas comerciais na estabilização proteica de vinhos brancos, uma vez que os vinhos sujeitos à instabilidade proteica são essencialmente brancos, devido ao seu baixo teor em polifenóis, pois os tintos e rosés raramente apresentam turvações devido a este tipo de instabilidade.

Sendo a bentonite um dos processos mais utilizados e mais efetivos para remover as proteínas do vinho, esta foi usada como termo de comparação às manoproteínas.

Assim, pretendeu-se:

- Efetuar ensaios com bentonite e outros produtos enológicos com o intuito de estabilizar os vinhos quanto à instabilidade proteica;
- Selecionar os produtos que estabilizam o vinho branco quanto à instabilidade proteica e efetuar a sua caracterização;
- Verificar a influência dos produtos selecionados na concentração de compostos fenólicos totais, flavonóides e não-flavonóides do vinho;
- Verificar a influência dos produtos selecionados na concentração dos ácidos fenólicos do vinho;
- Verificar a influência dos produtos selecionados na cor, características cromáticas e potencial de acastanhamento do vinho;
- Verificar a influência dos produtos selecionados nas características sensoriais dos vinhos.

A dissertação encontra-se organizada em 4 capítulos. Para além da introdução, na qual é feito um enquadramento teórico e são definidos os objetivos do trabalho, no capítulo 2, de revisão bibliográfica, são abordados os processos alternativos para a estabilização das proteínas no vinho, sendo tecidas considerações sobre o uso de bentonites e manoproteínas. No capítulo 3, encontram-se referidos os materiais e

métodos utilizados neste trabalho, os ensaios com bentonites e diferentes tipos de manoproteínas na estabilização dos vinhos, os efeitos nos compostos fenólicos, cor e nas características cromáticas e sensoriais nos vinhos tratados e ainda feita uma caracterização das manoproteínas comercias usadas. Neste capítulo, procede-se ainda à discussão dos resultados, já que à semelhança do anterior, aparece na forma de artigo. Finalmente no capítulo 4, são sintetizadas as principais considerações alcançadas neste estudo.

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2.Alternative processes for wine protein stabilization: A review

2.1 Abstract

The problem of haze in wine, depends of several factors, but grape proteins are normally major cause, they are designed pathogenesis related proteins and have ability to resist to proteolysis at wine pH. Stability tests are important to establish susceptibility to protein instability and to ensure effectiveness of selected method for protein stabilization. Among them, heat test correlate well forced precipitate with natural precipitate. The most commonly method to remove unstable white wine proteins is adsorption by sodium bentonite; however, other methods have been studied. Here, we discussed proteins stability tests and alternative process for wine protein stabilization, namely regarding the use of bentonites and mannoproteins.

Keywords: white wine, haze, unstable protein, protein stability tests, bentonite, mannoproteins.

2.2 Introduction

Wine is a complex matrix composed by more than 800 compounds; some of them not fully identified (Mijares and Sáez, 2000). Proteins are present in wine at low concentration, depending of content and composition on grape variety and maturity as well as on the winemaking process (Sauvage et al., 2010). These compounds could be responsible for colloidal instability and haze of wines (Waters et al., 2005; Sauvage et al., 2010). Therefore, knowledge of grape or wine protein fractions is essential, since some of they could be responsible for turbidity before or after bottling.

In white wine these issues are more pressing, since clarity is an essential quality feature required by consumers. The heat under bottled wine provoke protein haze, that is a common problem for markers of white wine (Hoj et al., 2000) but does not affect the olfactory and gustatory characteristics of the wine (Batista et al., 2009), however deposit formation or haze in bottled wines affect immediately its commercial performance, making them unacceptable for consumers (Sauvage et al., 2010).

The most important proteins related with white wine instability are pathogenesis-related proteins of *Vitis vinifera*, these include chitinases and thaumatin-like proteins (Robinson and Davies, 2000; Ferreira et al., 2002). These proteins slowly denaturate and aggregate during wine storage, giving a light dispersing haze (Waters et al., 1993).

Protein instability is currently prevented by removing proteins using fining agents. Fining agents are substances normally with electric charge (negative or positive), that are introduced in wine, which immediately flocculate and precipitate the particles with opposite electrical charge responsible for wine turbidity. This process is known as fining (Cardoso, 2007). Bentonite addition is the most commonly used process to prevent protein instability in white wine, by using the right dose, determined by stability tests (Lambri et al., 2012a). However, bentonite fining could affect wine quality under some conditions, like the removal of colour, flavour and aroma compounds (Waters et al., 1996; Høj et al., 2000), changing in this way wine sensory characteristics. Consequently, alternative techniques for bentonite fining have been studied, such as ultrafiltration (Hsu et al., 1987; Flores, et al., 1990), addition of proteolytic enzymes (Feuillat and Ferrari et al., 1982; Waters et al., 1992, Dizy and Bisson, 1999), flash pasteurization (Francis et al.,1994; Pocock et al., 2003), alternative adsorbents (silica gel, hydroxyapatite and alumina) (Sarmento et al. 2000b), zirconium oxide treatment

(Pashova et al., 2004; Salazar et al., 2006), natural zeolites (Mercurio et al., 2010), chitin (Vincenzi et al., 2005b) and the use of some mannoproteins (Gonzales-Ramos et al. 2008).

2.3 Characterization of white wine proteins

Proteins are the major nitrogenous compounds in wine and their concentration in unfined wine ranged from 15 to 300 mg/L (Ferreira et al., 2002; Waters et al., 2005; Batista et al. 2009), but may up to 700 mg/L (Vincenzi et al., 2005a). The wine proteins are composed by different protein fractions and the concentration of each fraction depends on grape variety, climate conditions in the region, soil, vineyard management, winemaking conditions, and others, that affect strongly the final protein content (Zoecklein, 1991; Pashova et al., 2004). Some of these proteins, even at low concentrations, are the principal responsible for protein instability and haze of white wines (Esteruelas et al., 2009b; Sauvage et al., 2010). The proteins responsible for instability survive throughout the winemaking process, because they are highly resistant to proteolysis and to the low must and wines pH (Ferreira et al., 2004).

Soluble proteins in grape and wine are globular (mainly albumins) and molecular mass (Mm) of these protein fractions is described to be distributed over a wide range (6 – 200 KDa) (Santoro, 1995) and isolectric point (pI) have been described from pI of 2.5 – 8.7 (Anelli, 1977; Yokotsuka et al. 1977; Heatherbell et al. 1985) (Table 2.1).Wine proteins have been considered a mixture of grape proteins, of *Vitis vinifera*, and in a minor extent, from autolyzed yeast of *Saccharomyces cereviseae* (Zoecklein, 1988; Ferreira et. al, 2002). Different methods have been used for grape and wine protein characterization (Table 2.2). The main source of wine proteins are grapes (Waters et al., 2005), which was demonstrated by immunological (Ferreira et al., 2000) and electrophoretic (Esteruelas et al., 2009b) methods.

Table 2.1 - Isoelectric point (pI) and Mm (kDa) identified in different protein fraction from grape and wine

Isoelectric Point (pI)		Molecular Mass (Mm)		Author
Grape	**Wine**	**Grape**	**Wine**	
	3.1-8.3			Correa et al. (1988)
	4.0-8.2		10.0-70.0	Dawes et al. (1990)
			15.5-69.0	Dorrestein et al. (1995)
	4.1-5.8	11.2-190.0		Hsu and Heatherbell (1987a)
5.6-7.6		19.0-100.0		Lamikanra (1987)
	4.6-8.8		12.0-41.0	Lamikanra and Inyang (1988)
			18.0-23.0	Moretti and Berg (1965)
	3.1-9.2		11.0-88.1	Murphy et al. (1989)
	3.0-5.6		14.0-94.0	Pueyo et al. (1993)
	3.2-9.0			Santoro (1994)
	3.6-9.0		6.0-200.0	Santoro (1995)
			10.0-50.0	Somers and Ziemelis (1973)
			10.0-64.0	Waters et al. (1990)
			21.0-65.0	Yokotsuka et al. (1991)
	2.5-9.7			Anelli (1977); Yokotsuka et al. (1977); Heatherbell et al. (1985)

Some authors claim, that haze is related to total protein concentration, and thus wines with high total protein content, showed also more tendency to become unstable (Mesquita et al., 2001; Ferreira et al., 2002). Nevertheless, other authors think that instability is not related to total protein concentration (Moretti and Berg, 1965; Ferreira et al., 2002), if each individual protein fraction behaves differently (Bayly and Berg, 1967; Hsu and Heatherbell, 1987a; Esteruelas et al., 2009a, Esteruelas et al., 2011), considering in this way that protein instability is caused by the presence of some specific protein fractions (Moretti and Berg, 1965; Fusi et al., 2010).

Table 2.2 - Some analytic methods used for grape and wine protein characterization

Analytic method	Author
Paper electrophoresis	Diemair et al. (1961)
PAGE	Moretti and Berg (1965); Bayly and Berg (1967); Correa et al. (1988); Pueyo et al. (1993); Santoro (1995)
SDS-PAGE	Yokotsuka et al. (1977); Correa et al. (1988); González-Lara et al. (1989), Waters et al. (1990); Dawes et al. (1994); Pueyo et al. (1993); Santoro et al. (1994); Dorrestein et al. (1995); Esteruelas et al. (2009a)
IEF	Anelli (1977); Correa et al. (1988); Gonzáles-Lara et al. (1989); Pueyo et al. (1993); Sauvage et al. (2010)
IEF-PAGE	Dawes et al. (1990;1994); Santoro (1995)
IEF-SDS-PAGE	Laminkara (1987); Laminkara e Inyang (1988)
IED-LDS-PAGE	Hsu and Heatherbell (1987a; 1987b); Hsu et al. (1987)
Capillary electrophoresis	Ledoux et al. (1992); (Luguera et al., 1997)
HPLC	Dubourdieu et al. (1986); Waters et al. (1990); Santoro (1995)
Immunologic	Ferreira et al. (2000)
MALDI-TOF	Sauvage et al. (2010)

PAGE – Polyacrylamide gel electrophoresis; SDS – Sodium dodecyl sulfate; IEF – Isoelectric focusing; HPLC – High performance liquid chromatography; MALDI-TOF – Matrix Assisted Laser Desorption Ionization – Time of Flight.

Denaturation of wine proteins lead to aggregation and flocculation resulting in a turbid suspension and finally formation of precipitates (Waters et al., 2005). Intrinsic or extrinsic alterations may be the cause of precipitation, such as pH, ethanol content, temperature and amount of phenolic compounds, and these reactions usually occur when wines are blended or during storage (Boulton, 1980; Sarmento et al. 2000a). Although, wine protein precipitation is considered a multifactorial process and haze may be controlled by non-protein factors, known as x factor (Batista et al., 2010). In this context, Waters et al. (2005) has reviewed experiments that show the influence of non-protein factors, such pH, ethanol content, metallic ions and polysaccharides. Batista et al. (2009) showed two mechanisms responsible for the heat-induced precipitation of wine proteins: one occurs to a high pH values, resulting of reduced protein solubility at its pI; other occurs at lower pH values, but also at other values, depending on the x factor.

2.4 Proteins responsible for wine haze

The first studies performed in wine proteins were done by Morreti and Berg (1965) and Bayly and Berg (1967); which suggested that proteins with low isoelectric point and low molecular mass were responsible for protein instability. According to Hsu et al. (1987b) the principal proteins responsible for instability in white wine have low molecular weight (12.6 kDa – 30 kDa) and pI (4.1 – 5.8), and besides glycoproteins are also important fractions contributing to protein instability in wines. This statement was later confirmed by Waters et al. (1991), which showed that protein fractions with those characteristics are more sensitive to high temperature and contribute to wine instability and haze.

More recent studies showed that proteins responsible for wine instability are pathogenesis-related *Vitis vinifera,* proteins. Due to their high quality, *Vitis vinifera,* is the most widely cultivated species for wine production, however is particularly susceptible to fungal diseases (Ferreira et al., 2004). As a defence mechanism against fungal attacks, pathogenesis-related proteins (PR) are synthesized during ripening (Waters et al., 2005), having a harmful action on parasites structures and repairing damage caused to the plant (Odjakova et al., 2001). Proteins pathogeneses-related are important in plant performance, such as development, disease resistance and general adaptation to stressful environment (Edreva, 2005). These proteins have the capacity to persist throughout the winemaking process, since they are resistant to proteolysis and are stable at acid pH of the wine (3.0 – 3.8) (Linthorst, 1991; Waters et al., 1996).

Proteins pathogeneses-related include 14 families (Van Loon and Van Strien, 1999), grouped on base in their similarity and function. In *Vitis vinifera* grapes, the two major PR proteins isolated from wine are thaumatin-like (PR-5 family) (Waters et al., 1996) and chitinases (PR-3 family) (Waters et al., 1996 and 1998). These proteins are also major soluble proteins from *Vitis vinifera* (Pocock et al., 2000; Falconer et al., 2010). They were synthesized during development regardless of variety, region and year (Waters et. al, 1996; Ferreira et al., 2000, Monteiro et al., 2001; Ferreira et al., 2004) and increased during ripening, therefore riper grapes are susceptive to protein haze (Pocock et al., 2000).

On the other hand, Sauvage et al. (2010) shows that vacuolar invertase (GIN_1), originated from the grape and glucanases, considered pathogenesis-related, also

influence haze formation (Ferreira et al., 2000). Esteruelas et al. (2009b) concluded that protein represents a small proportion of natural protein precipitate, which is also include phenolic compounds and polysaccharides. Proteins with a molecular weight between 18 kDa and 26 kDa makes up most of the precipitate and that all unstable proteins have a pI between 4.2 – 5 0. The presence of *Vitis Vinifera* thaumatin-like protein 1 (*Vv*TL1) bands confirmed the participation of these kind of proteins in turbidity and β-(1,3)-glucanase and ripening-related proteins, Grip22 precursor, have also been detected in natural protein haze of white wines (Waters et al. 1996).

2.5 Proteins stability tests

Stability tests are used frequently in the industry to estimate the haze potential of a white wine, at protein level, before bottling, and find out right doses of fining agent required, to prevent instability (Sarmento et al., 2000a). There are many stability predictive methods to determine the wine stability, namely heat test (Ribéreau and Peynaud, 1961; Berg and Akiyoshi, 1961; Pocock and Rankine, 1973), the use of protein precipitant such as trichloroacetic acid (Berg and Akiyoshi, 1961, Boulton, 1980), phosphomolybdic acid, also called bentotest (Rankine and Pocock,1971), ethanol (Boulton, 1980) and tannin (Mesrob et al., 1983). They can be classified in accordance with their mechanisms of action (Boulton, 1980; Mesrob et al., 1983; Dawes et al., 1994; Sarmento, 2000a, Esteruelas, 2009a), as heat denaturation, total protein assays, chemical denaturation, decrease solubility, based in stimulation of protein precipitation, assuming that haze caused may occur during wine storage (Sarmento et al., 2000a). According to Esteruelas et al. (2009a), all these tests produce different precipitates comparing with natural precipitation, which are considered not a perfect reproduction of natural phenomenon, as well as different tests involves different doses of fining agent to achieve stability. Also, Esteruelas et al. (2009a) concludes that forced precipitation leads to an increase in protein content, polysaccharides and polyphenols relative to precipitate obtained naturally, namely precipitate proteins that otherwise would not appear, including the fractions with molecular weight ranging between 22 – 25 kDa, probably chitinases.

2.5.1 Heat-Test

Heat test still the most widely used in industry, because it is probably the most reliable to eventual effects of storage haze/sediment formation in bottle. This test is used to simulate the formation of protein turbidity and can be considered appropriate to determinate right dose of fining agent required to remove heat-unstable proteins, also is the less affected by other wine components (Sarmento, 2000a). However, Sauvage et al. (2010) conclude that heat-test may induce the precipitation of almost all wine proteins, leading to an overestimation of fining agent doses necessary for stabilization.

This test is based on sample heating at high temperature over a period of time. All heat-test versions are based on acceleration processes of condensation, oxidation and phenolic compounds precipitation with proteins, at high temperatures (Sarmento, 2000a). Since different wines present different behaviours of protein precipitation, wine proteins may precipitate at different temperatures (Hsu, 1986) but, the most used is Pocock and Rankine (1973) method, which after combination of various temperatures and denaturations, submits wine at 80º C over a period of 6 hours. Ribéreau-Gayon and Peynaud (1961) recommended that wine should be heated to 80ºC for 10 min. However, Esteruelas et al. (2009a) affirm that at 90ºC for 1 hour, forms a precipitate with similar natural precipitate composition, comparing with other test. The sensitivity of different protein fractions to temperature is undetermined, leading to doubt if more sensitive fractions to low temperatures along time may precipitate, which is verified, for example, at storage conditions (Sarmento, 2000a). Although the generalized use of this test, the great disadvantage is the time consumed.

2.5.2 Trichloroacetic acid test

Trichloroacetic acid test (TCA) is based on chemical destruction of protein structures at pH below 1, being able to precipitate all proteins presents in wine, coming closer to the total protein content (Boulton, 1980).

Trichloroacetic acid test consists in adding 1 mL of TCA solution at 55% to 10 mL of wine followed by heating in a water bath at 100 º C, for a reaction period of 15 minutes at room temperature before observation (Hsu, 1986). According to Berg and

Akiyoshi (1961) this test can be correlated with protein stability; however, at industrial level do not get satisfactory results.

2.5.3 Tannin test

Tannin precipitation test is based on the hypothesis that wine proteins may precipitate during storage, binding with phenolic compounds with high molecular weight, giving information about wine protein capacity to be precipitated by these compounds, in this case, tannins (Sarmento, 2000a). This test is influenced by several intrinsic wine factors, namely, pH, total protein, iron concentration, potassium and copper, not being good indicator of fining agent amount (Sarmento, 2000a; Esteruelas, 2009a).

2.5.4 Bentotest

Bentotest is a solution of phosphomolybdic acid in HCl which precipitates wine protein, by neutralizing the protein molecular charge, leading to aggregation with heavy ion molybdenum (Hsu, 1986). This procedure has the ability to precipitate all the proteins in the sample, being mainly used to estimate the bentonite addition. This test has the advantage of to be quick; however since it is more sensitivity than the heat test leads to overfining (Hsu, 1986).

2.5.5 Ethanol test

Ethanol test is based on reducing the dielectric constant, which reduced the protein solubility (Lehninger, 1981), leading to precipitation of soluble fractions at wine pH. This test is significantly influenced by total protein content, pH and calcium concentration, which may lead to differences in the development of wine turbidity.

2.6 Wine protein stabilization treatments

2.6.1 Bentonite fining

Bentonite has been used as a clarifying agent in wine for many years. This fining agent is the most common treatment used in oenology to reduce the risk of protein haze in wine (Ferreira et al., 2002). The adsorption of wine proteins by bentonite is a cation-exchange process (Boulton et al., 1996). Bentonite treatment efficiency depends on bentonite type, level of bentonite addition, temperature, pH and wine composition (Ribéreau-Gayon et al., 2006).

Bentonites are complex aluminum hydrate-silicates, belonging to montmorillonites group (Ribéreau-Gayon et al., 2006). Montmorillonites structure consists in two tetrahedral silicon oxide sheets and one octahedral aluminium hidroxide sheet, combined in a structural unit (Catarino et al., 2004) with exchangeable cationic components (Zoecklein, 1988). This exchangeable cations, Ca^{2+}, Na^{+}, and Mg^{2+}, determine the bentonite type (Lambri et al., 2010) splitting into three groups, sodium, calcium and magnesium bentonites (Catarino et al., 2004). Other cations are present such as K^{+}, Fe^{2+} and Cu^{+}, but in lower extent (Marchal et al., 1995) and this cation ratio depends on bentonites.

Calcium and sodium bentonites are the predominant forms, but sodium is still the most widely used, since they swell more than calcium bentonites (Caratario et al., 2004). Therefore, swelling can potentially increase surface area available for wine protein adsorption (Boulton et al., 1996) improving more capacity to remove suspended colloids, like positively charged proteins (Blade and Boulton, 1988; Zoecklein, 1988; Sarmento et al., 2000b). Processing time of sodium bentonite is lower than calcium, but amount of sediment is higher, while calcium bentonite causes more compact sediment. In order to improve the adsorption properties of calcium bentonites, they are activated with sodium carbonate (Na_2CO_3) at 80 °C (Blade and Boulton, 1988), obtaining calcium activated bentonites whose, properties are the same, or even better than sodium bentonites (Catarino et al., 2004).

The method to prepare bentonite significantly affects their ability to remove wine proteins (Zoecklein, 1988). Bentonite, after hydration (with water or wine), has capacity

to expand, increasing their surface area and forms a gel with a strong negative charge at wine pH. These negative charged bentonites interact electrostatically with positively charged wine colloids, in particular proteins, leading to flocculation (Lambri et al., 2010; Sauvage et al., 2010).

The adsorption of proteins can be affected by competition with cations (K^+, Ca^{2+}, Na^+, and Mg^{2+}), pH and ethanol content of solution matrix (Blade and Boulton, 1988). According to Hsu et al. (1987b), pre-hydrated bentonite at high temperature, low pH, high level of alcohol and low tannin, produces better results in clarification. Lambri et al. (2010) showed that different pH values change the efficacy of bentonite in adsorption, because more protein was removed at pH 3.60 than pH 3.30, this was related with competition between hydrogen ions and protein.

The process of bentonite treatment involves three physical reactions: dispersion of the agent, adsorption of the solutes and sedimentation of the complex (Blade and Boulton, 1988). Bentonite removes proteins by charge-charge interaction forming complexes which can be removed by filtration. Bayly and Berg (1967) conclude that removal of protein fractions by addition of bentonite did not occur in equal proportion but removes high charged protein fractions first. Bentonite removes first proteins with high pI (5.8 – 8.0) and intermediate MW (32 kDa – 45 kDa). However, in a 2-dimentional gel electrophoresis Hsu et al. (1987b), showed that to stabilize wine it is necessary to remove proteins with lower pI (4.1 – 5.8) and lower MW (12.6 kDa and 20 – 30 kDa) which include glycoproteins who represent a major fraction of proteins. This hypothesis is supported by Lambri et al. (2012b), which using five different types of activated sodium bentonite, showed that different bentonite labels can selectively remove specific proteins responsible for the turbidity after heating.

Dawes et al. (1994) found that bentonite was not selective on pI base, thus bentonite fining removed all protein fractions. Ferreira et al. (2002) and Lambri et al. (2010) claim that bentonite is not specific for proteins, and may also remove other charged species or aggregates. The presence of certain colloids is necessary, because they confer mouthfeel to the wine, and contribute to the fixation of aromatic compounds (Achaerandio et al., 2001). However, since bentonite is not specific, could also interact with aromatic compounds (Moio et al., 2004), reducing the wine volatile molecules, resulting in a loss of aroma and flavour (Lambri et al., 2010). Most of odor-active molecules are indirectly removed via deproteinization, and only a few odor-active

molecules are directly removed through adsorption (Lambri et al., 2010). Therefore, excessive amount of bentonite can cause a negative effect on wine organoleptic characteristics. It has also been described that large quantity of lees produced by bentonite fining contains 5 to 20% of the wine volume, resulting in wine lose (Lagace and Bisson, 1990). All of these reasons have led to search for alternatives to bentonite fining.

2.7 Alternative protein stabilization treatments

2.7.1 Adsorbents

The use of adsorbents has been investigated to stabilize wine proteins. It was demonstrated that zirconium oxide in powder shows ability to adsorption, of unstable proteins on the surface (Pashova et al., 2004), stabilizing the wine by removing preferentially protein fractions between 20 – 30 kDa. The use of this adsorbent achieved stability with minor negative impact (Salazar et al., 2010) on physicochemical and sensory characteristics (Pashova et al., 2004; Salazar et al., 2006). Also, Marangon et al. (2010) showed that white wine are stabilized, removing the unstable proteins, through adsorption by zirconia after treatment with 25 g L^{-1} during 72 h, however, wine present slightly lower fruit aroma and flavour intensity. Sarmento et al. (2000b) evaluate the capacity of different materials such as, swelling clays, low-swelling clay, ion-exchange resins, aluminia, hydroxyapatite, and silic gel as alternative to remove wine proteins, and results show that some ion-exchange resins have good potential to adsorb proteins like swelling clays, low-swelling clays and silica gel.

Mercurio et al. (2010) also propose alternative adsorbent, natural zeolites. Natural zeolites have a large external surface, negatively charged, that permit interactions with other cations, or polar molecules, unable to enter in their microporous structure. High zeolitizes tuff/wine ratios permit protein stabilization, and treatment with zeolite-rich powder reduce potassium ion significantly, improving the tartaric stability (Mercurio et al., 2010). Another advantage results from no affecting the concentrations of the most representative phenolic compounds, that is the taste and aromatic quality is not

significantly changed after treatment (Wyss et al., 2005). Adsorption of proteins by immobilised phenolic compounds on agarose chromatography resins has also been used to stabilize wine proteins (Power et al., 1988).

2.7.2 Ultrafiltration

Membrane ultrafiltration with different molecular mass cut-offs has been studied with the aim to resolve wine protein stability problems (Ferreira et al., 2002), this technique is based in capacity of membrane cut-offs, ranging between 1 – 100 kDa, and splitting mainly molecules with high molecular mass. However, the use of ultrafiltration to deal with problem of white wine turbidity has been relatively limited because is not known the potential removal of polysaccharides, or other macromolecules, that may be essential to the wine quality (Gonçalves et al., 2001).

Hsu et al. (1987) have investigated the effect of ultrafiltration and have used membranes with different cut-off capacity, ranging between 50 – 10 kDa. Using a membrane between 10 – 30 kDa cut-off capacities it was possible to remove 99% of wine proteins. However, proteins with MM between 12.6 – 30.0 kDa tend to cross the membrane even with 10 kDa cut-off capacity (Flores et al., 1990). These authors have also verified that wine treated with ultrafiltration has a considerably reduction in colour (A_{420}), total phenols and reduction in aromatic compounds changing the wine aromatic profile because macromolecules may be retained in the membranes (Miller et al., 1985; Fuillat et al., 1987;). Gonçalves et al. (2001) affirm that an ultrafiltration membrane with a cut-off of 100 kDa, may be an alternative for wine clarification, in terms of ratability, wine quality and tartaric stability. However, the efficiency of ultrafiltration depends of the wine composition. High costs in equipment and running associated to aromatic compounds loss, have made membrane ultrafiltration unattractive to the wine industry as an alternative to remove unstable proteins.

2.7.3 Chitin

The class IV chitinases are one of the principal responsible for haze formation (Sauvage et al., 2010). Studies carried out by Vincenzi et al. (2005b) reveal that chitin, a linear polymer from cell wall of yeast (Klis et al., 2006), remove specific wine protein, namely class IV chitinase from grape involved in white wine instability. This process reduced 80% of the haze induced by the heat test, which correspond in 29% of reduction of the wine protein content. However, effects on organoleptic quality were not known because this study not includes a sensory or a chemical analysis of wine aroma compounds after chitin treatment.

2.7.4 Proteolytic Enzymes

The use of proteolytic enzymes has been studied as an alternative technique to remove wine proteins, through enzymatic hydrolysis into small peptides and their components (Ferreira et al., 2002). This investigation used endogenous and exogenous proteolytic enzymes, such as grape proteases (Cordinnier et al., 1968), yeast proteases (Ledoux et al., 1992) and exogenous proteases (Modra, 1989).

Studies demonstrated that most proteins present in wine are pathogenesis-related proteins, for that reason they have the capacity to persist throughout the winemaking process resisting to proteolysis (Linthorst, 1991; Ferreira et al., 2002). However, Pocock et al. (2003) demonstrated that proteolytic enzymes, from different yeast strains were active in wine at high temperature at a small period of time (90 ºC for 1 min. or 45 ºC for 1 day), decreasing considerably wine protein concentration. However, white winemaking temperature is approximately 15º C, and at this temperature proteolytic enzymes are ineffective to hydrolyze wine proteins responsible for wine haze (Waters et al., 1992; Ferreira et al., 2002; Waters et al., 2005). The difficulty in removing proteins may be related to the presence of polysaccharides, who can act as protective colloids, avoiding the removed of unstable wine proteins (Waters et al., 1992). Interesting were the results of Waters et al. (1992), who showed that through the addition of an enzyme, on isolated protein fractions its demonstrated instability, degradation do not occured,

confirming resistance to proteolysis, with or without other macromolecules, so unstable proteins are not hydrolyzed with proteases treatment.

2.7.5 Flash Pasteurisation

Flash pasteurisation consists in heating wine to 90 ºC for a few seconds following fast cooling. This high speed process is less liable to affect adversely the organoleptic characteristics of wine (Francis et al., 1994; Ribéreau-Gayon et al., 2006). Pocock et al. (2003) proved that with short term heating at 90 ºC reduced the requirement of bentonite between 50-70% for the untreated wines, without affecting sensory profile of wines.

2.7.6 Mannoproteins

The use of mannoproteins in oenology has been proposed in order to reduce or eliminate bentonite application or other treatments. This method is often chosen considering there beneficial properties in protein stabilization and haze reduction in white wine, however they could also exert a positive effect on the wine quality (Waters et al., 1993; Waters et al., 1994b; Gonzales-Ramos et al., 2008).

According to Gonçalves et al. (2002), 32.2% of total polysaccharides present in white wine are mannoproteins. They originates in the outer layer of yeast cell wall, namely, *Saccharomyces cerevisiae*, constituting 35 – 40% of the cell wall, these polysaccharides are glycoproteins highly glycosylated, and covalently linked to a amorphous matrix of β-1,3-glucan (Klis et al., 2002) and contain 10 – 20 % of protein and 80 % of D-mannose related with D-glucose and N-acetyglucosamine (Rodrigues et al., 2012a,b). Mannoproteins for oenological use are extracted from purified yeast cell wall, by enzymatic extraction, using β-glucosidase exo-1,3 EC 3.2.1.58 for glucans digestion, or by physical and chemical processes, such as heat treatment of yeast wall at height temperatures (120 °C) and sterilization system with citrate buffer at pH 7, respectively (OENO 26/2004).

Several studies have been made to find the main proprieties that make mannoproteins important to winemaking order (Caridi, 2006). The major functions are adsorb ochratoxin A (Batista et al., 2004), which is a mycotoxins group from several fungi; enhance malolactic bacteria growth (Guilloux et al., 1995; Rosi et al., 1999); inhibit tartaric salts crystallisation (Moine-Ledoux et al., 2002); prevention of haze (Waters et al., 1994a; Waters et al., 2005; Gonzales-Ramos et al., 2006); promote flocculation and yeast autolyses in sparkling wines (Nunez et al., 2006); interact with phenolic compounds (Vasserot et al., 1997; Escot et al., 2001; Riou et al., 2002; Poncet et al., 2007); interact with some aromas (Lubbers et al., 1994; Wolz, 2005 ; Charlier et al., 2007); improve sensory characteristics such as reduce astringency, increment sweetness and roundness (Escot et al., 2001; Vidal et al., 2004; Guadalupe et al., 2007and 2010).

Mannoproteins are heterogeneous proteins with a molecular weight between 5 – 400 kDa, however, Waters et al. (1994a) identified a mannoprotein with 420 kDa which was composed by 30% polypeptide and 70% carbohydrate, of which 98% was mannan. Although Waters et al. (1993) have demonstrated that wine mannoproteins protect unstable proteins, preventing wine turbidity when wine was submitted at high temperatures. These authors verified that these actions do not prevent the precipitation of the proteins, instead particle size decrease, justifying in this way the wine stabilization.

Although, mannoproteins with low molecular weight, such as invertase (32 kDa) (Moine-Ledoux et al., 1999), offer greater protein stability to the wine, interaction with other wine components lead to improvement in quality. Different glycoproteins have proved their protective effect against haze, including yeast invertase (Moine-Ledoux et al., 1999; Dupin et al. 2000), wine arabinogalactan proteins (Waters et al., 1994b), gum arabic and arabinogalactan proteins from apples (Pellerin et al., 1994).

2.8 Conclusion

White wine protein instability is related with external conditions, namely high temperatures exposition, leading to protein precipitation.

Technologies of white wine clarification require detailed knowledge about proteins and other wine compounds, as well the interactions that occur between them. The principal proteins presents in the wine, responsible for haze formation, are designed pathogenesis-related and are highly resistant to proteolysis. Bentonite remains the most efficient method to remove unstable proteins from white wine; however, methods to assess the amount of fining agent to be added are considered insensitive resulting in imprecise amount estimation.

Disadvantages resulting from bentonite application leads to development of alternative methods, however none of them is able to eliminate effectively this problem. It is necessary further deepen the knowledge concerning characteristics of wine colloids, and their interactions to develop viable alternatives with less impact on wine characteristics.

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3. Potential alternatives to bentonite for white wine stabilization: Effects on physicochemical and sensory characteristics

[Under submitting process]

3.1 Abstract

Fining with sodium bentonite still the most commonly used process to stabilize wine against protein instability. However, bentonite is not selective for instable proteins and could modify the physicochemical and sensory characteristics of wine, impairing its qualities. Therefore, the focus of this work was to compare the efficiency of different bentonites and mannoproteins that could stabilize white wine proteins.

Some trials were performed in white wine, with several different products available in the market, and results showed that sodium bentonite and mannoproteins were the ones that best increased protein stability. Consequently, several mannoprotein additives were chosen and characterized concerning their sugar composition and protein content. The effects of different types of bentonite and mannoproteins on wine protein stability, phenolic compounds (total phenols, flavonoids, non-flavonoids and phenolic acids), browning potential, colour, chromatic characteristics and sensory characteristic were evaluated. This study shows that bentonite is efficient in white wine protein stabilization; however, some mannoproteins could also be used as alternative to bentonite to stabilize white wine proteins because, besides an increase in protein thermal stability, and improvement on sensorial characteristics were also observed.

Keywords: white wine, protein instability, fining, bentonite, mannoproteins, phenolic compounds, sensory attributes

3.2 Introduction

Proteins are one of the principal compounds present in white wine, responsible for colloidal instability and clarity of these wines (Esteruelas et al., 2009a; Sauvage et al., 2010; Lambri et al., 2012b). Even protein haze does not affect the olfactory and gustatory characteristics of white wine (Batista et al., 2009), in commercial bottled wines, haze is considered a defect making them unacceptable for consumers (Sauvage et al., 2010).

Wine protein fractions and their concentration in wine depends on some factors, such as grape variety, climate conditions, soil type, growth environments in the vineyard, grape maturity and winemaking process (Pashova et al., 2004; Sauvage et al., 2010). Haze may result by intrinsic or extrinsic induced changes, such as in pH, ionic strength, ethanol content and storage temperature (Boulton, 1980). Alterations in these parameters can lead to wine protein denaturation that aggregate and flocculate resulting in a turbid suspension and finally formation of amorphous precipitates (Ferreira et al., 2002, Waters et al., 2005).

Fusi et al. (2010) considered that protein instability is caused by the presence of specific proteins, and differently behaviour of each individual protein fraction (Bayly and Berg, 1967; Hsu and Heatherbell, 1987a; Esteruelas et al., 2009a; Esteruelas et al., 2011). According to Hsu and Heatherbell (1987b), protein fractions with low molecular weight (12.6 kDa – 30 kDa) and low pI (4.1 – 5.8) are the major contribute to wine instability.

The principal proteins able to induce haze have been identified in forced precipitate caused by heat, and are denominated pathogenesis-related, that include thaumatin-like proteins and chitinases, being the most abundant in wine (Waters et al.,1996; Robinson and Davies, 2000; Falconer et al., 2010). These proteins are synthesized during the ripening as a defence mechanism against fungal attacks (Waters et al., 2005), they persist throughout the winemaking process, resisting to proteolysis and being stable at acid pH (Linthorst, 1991).

To prevent protein instability, proteins were usually removed using fining agents, which are substances added to wine, that flocculate and precipitate the particles (proteins) responsible for wine turbidity (Cardoso, 2007). Bentonite, a montmorillonite clay, has been used as clarify agent in wine for many years. It is the most commonly

used process in the wine industry to prevent protein instability in white wine, using the right dose, determined by stability tests (Lambri et al., 2012a). However, the efficiency of bentonite fining depends of the bentonite type, dose, wine temperature, pH and wine composition (Ribéreau-Gayon et al., 2006). Ferreira et al. (2002) and Lambri et al. (2010) claim that bentonite is not specific for proteins, and may remove other charged species or aggregated. Therefore, bentonite fining could affect the wine quality such as removal of colour, flavour and texture compounds (Høj et al., 2001) changing the sensory properties.

Consequently, alternative techniques for bentonite fining have been studied such as ultrafiltration (Hsu Heatherbell., 1987b; Flores, et al., 1990), addition of proteolytic enzymes (Feuillat and Ferrari et al., 1982; Waters et al., 1992; Dizy and Bisson, 1999), flash pasteurization (Francis et al.,1994; Pocock et al., 2003), alternative adsorbents (Sarmento et al. 2000b), zirconium oxide treatment (Pashova et al., 2004; Salazar et al., 2006), natural zeolites (Mercurio et al., 2010) and the use of some mannoproteins (Gonzales-Ramos et al., 2008). About this later, some studies verified that mannoproteins improved wine chemical stability and sensorial quality (Waters et al., 1994; Vidal et al., 2004; Gonzales-Ramos et al., 2006).

Thus, the main objective of this study was to evaluate potential alternatives to bentonite for white wine stabilization. Based on these previous results, the further objective was to compare the effectiveness of different mannoproteins to different bentonites and to assess their effects on phenolic compounds, as well as on chromatic and sensorial characteristics.

3.3 Material and methods

3.3.1 Characteristics of the wines

For the initial evaluation white wine with following characteristics was used (wine 1): Alcohol content (% v/v) 14.8, specific gravity (20ºC) (g/mL) 0.9867, titratable acidity (g/L tartaric acid) 5.2, pH 3.4, volatile acidity (g/L acetic acid) 0.31, protein stability heat test 24.4 NTU.

For the second experiment a young white wine from Douro Valley 2011 vintage was used (wine 2). The main characteristics of the wine were as follows: Alcohol content (% v/v) 14.2, specific gravity (20ºC) (g/mL) 0.9890, titratable acidity (g/L tartaric acid) 5.5, pH 3.3, volatile acidity (g/L acetic acid) 0.31, protein stability heat test 7.1 NTU.

3.3.2 Analysis of conventional oenological parameters

Alcohol, specific gravity, pH, titratable acidity and volatile acidity were analysed using a Bacchus micro (Figure 3.1).

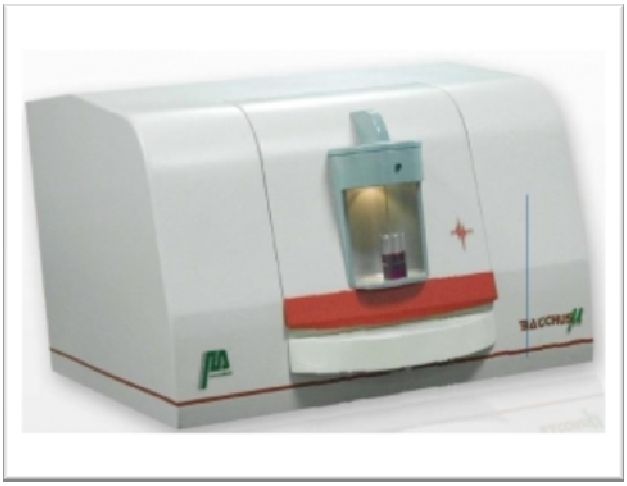


Figure 3.1 - Bacchus micro, used for oenological analysis in this work

3.3.3 Fining experiments

The experiments for initial evaluation of alternatives for white wine protein stabilization involved the addition of different fining agents and additives, on wine 1 (Figure 3.2). The fining products tested were natural sodium bentonite, tannins, CMC, pectolytic enzyme, chitosan, silica gel, polysaccharides and mannoproteins, and were tested at medium and high doses (Table 3.1). The oenological products were prepared to the manufacturer's specifications. Wine containing no oenological products was used as a control. The oenological products were thoroughly mixed, added to each treatment and allowed to remains in contact with the wine in 50 mL flasks at 20ºC during 7 days. Samples were then centrifuged at 537.6 g for 10 min before analysis. All experiments were run in duplicate.

Table 3.1 - Oenological additives and doses used in first wine for fining experiments (high dose is the maximum recommended by manufacture)

Codes	Oenological product	Recommended dosage g/hL	Medium dosage g/hL
S1	Silica gel	25-75	50
T1	Tannins	3 – 10	6.5
T2		3 – 10	6.5
T3		2 – 8	5
T4		5 – 10	7.5
CMC1	Carboxylmethylcellulose	5 – 10	7.5
CMC2		25 – 50	37.5
CMC3		100 – 200 mL/hL	50
B1	Bentonite	20 – 120	70
Q1	Chitosan	100	50
E1	Enzyme	2– 4	3
M1	Mannoprotein	0.5 – 5	2.75
M2		10 – 40	25

A second experiment was performed using different commercial types of bentonites and mannoproteins on wine 2 (Figure 3.2). They were used five bentonites (P, Br, PN, M, Vy) (Figure 3.3A and Table 3.2), and eleven types of mannoproteins (NS, VP, BM, Mb, B150, BB, NF, B20, PG, V, BA) (Figure 3.3B and Table 3.3) with different molecular weight and extractions processes (chemical and enzymatic).

Table 3.2 - Composition of commercial bentonites used in this work, according manufacture

Bentonites	Composition
P	Sodium and calcium
Br	Activated sodium and calcium
PN	Natural sodium
M	Activated calcium
Vy	Natural calcium

Medium concentration of bentonites and high concentration of mannoproteins were prepared to the manufacture´s specifications (Table 3.4). Wine containing no oenological products was used as a control and the experiment was run as before. All analyses were performed in duplicate.

Table 3.3 - Composition of commercial mannoproteins used in this work, according manufacture

Mannoproteins	Composition
NS	Prepared from yeast walls;
VP	Formulation made of yeast cell wall polysaccharides and peptides;
BM	Extracted from cell wall of yeast via enzymatic;
Mb	Mannoprotein from yeast cell walls;
B150	Prepared based on yeast cell, molecular weight 150 kDalton;
BB	Specific preparation of yeast cell walls and mannoproteins;
NF	Prepared from yeast walls, purified with pectolytic enzyme;
B20	Prepared from yeast, rich in polysaccharides and nitrogen compounds with low molecular weight;
PG	Prepared from specific yeast walls;
V	Polysaccharides extracted from yeast cell walls, highly purified;
BA	Prepared based on cell walls from ,yeast with high enzymatic activity β-glucosidase;

Table 3.4 - Bentonites and mannoproteins doses used in this work (high dose is the maximum recommended by manufacture)

Oenological products	Codes	Recommended dosage g/hL	Medium dosage g/hL
Bentonites	P	10 – 40	25
	Br	50 – 200	125
	PN	40 – 120	80
	M	10 – 20	15
	Vy	40 – 100	70
Mannoproteins	NS	30	
	VP	1 – 5	
	BM	5 – 10	
	Mb	10 – 40	
	B150	40	
	BB	5 – 10	
	NF	5 – 40	
	B20	40	
	PG	5 – 40	
	V	0.5 – 5	
	BA	40	

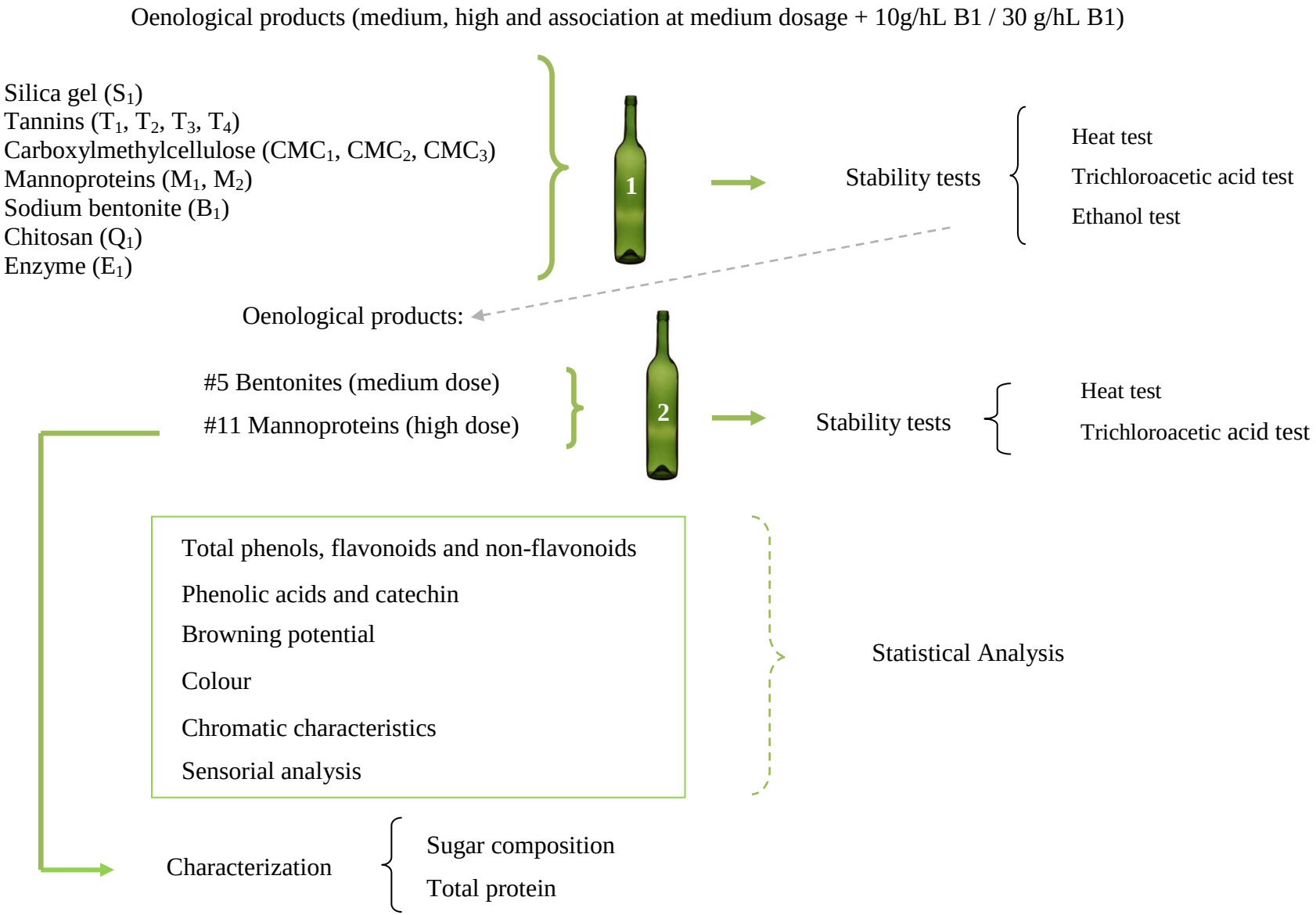
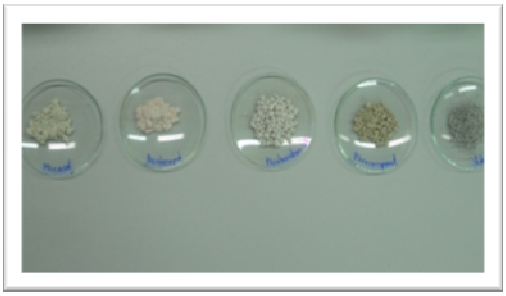


Figure 3.2 - Esquematic procedure of the experiment developed in this work

A



B

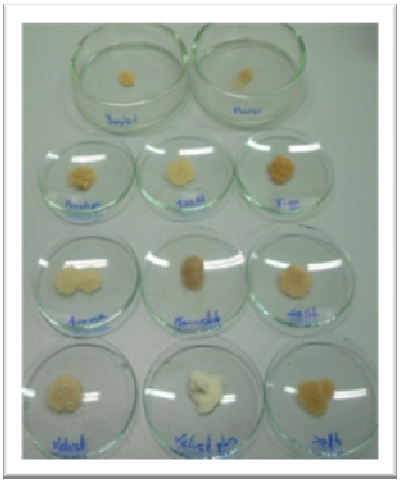


Figure 3.3 - Commercial bentonites (A) and mannoproteins (B) used in fining experiments

3.3.4 Commercial mannoprotein characterization

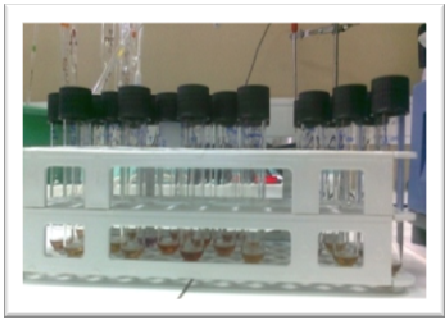
3.3.4.1 Sugar

Commercial mannoproteins were characterized concerning their sugar composition and concentration by anion-exchange chromatography with pulsed amperometric detection, after acid hydrolysis.

Two sequential acid hydrolysis were performed, with and without Saeman hydrolysis, in order to obtain the amount of insoluble polysaccharide present in these commercial mannoprotein. For Saeman hydrolysis, each sample (5 mg) was treated during 3 hours at room temperature, with 400 µL of H_2SO_4 (72%) (mixing every 15 min.) (Figure 3.4A, B). After this time 4.4 mL of water were added and the material was hydrolysed during 2.5 hours at 100 °C (Figure 3.4C). After cooling, 500 µL of 2-desoxiglucose (0.5 mg/mL, internal standard) was added. The second hydrolysis was performed in the same way without the Saeman hydrolysis.

For chromatographic analysis 400 µL of each sample were diluted with 4600 µL of water into vials. Quantification was performed by the internal standard method using calibration curves of fucose, rhamnose, arabinose, galactose, glucose, mannose, xylose, galacturonic and glucoronic acid standards.

A



B

C

Figure 3.4 - Samples treated with H_2SO_4 (Saeman Hydrolysis) (A); Mix samples (Saeman Hydrolysis) (B) and samples submited at 100ºC (Saeman and acid Hydrolysis) (C)

Sugar separation was performed with a CarboPac PA-20 column (150 mm × 3 mm) with a CarboPac PA20 pre-column (Dionex) using eluent A - 1.25 mM NaOH solution containing 2 mM $Ba(OH)_2$, eluent B - 400 mM sodium acetate containing 2 mM $Ba(OH)_2$ and eluent C - 500 mM NaOH containing 2 mM $Ba(OH)_2$. The eluent was kept under nitrogen all times to reduce carbonate build up and biological contamination. The injection volume was 5 μL, the flow rate was 0.3 mL/min and the column temperature was maintained at 35ºC during the run. The following elution program was used: 0-19 min., 100% A, increase to 50% B until 27 min. and maintained until 37 min.; increase to 40% C and decreasing to 0% B until 47 min. and maintained until 57 min. The column was conditioned with 100 % A during 15 min. before injection. The sugar analysis was performed by anion-exchange chromatography (Figure 3.5) equipped with electrochemical detector of Au working electrode, Ag/AgCl reference electrode, and Ti counter electrode. The ED cell waveform was +0.1 V from 0.00 to 0.40 s, then –2.0 V from 0.41 to 0.42 s, and a ramp –2.0 to +0.6 V from 0.42 to 0.43 s, followed by –0.1 V from 0.44 to 0.50 s (end of cycle). The integration region was from 0.2 s to 0.4 s

All analyses were performed in duplicate.

Figure 3.5 - Dionex ICS 3000 used in this work to quantify sugar from commercial mannoprotein

3.3.4.2 Protein concentration

Total nitrogen was determined by the Kjeldahl method based on mineralization, distillation and titration with 0.1 N HCl (Manfredini, 1989; OIV, 2006b) (Figure 3.6). Total protein content was determinate as Kjeldahl nitrogen multiplied by 6.25 (P = N x 6.25).

Figure 3.6 - Kjeldahl distiller used in this work to determinate total protein of commercial mannoproteins

3.3.5 Protein Stability tests

3.3.5.1 Heat test

Wines were heated at 80°C during 30 min. (Figure 3.7A) and then cooled at room temperature. All wines were previously filtered. Wine turbidity was measured in nephelometric turbidity unit (NTU), using a LP 2000 Turbidity Meter (Figure 3.7B). If the difference (ΔNTU) in nephelometric turbidity unit (NTU), between the heated and unheated samples was lower than 2 NTU units, mean that the wine sample is stable (Pocock and Rankine 1973). All analyses were performed in duplicate.

3.3.5.2 Tricloroacetic acid test (TCA)

One mL of tricloroacetic acid (55%) was added to 10 mL of each wine sample. The samples were heated in a water bath at 100ºC during 2 min, all wines were previously filtered. Induced turbidity was then measured in nephelometric turbidity unit (NTU) (with NTU < 19 mean stability) (Figure 3.7B) at room temperature (Berg and Akihoshy, 1961). All analyses were performed in duplicate.

3.3.5.3 Ethanol test

Two mL of ethanol (77%) were added to 20 mL of each wine sample at 5ºC. Induced turbidity was then measured in nephelometric turbidity unit (NTU) (with NTU < 10 mean stability) (Figure 3.7B) at room temperature (Boulton, 1980). All analyses were performed in duplicate.

A

B

Figure 3.7 - Water bath (A) and Nephelometer LP 2000 Turbisity Meter (B) used for proteins stability tests.

3.3.6 Quantification of flavonoid phenols and non-flavonoid phenols

The phenolic content of the wines was determined using the absorbance at 280 nm before and after precipitation of the flavonoid phenols, through reaction with formaldehyde, according to Kramling & Singleton (1969).

Using this method, flavonoid, non-flavonoid and total phenols in the wines were quantified. The results were expressed as gallic acid equivalents by means of calibration curves with standard gallic acid (Sigma).

The polyphenolic content was also determined by a spectrophotometric method, using a Schimadzu UVmini-1240 spectrophotometer (Schimadzu, Kyoto, Japan) (Figure 3.8), and expressed as a total phenolic index (TPI=A_{280nm} x dilution factor). All analyses were performed in duplicate.

Figure 3.8 - Shimadzu UVmini-1240 Spectrophotometer used in this work

3.3.7 Browning potential

Test tubes were filled with 10 mL of the wine to be tested. Control and test samples were spared thoroughly with nitrogen and oxygen, respectively. All tubes were sealed hermetically and maintained at 55 ºC for 5 days (Figure 3.9). The test was conducted on treated and untreated wine, and the browning value difference was calculated by measuring the increase in $A_{420\ nm}$, using a Schimadzu UVmini-1240 spectrophotometer (Schimadzu, Kyoto, Japan) as recommended by Singleton and Kramling (1976). All analyses were performed in duplicate.

Figure 3.9 - Oven used in this work for browning potential determination

3.3.8 Chromatic characterization

The absorption spectra of wine samples were recorded with a Schimadzu UVmini-1240 spectrophotometer (Schimadzu, Kyoto, Japan) scanned from a range of 380 nm to 770 nm, using 1 cm path length quartz cells. Data were collected to determine a measure of L* (lightness), a* (redness), and b* (yellowness) coordinates using the CIELab method according to Organisation International de la Vigne et du Vin (OIV, 2006a).

The Chroma [$C^* = [(a^*)^2 + (b^*)^2]^{1/2}$] and hue-angle [$h^o = \tan g^{-1}(b^*/a^*)$] values were also determined. To distinguish the colour more accurately, the difference was calculated using the following equation: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$ and reported in CIELab units. This allows reliable quantification of the overall colour difference a sample, when compared to a reference sample (unfined sample). Colour differences can be distinguished by the human eye when the difference between ΔE*

values are greater than two units (Spagna et al., 1996). All analyses were performed in duplicate.

3.3.9 Phenolic acids and flavonoid profile

Phenolic acids and flavonoids were performed by HPLC with a diode-array detector (Figure 3.10). The column was a reverse phase C18 column (25cm, 4.5mm diameter, 5 μm particles). The eluent was constituted by 5% aqueous formic acid (solvent A) and methanol (solvent B). The elution program was the following: 5% of B from zero to 5 min. followed by a linear gradient up to 65% of B until 65min and from 65 to 67min down to 5% of B. The flow rate was 1mL/min. Detection was performed from 200 to 650 nm with injection volume 25μL. The identification was made considering their retention times and UV spectra. The chromatograms were recorded at 280 and 325 nm for phenolics in general. All analyses were performed in duplicate.

Figure 3.10 - Dionex UltiMate 3000 HPLC used to quantify phenolic acid and flavonoids, in this work

3.3.10 Colour analysis

Colour was determined by measuring absorbance at 420 nm (10 mm cell) using a Schimadzu UVmini-1240 spectrophotometer (Schimadzu, Kyoto, Japan) in line with the Organisation International de la Vigne et du Vin methods (OIV, 2006a). All analyses were performed in duplicate.

3.3.11 Sensory evaluation

The sensory analysis was performed by a trained panel of seven members. The samples were stored at appropriate light and temperature conditions (20 ºC). Samples were presented to the panel in tasting glasses marked with three digits in a randomised order. Fifteen attributes were selected: visual (limpidity, colour), aroma (aroma intensity, fruity, floral, vegetable, oxidised, chemist) and taste (sweetness, acidity, bitterness, flavour intensity, body, balance, persistence). The attributes were quantified using a ten-point intensity scale (ISO 4121, 2003). A total sensory score was calculated for each wine as the sum of an average score of visual, aroma and taste attributes. All evaluations were conducted from 10:00 to 12:00 A.M. in an individual booth (ISO 8589, 2007) and according to standardized procedures (ISO 3591, 1977).

3.3.12 Statistical analysis

The data are presented as mean ± standard deviation. Statistical analyses were carried out using Statistica 7 software (Statsoft, OK, USA) program. Kolmogorov-Smirnov was used to test normal variable distribution and two-way ANOVA was used to compare both physicochemical and sensory data. Homogeneity of variance could be assumed based on Levene test.

Tukey honestly significant difference (HSD, 5% level) test was applied to physicochemical data to determine significant differences between the stability treatments. Duncan’s multiple range test (MRT) was applied to sensory data to

determine significant differences between the fining treatments. The model was statistically significant when *p* values were less than 0.05.

Principal component analysis (PCA) was carried out to indentify patterns between wine treatments and sensorial analysis.

3.4 Results and discussion

3.4.1 Effect of different types of bentonite and mannoproteins on white wine protein stability

The initial experiments were performed with different fining agents and oenological additives, with the purpose to search for alternatives to bentonite that could stabilize wine protein instability. Also we search the correct amount of oenological product able to stabilize the wines.

Results of protein stability obtained in these trials by protein stability tests, are shown in Table 3.5. Protein stability was assayed by three different tests; the heat test, trichloroacetic acid test (TCA test) and ethanol test.

The results showed that mannoprotein (M_1 and M_2), as well as enzyme and chitosan, increase protein stability, by the heat test, when applied at the highest concentration recommended by manufacturer. As expected sodium bentonite (B_1 at medium and high dose) also increase protein stability. Stability tests can be classified in accordance with their mechanisms of action. The first test provides information about protein thermal denaturation, the second test, by using a strong acid test (trichloroacetic acid) promotes a chemical protein desnaturation, being able to precipitate all proteins present in wine and the third test is based on reducing the dielectric constant, which reduced protein solubility. Thus, heat test still the most widely used in industry, because it is very reliable for providing information about protein thermal stability.

In this initial evaluation, it was also studied the association of diverse fining agents, at medium concentration, with bentonite B1, at 10 g/hL or 30 g/hL, in order to improve wine protein stability (Table 3.6). However, the results obtained with this experiment are not an alternative, because with 10 g/hL of bentonite the protein stability could not

be achieved and, although with 30 g/hL of bentonite, all the assays were stable regard protein stability.

Table 3.5 - Protein stability tests performed in white wines added with diverse oenological products, at two doses

	Stability test					
	Heat test		TCA test		Ethanol Test	
Doses	Medium	High	Medium	High	Medium	High
C	+	+	+	+	-	-
T_1	+	+	+	+	-	-
T_2	+	+	+	+	-	-
T_3	+	+	+	+	+	-
T_4	+	+	+	+	+	+
S_1	+	+	+	+	-	-
B_1	-	-	-	-	-	-
M_1	+	-	+	+	-	-
M_2	+	-	+	+	-	-
CMC_1	+	+	+	+	-	-
CMC_2	+	+	+	+	-	-
CMC_3	+	+	+	+	-	-
E_1	+	-	+	+	-	-
Q_1	+	-	+	+	-	-

Stability tests: unstable (+), stable (-); Untreated wine (C), tannins (T_1,T_2,T_3,T_4), silic gel (S_1), bentonite (B_1), mannoprotein (M_1,M_2), carboxylmethylcellulose (CMC_1, CMC_2, CMC_3), enzyme (E_1), chitosan (Q_1).

Based on these results a trial with wine 2 was performed using five different types of bentonite and eleven commercial mannoproteins in order to evaluate how mannoprotein could stabilize white wine protein instability (Table 3.2 and Table 3.3). The concentrations tested were based on previous results, since at low concentration of bentonite the protein stability is already achieved, and with mannoproteins it is needed highest concentrations.

Protein stability was assayed using only two different methods, giving complementary information, the heat test and TCA test. The results obtained with these protein stability are shown in Table 3.7.

Table 3.6 - Protein stability tests performed in white wine added with diverse oenological products (at medium concentration) in association with bentonite (10 g/hL or 30g/hL)

	Bentonite 10 g/hL			**Bentonite 30 g/hL**		
	Heat test	TCA test	Ethanol test	Heat test	TCA test	Ethanol test
C	+	+	-	+	+	-
B	+	+	-	-	-	-
T_1	+	+	-	-	-	-
T_2	+	+	-	-	-	-
T_3	+	+	-	-	-	-
T_4	+	+	-	-	-	-
S_1	+	+	+	-	-	-
M_1	+	+	-	-	-	-
M_2	+	+	-	+	-	-
CMC_1	+	+	-	-	-	-
CMC_2	+	+	-	-	-	-
CMC_3	+	+	-	-	-	-
E_1	+	+	-	-	-	-
Q1	+	+	-	-	-	-

Stability tests: unstable (+), stable (-);
Untreated wine (C), bentonite (B_1); tannins (T_1, T_2, T_3, T_4), silic gel (S_1), mannoprotein (M_1, M_2), carboxylmethylcellulose (CMC_1, CMC_2, CMC_3), enzyme (E_1), chitosan (Q_1).

All bentonites stabilize the wine by the heat test, with an exception in the TCA test for bentonite P, this results showed the known efficient of bentonite on stabilizing wine proteins. Considering mannoproteins it was observed high thermal protein stability since 9 onto 11 studied mannoproteins stabilize the wine by the heat test (Table 3.7).

In opposite, in all the trials with mannoproteins the results obtained with TCA test were unstable, which was relative expected, because an increase in wine proteins concentration could occurred after mannoprotein addition.

Regarding the volume of lees in the flask bottom, of the experiments, bentonites produced more lees than mannoproteins. However, among the bentonites differences were observed, namely a reduced volume of less was achieved for the bentonite

obtained by a sodium and calcium bentonite (P) followed by activated calcium bentonite (M), natural calcium (Vy) and natural sodium (PN) bentonite, activated sodium and calcium bentonite (Br). All mannoproteins present a little volume of lees. It is important to select fining agents that have a reduced volume of lees in order to decrease the wine loss (Lagace and Bisson, 1990).

Table 3.7 - Proteins stability tests performed in white wines treated with diverse bentonites and mannoproteins.

	Bentonite			**Mannoprotein**	
	Heat test	**TCA test**		**Heat test**	**TCA test**
Doses	Medium			High	
C	**+**	**+**	**C**	+	+
P	**-**	**+**	**NS**	-	+
Br	**-**	**-**	**VP**	-	+
PN	**-**	**-**	**BM**	-	+
M	**-**	**-**	**Mb**	-	+
Vy	**-**	**-**	**B150**	+	+
			BB	-	+
			NF	-	+
			B20	-	+
			PG	+	+
			V	-	+
			BA	-	+

Stability tests: unstable (+), stable (-);
Untreated wine (C), sodium and calcium bentonite (P), activated sodium and calcium bentonite (Br), natural sodium bentonite (PN), activated calcium bentonite (M), natural calcium bentonite (Vy), mannoproteins (NS, VP, BM, Mb, B150, BB, NF, B20, PG,V, BA).

3.4.2 Mannoprotein characterization

Commercial mannoproteins used in this work were characterized concerning their sugar composition and concentration, as well as their protein content (Table 3.8), in order to better understand the relationship between mannoprotein composition and effectiveness in protein stabilization. The results show that sugar identified and quantified in mannoproteins studied, besides mannose (17.4 to 41.9 g/100g), were also

fucose (0.7 to 1.6 g/100g), arabinose (0.0 to 1.7 g/100g), galactosamine (0.2 to 0.4 g/100g), glucose (6.8 to 41.4 g/100g) and galactose (0.0 to1.7 g/100g). Regarding protein concentration of the commercial mannoproteins studied, it was shown that it ranged from 10.4 g/100g to 44.4 g/100g of mannoproteins (Table 3.8).

Through the sugar characterization of commercial mannoproteins was possible verify that mannoproteins with less percentage of mannose (mannoproteins B150 and PG, with 20.1 g/100g and 19.4 g/100g, respectively) are less effective in stabilizing the wine against protein instability (Table 3.7). This result suggests that effectiveness of mannoproteins to stabilize instable wine proteins, depend on the amount of mannose present in the mannoprotein, being more effective when the percentage of mannose is higher. However, the protein concentration of mannoproteins, as already mentioned, may increase wine protein concentration. After determination of total protein, by Kjeldahl method, the results showed that mannoproteins B150 and PG, presented high values of proteins (37.4 g/100g and 37.1 g/100g of mannoprotein, respectively). Theses values coupled with low concentration of mannose, could justify the instability in wine treated with these mannoproteins.

Table 3.8 - Sugar and total protein present in mannoproteins obtained by chromatography (mean ± SD) and Kjeldahl method

	Mannoprotein sugar composition														
	Fuc		**Ara**		**$GlcNH_2$**		**Gal**		**Gluc**		**Man**		**Total sugar g/100g**	**Soluble sugar g/100g**	**Insoluble Sugar g/100g**	**Total Protein g/100g**
Mannoprotein	H. S	H. A	H. S	H. A	H. S	H. A	H. S	H. A	H. S	H. A	H. S	H. A				
NS	1.0±0.1	1.0±0.0	0.0±0.0	0.0±0.0	0.2±0.0	0.1±0.0	0.1±0.1	0.1±0.0	28.7±1.8	20.2±0.3	20.7±1.3	22.9±0.4	50.3±3.3	43.8±0.0	6.85	44.4
Vp	0.9±0.0	0.7±0.2	0.0±0.0	0.0±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.1±0.0	19.6±0.2	19.8±0.0	26.1±0.5	26.6±0.2	46.9±0.7	47.0±0.7	1.30	40.9
BM	0.9±0.1	0.5±0.0	0.1±0.0	0.0±0.0	0.3±0.0	0.2±0.0	0.0±0.0	0.0±0.0	6.8±1.2	6.1±0.1	41.9±0.0	42.6±0.2	49.9±1.1	49.2±0.5	0.64	17.6
Mb	0.9±0.6	0.5±0.2	0.0±0.0	0.1±0.0	0.4±0.0	0.4±0.0	0.0±0.0	0.0±0.0	15.7±0.6	15.7±0.8	38.6±1.5	37.8±1.4	55.8±0.2	54.3±0.1	1.51	10.4
B150	0.7±0.0	1.0±0.2	1.7±0.1	1.7±0.0	0.2±0.0	0.1±0.0	1.7±0.1	1.6±0.1	40.2±2.1	35.3±2.7	20.1±1.3	19.8±1.2	64.5±3.6	59.1±3.3	5.48	37.4
BB	0.7±0.0	0.9±0.3	0.0±0.0	0.0±0.0	0.2±0.0	0.2±0.0	0.0±0.0	0.0±0.0	4.4±0.2	4.2±0.4	35.4±1.0	32.4±3.4	40.7±0.8	37.3±3.6	3.43	26.0
NF	0.8±0.0	0.6±0.1	0.0±0.0	0.0±0.0	0.2±0.0	0.1±0.0	0.0±0.0	0.0±0.0	41.4±0.2	29.7±0.2	21.6±0.4	20.8±0.3	64.0±0.2	50.9±0.5	13.16	38.3
B20	1.6±0.0	0.6±0.2	1.9±0.2	1.6±0.1	0.2±0.0	0.1±0.0	1.9±0.1	1.6±0.2	29.5±2.3	21.8±1.7	17.4±0.1	17.0±1.7	52.3±2.7	42.5±5.2	9.85	44.0
PG	0.9±0.3	1.4±0.0	0.0±0.0	0.0±0.0	0.2±0.0	0.2±0.0	0.0±0.0	0.0±0.0	45.5±1.4	42.1±0.0	19.4±1.0	19.4±0.0	66.0±0.1	62.3±1.0	3.63	37.1
V	0.9±0.0	0.7±0.1	0.0±0.0	0.0±0.0	0.2±0.0	0.2±0.0	0.0±0.0	0.0±0.0	38.6±1.4	38.0±2.0	40.8±1.0	41.7±0.2	80.5±2.4	80.3±1.7	0.24	26.3
BA	1.3±0.6	1.5±1.0	1.7±0.1	1.8±0.1	0.2±0.0	0.1±0.0	1.7±0.1	1.7±0.0	31.9±3.1	33.2±1.4	17.7±0.6	20.2±0.6	54.4±2.9	57.9±3.0	2.40	42.1

Mannoproteins (NS, VP, BM, Mb, B150, BB, NF, B20, PG,V, BA); Fuc – Fucose, Ara – arabinose , $GlcNH_2$ – galactosamine, Gal – galactose, Glu – glucose, Man – mannose; H:S – Saeman Hydrolysis ; H.A – Acid Hydrolysis.

3.4.3 Effect of different types of bentonites and mannoproteins on the browning potential, total phenols, non-flavonoid and flavonoid compounds

Results of total polyphenol index, total phenols, flavonoids, non-flavonoids and browning potencial of white wine treated with bentonites and mannoproteins were presented in Table 3.9.

The results indicate that all bentonites tested had no significant effect on total phenols concentration, this results are in acordance with Hsu et al. (1987b), the same occured for flavonoids and non-flavonoids. The white wine treated with some mannoproteins had a decrease in total phenols concentration, in flavonoids and in non-flavonoids, with exception for white wines treated with NS, NF and PG.

The results obtained for the browning potential showed a decrease after bentonite application, mainly with bentonite Br and PN; also all mannoproteins decreased the browning potential, specifically NF and B20 (Table 3.9). The oxidation of phenols, such as catechins and proanthocyanidins, may occur when wine is exposed to oxygen. Oxidation can have an impact on wine colour and lead to browning of the wine (Zoecklein et al., 1995; Ribéreau-Gayon et al., 2006).

Table 3.9 - Total polyphenol index (TPI), total phenols, flavonoids, non-flavonoids, browning potencial of both untreated and treated white wine (mean ± SD)

	TPI	Total phenols (mg/L gallic acid)	Flavonoids (mg/L gallic acid)	Non-flavonoids (mg/L galic acid)	Brownig potential
C	6.53±0.11^{a}	24.6±0.32^{a}	12.9±0.30^{a}	11.7±0.03^{a}	0.045±0.003^{a}
Bentonite					
P	6.86±0.05^{a}	25.6±0.14^{a}	14.3±0.11^{ab}	11.3±0.03^{a}	0.020±0.001^{c}
Br	6.70±0.03^{a}	25.3±0.12^{a}	14.1±0.03^{ab}	11.1±0.10^{a}	0.013±0.003^{d}
PN	6.77±0.04^{a}	25.3±0.10^{a}	13.8±0.44^{ab}	11.6±0.34^{a}	0.015±0.002^{d}
M	6.77±0.07^{a}	25.3±0.21^{a}	14.2±0.01^{ab}	11.2±0.22^{a}	0.022±0.001^{c}
Vy	6.47±0.09^{a}	24.5±0.26^{a}	13.4±0.22^{ab}	11.0±0.05^{a}	0.033±0.002^{b}
Mannoprotein					
NS	6.63±0.12^{a}	24.9±0.33^{a}	13.8±0.29^{a}	11.2±0.04^{a}	0.018±0.004^{b}
VP	5.71±0.06^{b}	22.3±0.17^{b}	12.1±0.18^{c}	10.2±0.01^{b}	0.012±0.003^{c}
BM	5.94±0.05^{b}	23.0±0.13^{b}	12.5±0.18^{c}	10.5±0.05^{b}	0.011±0.003^{c}
Mb	5.87±0.03^{b}	22.8±0.08^{b}	12.5±0.09^{c}	10.2±0.01^{b}	0.025±0.004^{b}
B150	5.76±0.01^{b}	22.5±0.04^{b}	12.3±0.15^{c}	10.2±0.20^{b}	0.016±0.003^{c}
BB	5.83±0.01^{b}	22.7±0.02^{b}	12.4±0.13^{c}	10.3±0.11^{b}	0.025±0.000^{b}
NF	6.63±0.05^{a}	24.9±0.15^{a}	13.6±0.13^{a}	11.2±0.03^{a}	0.007±0.003^{d}
B20	5.79±0.05^{b}	22.5±0.14^{b}	12.9±0.33^{d}	9.6±0.47^{c}	0.008±0.001^{d}
PG	6.49±0.02^{a}	24.5±0.06^{a}	13.3±0.03^{a}	11.2±0.03^{a}	0.019±0.000^{b}
V	5.76±0.06^{b}	22.5±0.17^{b}	12.4±0.18^{c}	10.1±0.01^{b}	0.019±0.004^{b}
BA	5.73±0.01^{b}	22.4±0.03^{b}	12.0±0.07^{c}	10.4±0.04^{b}	0.017±0.001^{b}

Untreated wine (C), sodium and calcium bentonite (P), activated sodium and calcium bentonite (Br), natural sodium bentonite (PN), activated calcium bentonite (M), natural calcium bentonite (Vy), mannoproteins (NS, VP, BM, Mb, B150, BB, NF, B20, PG,V ,BA). Different letters for statistical different means, $p<0.05$

3.4.4 Effect of different types of bentonites and mannoproteins on phenolic acids and flavonoid

Phenolic acids are present in white wine usually combined with other molecules ranging their concentration among 10-20 mg/L (Batista et al., 2010) proving mainly from the grape pulp (Basha et al., 2004), they include cinamic and benzoic acids and are one of the major classes of compounds in *Vitis vinifera* (Zoecklein et al., 1995). Table 3.10 shows the results obtain by HPLC analyses of phenolic acids and flavonoids (catechin) of the white wine, before and after treatment with different bentonites and

mannoproteins. In general bentonites do not influence significantly the phenolic acids and well as catechine in the fined wines, with exception of bentonite Vy which decrease significantly gallic acid. Such decrease may be related to the interaction of compounds with proteins and precipitate or may transform into other compounds through esterification, glycolisation and oxidation (Esteruelas et al., 2011). Mannoproteins, also do not induced significant changes in these compounds, with exception for mannoprotein NF that decreased the cafeic acid. These results obtained for phenolic acids and chatechin are in accordance with previous results presented in table 3.9.

The phenolic compounds, ferulic acid, etil caffeic and etil coumaric were present in minor quantity in this wine and remained unchanged after treatment. In turns, the increase observed in same phenolic compounds may be related to the hydrolysis of other compounds (Esteruelas et al., 2011).

Table 3.10 - Phenolic acids and flavonoid (% area) obtained by HPLC of both untreated and treated white wine with bentonite and mannoproteins (mean ± SD)

		Bentonite					Mannoprotein										
	C	P	BR	PN	M	Vy	NS	VP	BM	Mb	B150	BB	NF	B20	PG	V	BA
Gallic acid	40.1±1.1^{bc}	36.1±2.9^{abc}	31.3±3.4^{ab}	31.8±2.1^{ab}	38.0±0.5^{abc}	28.4±0.5^{a}	36.9±7.2^{abc}	41.2±0.5^{bc}	41.7±2.6^{bc}	41.2±4.2^{bc}	44.1±2.9^{c}	40.0±0.9^{bc}	39.6±1.4^{bc}	40.3±0.0^{bc}	41.6±1.5^{bc}	41.0±0.6^{bc}	41.3±3.4^{bc}
Catechin	12.0±0.7^{ab}	18.9±1.8^{b}	15.1±0.2^{ab}	12.3±1.6^{ab}	12.6±0.8^{ab}	11.9±0.5^{ab}	12.4±3.7^{ab}	15.0±4.0^{ab}	10.1±1.4^{ab}	9.3±0.1^{a}	10.8±0.2^{ab}	10.1±0.9^{ab}	16.9±0.2^{ab}	13.5±2.8^{ab}	9.2±3.6^{a}	13.4±2.5^{ab}	13.3±3.4^{ab}
Trans-caftaric acid	27.4±0.2^{a}	25.5±2.9^{a}	30.6±1.9^{a}	32.6±0.3^{a}	28.2±0.6^{a}	30.9±2.2^{a}	28.5±4.6^{a}	25.1±2.0^{a}	28.1±0.1^{a}	28.9±2.4^{a}	25.4±1.5^{a}	29.1±1.5^{a}	24.4±2.1^{a}	25.4±3.2^{a}	28.2±1.6^{a}	25.8±1.2^{a}	25.1±1.4^{a}
2-S-glutathionyl caftaric acid	9.7±0.4^{a}	10.0±1.6^{a}	10.9±0.6^{a}	11.5±0.1^{a}	10.1±0.1^{a}	10.8±2.5^{a}	10.7±2.9^{a}	8.9±0.7^{a}	9.7±0.2^{a}	10.1±0.5^{a}	9.1±0.3^{a}	10.2±0.7^{a}	9.4±0.1^{a}	9.5±0.1^{a}	10.0±0.2^{a}	9.5±0.8^{a}	9.4±0.4^{a}
Coutaric isomeric acid	3.4±0.1^{a}	3.0±0.0^{a}	3.8±0.2^{a}	3.6±0.4^{a}	3.5±0.1^{a}	10.7±6.2^{b}	3.9±1.8^{a}	3.0±0.3^{a}	3.1±0.4^{a}	3.4±0.8^{a}	3.6±0.7^{a}	3.3±0.1^{a}	3.7±0.1^{a}	3.6±0.4^{a}	3.5±0.1^{a}	3.2±0.3^{a}	3.5±0.5^{a}
Coutaric acid	2.1±0.0^{a}	1.5±0.4^{a}	2.4±0.1^{a}	2.5±0.1^{a}	2.2±0.0^{a}	1.7±1.6^{a}	3.0±1.2^{a}	1.9±0.1^{a}	2.2±0.0^{a}	2.3±0.0^{a}	2.0±0.1^{a}	2.3±0.2^{a}	2.4±0.2^{a}	2.4±0.4^{a}	2.2±0.0^{a}	2.0±0.2^{a}	2.5±0.5^{a}
Caffeic acid	2.3±0.1^{b}	2.2±0.4^{b}	2.5±0.1^{b}	2.2±0.4^{b}	2.3±0.0^{b}	2.4±0.3^{b}	1.6±0.0^{ab}	2.1±0.2^{b}	2.0±0.3^{b}	1.8±0.1^{ab}	2.1±0.0^{b}	2.0±0.4^{b}	0.8±0.3^{a}	2.3±0.0^{b}	2.3±0.1^{b}	2.2±0.2^{b}	2.2±0.2^{b}
4-hidroxicumaric acid	1.2±0.0^{a}	1.2±0.2^{a}	1.4±0.1^{a}	1.5±0.1^{a}	1.3±0.0^{a}	1.3±0.3^{a}	1.3±0.1^{a}	1.1±0.1^{a}	1.1±0.0^{a}	1.3±0.0^{a}	1.1±0.0^{a}	1.2±0.0^{a}	1.2±0.0^{a}	1.2±0.0^{a}	1.2±0.0^{a}	1.2±0.2^{a}	1.2±0.1^{a}
Ferulic acid	0.4±0.0^{a}	0.3±0.0^{a}	0.4±0.0^{a}	0.4±0.0^{a}	0.4±0.0^{a}	0.4±0.0^{a}	0.4±0.1^{a}	0.4±0.0^{a}	0.4±0.0^{a}	0.4±0.0^{a}	0.4±0.1^{a}	0.4±0.0^{a}	0.4±0.0^{a}	0.4±0.1^{a}	0.4±0.0^{a}	0.4±0.1^{a}	0.3±0.0^{a}
Etil caffeic	0.9±0.0^{a}	0.8±0.0^{a}	1.0±0.0^{a}	1.0±0.1^{a}	0.9±0.0^{a}	1.0±0.2^{a}	0.8±0.1^{a}	0.8±0.1^{a}	0.8±0.1^{a}	0.8±0.0^{a}	0.8±0.0^{a}	0.9±0.0^{a}	0.8±0.1^{a}	0.8±0.2^{a}	0.9±0.0^{a}	0.9±0.1^{a}	0.8±0.1^{a}
Etil coumaric	0.5±0.0^{a}	0.4±0.0^{a}	0.6±0.0^{a}	0.6±0.0^{a}	0.5±0.0^{a}	0.5±0.1^{a}	0.5±0.1^{a}	0.5±0.0^{a}	0.5±0.0^{a}	0.5±0.0^{a}	0.5±0.0^{a}	0.5±0.0^{a}	0.4±0.1^{a}	0.5±0.1^{a}	0.5±0.0^{a}	0.5±0.1^{a}	0.4±0.1^{a}

Untreated wine (C), sodium and calcium bentonite (P), activated sodium and calcium bentonite (Br), natural sodium bentonite (PN), activated calcium bentonite (M), natural calcium bentonite (Vy), mannoproteins (NS, VP, BM, Mb, B150, BB, NF, B20, PG,V, BA). Different letters for statistical different means, $p<0.05$

3.4.5 Effect of different types of bentonites and mannoproteins on the white wine colour and chromatic characteristics

All types of bentonites decreased significantly wine colour ($A_{420\ nm}$), being the lowest values for the wine fined with natural sodium bentonite (PN); in opposite, the addition of all most mannoproteins increase the wine colour, principally mannoprotein B150 and BA (Table 3.11).

Lightness was maintained, or improved, in all wines with exception of the wine treated with mannoprotein VP. Results show that the a* values are negative and b* values are positive, which means that the colour of the wine are positioned at 2° quadrant of the colour space defined by the variables (-a *) and (+ b *) where is positioned the colour green to yellow, which means that these wines have a yellow-green matrix. The value for b* (yellowness) decreased significantly with all bentonites and with some mannoproteins (B20, V, BA), these results are in accordance with the results obtained for wine colour. The hue-angle (h^o) values increased after addition of bentonite indicating that some yellow pigments were removed. The same occurred with one mannoproteins (B20), while, others decreased the hue-angle, this observation could indicate that some mannoprotein increment yellow pigmentation (Cosme et al., 2012).

The colour variation (ΔE*) which is the geometric mean of ΔL*, Δa* and Δb*, can be visually discriminated by the human eye when it is greater than 2 CIELab units. Between each wine and the untreated wine, this value was obtained only for the wine treated with mannoprotein VP, which means that the colour of this wine could be distinguished by the human eye.

Table 3.11- Chromatic characteristics and colour of both untreated and treated white wine (mean ± SD)

	L*(%)	a*	b*	*h*°	C*	ΔE*	Colour
Bentonite							
C	98.4± 0.2^{a}	-1.19±0.07^{b}	3.36±0.01^{b}	109.52±1.03^{a}	3.56±0.03^{b}		0.0188±0.001^{b}
P	99.4± 0.1^{b}	-1.31±0.08^{ab}	2.96±0.01^{a}	113.86±1.27^{b}	3.24±0.05^{a}	0.90±0.08^{a}	0.0111±0.000^{a}
Br	99.4± 0.2^{b}	-1.28±0.01^{ab}	2.92±0.06^{a}	113.59±0.52^{b}	3.19±0.05^{a}	0.91±0.22^{a}	0.0102±0.003^{a}
PN	99.4± 0.0^{b}	-1.33±0.01^{ab}	2.76±0.08^{a}	115.77±0.39^{b}	3.06±0.08^{a}	1.04±0.07^{a}	0.0078±0.000^{a}
M	99.7± 0.1^{b}	-1.35±0.03^{ab}	2.89±0.18^{a}	115.12±1.81^{b}	3.19±0.15^{a}	1.22±0.20^{a}	0.0102±0.000^{a}
Vy	99.9± 0.1^{b}	-1.42±0.01^{a}	2.93±0.02^{a}	115.82±0.28^{b}	3.25±0.02^{a}	1.45±0.05^{a}	0.0122±0.001^{a}
Mannoprotein							
C	98.4±0.2^{b}	-1.19±0.07^{bc}	3.36±0.01^{bc}	109.52±1.03^{cde}	3.56±0.03^{ab}		0.0188±0.001^{a}
NS	98.7±0.0^{b}	-1.27±0.03^{abc}	3.78±0.01^{ef}	108.57±0.45^{cd}	3.99±0.00^{cde}	0.47±0.02^{a}	0.0310±0.002^{b}
VP	92.2±0.0^{a}	-0.89±0.00^{d}	6.13±0.03^{h}	98.26±0.04^{a}	6.19±0.03^{g}	6.96±0.00^{d}	0.0384±0.000^{c}
BM	99.7±0.0^{c}	-1.30±0.02^{abc}	3.79±0.01^{ef}	108.89±0.25^{cd}	4.00±0.01^{cde}	1.18±0.04^{bc}	0.0333±0.000^{b}
Mb	98.5±0.0^{b}	-1.23±0.04^{bc}	4.61±0.18^{g}	104.90±0.14^{b}	4.77±0.18^{f}	1.26±0.18^{bc}	0.0334±0.002^{cd}
B150	99.9±0.1^{c}	-1.33±0.04^{abc}	3.99±0.08^{f}	107.03±1.80^{bc}	4.20±0.09^{e}	1.74±0.24^{c}	0.1140±0.001^{f}
BB	99.9±0.0^{c}	-1.38±0.04^{abc}	3.53±0.04^{cd}	111.38±0.79^{def}	3.79±0.02^{bc}	1.45±0.00^{bc}	0.0325±0.000^{b}
NF	99.2±0.7^{bc}	-1.40±0.12^{ab}	3.89±0.00^{ef}	109.72±1.57^{cde}	4.13±0.04^{de}	0.93±0.40^{ab}	0.0429±0.001^{d}
B20	99.9±0.0^{c}	-1.47±0.03^{a}	3.26±0.07^{ab}	114.27±0.05^{f}	3.58±0.08^{ab}	1.44±0.01^{bc}	0.0446±0.001^{d}
PG	98.4±0.1^{b}	-1.26±0.05^{bc}	3.67±0.06^{de}	108.91±1.00^{cd}	3.87±0.04^{cd}	0.37±0.06^{a}	0.0210±0.001^{a}
V	99.9±0.0^{c}	-1.24±0.04^{bc}	3.17±0.01^{ab}	111.39±0.62^{def}	3.40±0.02^{a}	1.43±0.00^{bc}	0.0391±0.001^{c}
BA	99.9±0.1^{c}	-1.27±0.01^{abc}	3.07±0.01^{a}	112.51±0.27^{ef}	3.32±0.00^{a}	1.38±0.08^{bc}	0.1045±0.000^{e}

Untrated wine (C), sodium and calcium bentonite (P), activated sodium and calcium bentonite (Br), natural sodium bentonite (PN), activated calcium bentonite (M), natural calcium bentonite (Vy), mannoproteins (NS, VP, BM, Mb, B150, BB, NF, B20, PG,V, BA); L*(%) - lightness, a* - redness, b* - yellowness,C* - Chroma, h^{o} - hue angle, ΔE* – total colour difference. The values corresponding to ΔE* were obtained taking as a reference the untreated wine (C). Different letters for statistical different means, $p<0.05$

3.4.6 Effect of the different types of bentonites and mannoproteins on sensory evaluation

In the wine there are present different chemical components and their composition and quantity may be responsible for sensory characteristics, and can also be changed throughout winemaking techniques, such fining treatment (Jones et al., 2008). White wines are usually fined to remove unstable proteins however this process may modify the level of wine volatile compounds (Hoj et al., 2001) because during fining process some wine compounds, concerning to sensory proprieties, can be loss. These compounds interact with proteins, because these last has the ability to fix aroma compounds, and when removed from wine creep some aromas. The effect of these two treatments (bentonite and mannoproteins) on sensorial characteristics depends on chemical nature, such as pH and ethanol, concentration of the volatile compounds and the amount and characteristic of proteins presents in wine (Lambri et al., 2010).

After sensory analyse no significant differences ($p < 0.05$) among the wines were observed, as shown in Table 3.12 were the average score of each attribute evaluate are presented for bentonite and mannoprotein treatment. The sensory profile of each treatment is shown graphically in figure 3.11 where the sum of the values assigned by the panellists for each attribute is marked on the corresponding axis. The centre of the figure represents the lowest point of the scale used in the evaluation, while the intensity increases from the centre to the periphery. Generally, attribute most pointed was the colour, acidity and flavour intensity.

To better understand the effect of the different treatments on wine sensorial attributes PCA (Principal Component Analysis) analysis was carried out (Figure 3.12).

In the PCA with the sensorial data from treatment with bentonite and manno proteins, the first component accounted for 97.08% of the total variance and the second component 0.90%, representing the first two factorial axes 97.98% of the total variance (Figure 3.12 C). In a PCA analysis, if both the first three components accumulate a relative high percentage of the total variation, in general above 70%, they satisfactory explain the variability among the samples tested (Mardia et al., 1979). Evaluating the projections of Figure 3.12 is possible to visualize the special distribution of the samples evaluated sensorally. Among the sensorial attributes assessed in the wines submitted to bentonite or mannoprotein treatments, we can discriminated three groups as followed:

group I with P, Br, PN, Vy, B150, BB; group II with VP, NF, B20, PG, BA and group III with NS, BM, Mb, V, M (Figure 3.12 C). The wines in group II include only mannoproteins, and were the highest scored. In turns, wines from group I and III, include bentonites and some mannoproteins, and were lower scored.

The formation of these three groups may be related to the composition of each oenological product added to the wine. Through the characterization of mannoproteins (Table 3.8), it was possible to verify, that the wines being in the highest scored group, wines treated with mannoproteins, are the ones with higher glucose (VP – 19.6 g/100g; NF – 41.4 g/100g; B20 – 29.5 g/100g; PG – 45.5 g/100g; BA – 31.9 g/100g) values.

Interactions between aroma compounds and fining agents may occur and change the volatility of aroma compounds by adsorption on the suspended solids, and change organoleptic characteristics of wine (Lubbers et al., 1993; Main and Morris, 1994; Puig-Deu et al., 1996). Bentonites have great affinity to remove nitrogenous compounds and volatile substances (Puig-Deu et al., 1996). The presence of polysaccharides normally had little effect on the intensities of the individual aroma attributes, with the exception for “estery” and “floral“ attributes (ethanol level) (Jones et al., 2008) and wine fortified with yeast mannoproteins were not sensorial different when compared with untreated wine used as control (Will et al., 1991). However in this work, the highest scored obtained in wines in group II, suggested that mannoproteins improved sensorial characteristics of wine.

Table 3.12 - Mean scores for each descriptor after sensorial evaluation of the wines before and after treatment with bentonite and mannoprotein (mean±SD)

Descriptors	Bentonite							Mannoprotein								
	C	P	Br	PN	M	Vy	NS	VP	BM	Mb	B150	BB	NF	B20	PG	V	BA
Colour	7±2^{a}	8±1^{a}	8±2^{a}	8±2^{a}	8±2^{a}	8±2^{a}	8±2^{a}	8±2^{a}	8±2^{a}	8±2^{a}	8±2^{a}	8±2^{a}	8±2^{a}	8±2^{a}	8±2^{a}	8±2^{a}	8±2^{a}
Limpidity	7±3^{a}	7±3^{a}	7±3^{a}	7±3^{a}	8±2^{a}	7±3^{a}	7±3^{a}	7±3^{a}	7±3^{a}	7±3^{a}	7±3^{a}	7±3^{a}	7±3^{a}	7±3^{a}	7±3^{a}	7±3^{a}	7±3^{a}
Aroma Intensity	7±2^{a}	6±1^{a}	6±1^{a}	7±1^{a}	6±2^{a}	6±1^{a}	6±1^{a}	6±1^{a}	6±1^{a}	6±1^{a}	6±1^{a}	7±1^{a}	7±1^{a}	7±1^{a}	7±1^{a}	7±1^{a}	6±1^{a}
Fruity	7±2^{a}	6 ±1^{a}	6±1^{a}	6±1^{a}	5±1^{a}	6±2^{a}	6±2^{a}	6±2^{a}	6±2^{a}	5±2^{a}	6±1^{a}	5±2^{a}	6±1^{a}	6±1^{a}	6±1^{a}	5±2^{a}	6±1^{a}
Floral	5±2^{a}	5±1^{a}	5±1^{a}	5±1^{a}	4±1^{a}	5±2^{a}	5±2^{a}	5±2^{a}	5±2^{a}	5±2^{a}	5±2^{a}	6±2^{a}	6±1^{a}	6±1^{a}	5±1^{a}	5±2^{a}	5±2^{a}
Vegetable	3±2^{a}	2±2^{a}	3±1^{a}	2±2^{a}	3±2^{a}	3±2^{a}	3±2^{a}	3±2^{a}	3±2^{a}	3±3^{a}	3±2^{a}	3±2^{a}	3±2^{a}	2±2^{a}	2±2^{a}	3±2^{a}	2±2^{a}
Oxideised	2±2^{a}	2±2^{a}	2±1^{a}	2±1^{a}	2±2^{a}	2±2^{a}	2±1^{a}	2±2^{a}	2±2^{a}	2±2^{a}	2±2^{a}	2±2^{a}	3±3^{a}	2±2^{a}	3±3^{a}	3±2^{a}	2±2^{a}
Chemist	3±2^{a}	2±2^{a}	3±2^{a}	2±1^{a}	3±2^{a}	2±2^{a}	3±2^{a}	3±2^{a}	3±2^{a}	3±3^{a}	2±2^{a}	3±2^{a}	2±2^{a}	2±2^{a}	3±2^{a}	3 ±2^{a}	2±2^{a}
Sweetness	5±2^{a}	5±2^{a}	4±2^{a}	4±2^{a}	5±2^{a}	4±2^{a}	5±2^{a}	5±1^{a}	5±2^{a}	5±2^{a}	4±2^{a}	5±2^{a}	5±2^{a}	5±2^{a}	5±2^{a}	5±2^{a}	5±1^{a}
Acidity	7±1^{a}	7±1^{a}	7±2^{a}	6±1^{a}	6±2^{a}	7±2^{a}	7±1^{a}	6±1^{a}	6±2^{a}	6±2^{a}	6±2^{a}	7±1^{a}	6±1^{a}	6±1^{a}	6±2^{a}	6±1^{a}	6±2^{a}
Bitterness	4±2^{a}	3±1^{a}	3±1^{a}	3±1^{a}	3±1^{a}	3±2^{a}	3±2^{a}	3±1^{a}	3±2^{a}	3±2^{a}	3±2^{a}	3±2^{a}	3±2^{a}	3±2^{a}	3±2^{a}	4±2^{a}	3±1^{a}
Flavour Intensity	6±2^{a}	6±1^{a}	6±1^{a}	6±1^{a}	6±1^{a}	6±1^{a}	6± 1^{a}	6±1^{a}	6±1^{a}	5±1^{a}	6±1^{a}	6±1^{a}	6±2^{a}	6±1^{a}	6±1^{a}	6±1^{a}	7±1^{a}
Body Balance	6±1^{a}	6±1^{a}	6±1^{a}	6±1^{a}	6±1^{a}	5±1^{a}	7±1^{a}	7±1^{a}	6±2^{a}	6±1^{a}	6±1^{a}	6±1^{a}	7±1^{a}	6±1^{a}	6±1^{a}	6±2^{a}	7±1^{a}
Flavour balance	6±1^{abc}	6±1^{abc}	5±1^{a}	6±1^{abc}	6±1^{abc}	6±1^{abc}	6±1^{abc}	7±2^{bc}	6±1^{abc}	6±1^{abc}	6±1^{abc}	6±1^{abc}	6±1^{abc}	6±1^{abc}	7±1^{ab}	5±2^{ab}	7±1^{c}
Persistence	6±2^{a}	6±1^{a}	6±1^{a}	6±1^{a}	6±1^{a}	6±1^{a}	6±1^{a}	6±1^{a}	6±1^{a}	6±1^{a}	6±1^{a}	6±1^{a}	7±1^{a}	7±1^{a}	6±1^{a}	6±1^{a}	6±1^{a}

Untreated wine (C), sodium and calcium bentonite (P), activated sodium and calcium bentonite (Br), natural sodium bentonite (PN), activated calcium bentonite (M), natural calcium bentonite (Vy), mannoproteins (NS, VP, BM, Mb, B150, BB, NF, B20, PG,V, BA). Means with the same superscript letter do not differ significantly for the descriptor evaluated (Ducan test, 5%)

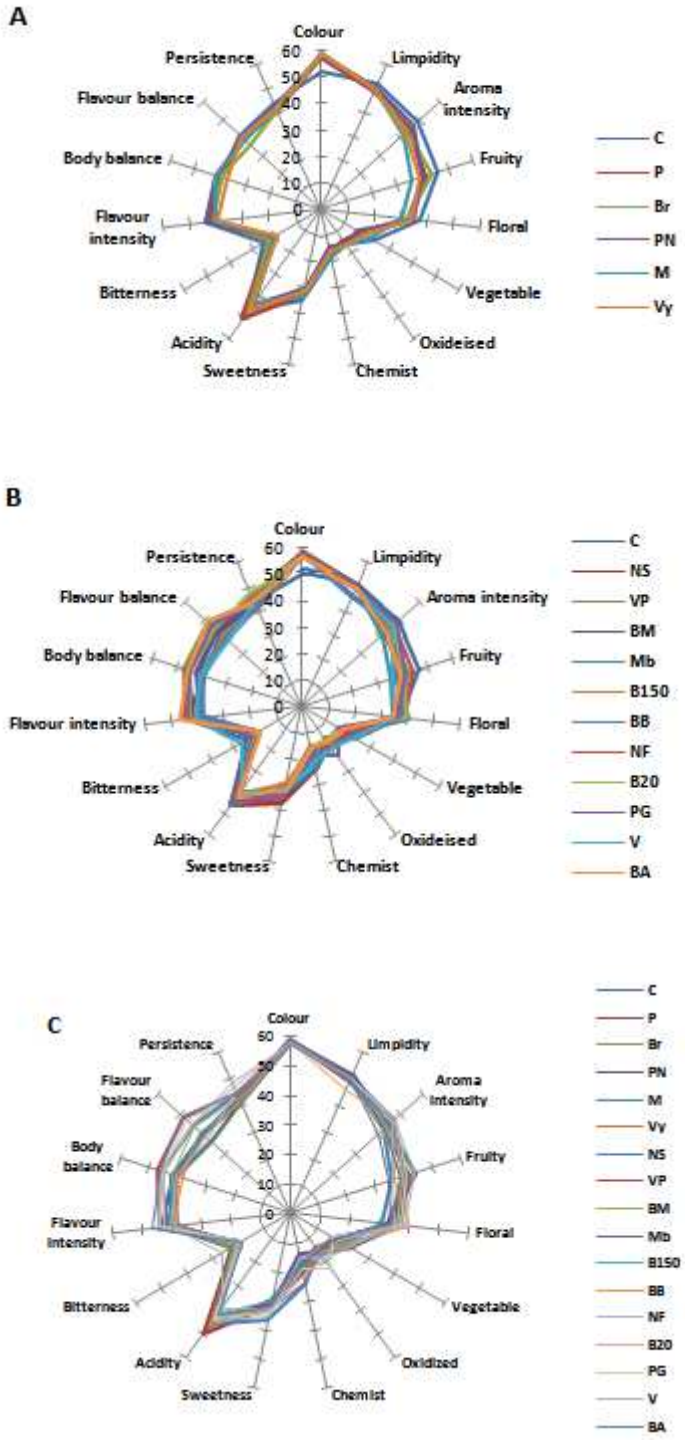
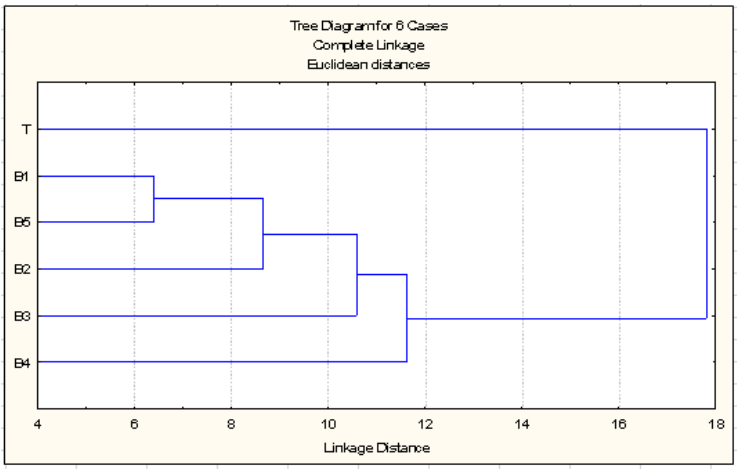


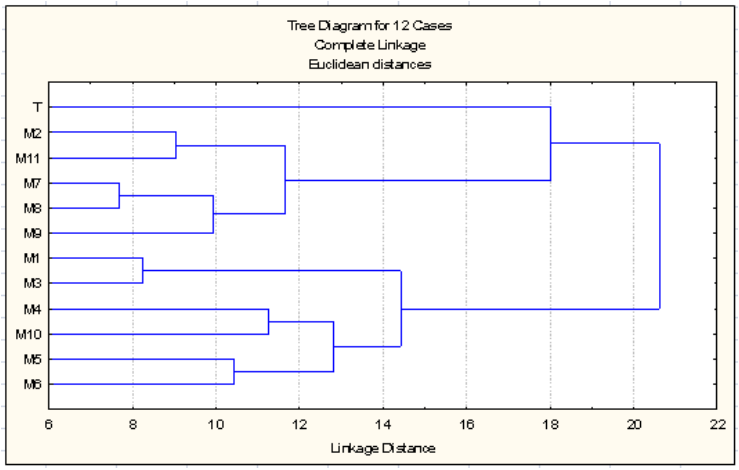
Figure 3.11 - Sensory profiles of white wine treated with bentonite and mannoprotein obtained by mean of scores given by the panellists. A – bentonite treatment, B – mannoprotein treatment, C – bentonite and mannoproteins treatment

Untreated wine (C), sodium and calcium bentonite (P), activated sodium and calcium bentonite (Br), natural sodium bentonite (PN), activated calcium bentonite (M), natural calcium bentonite (Vy), mannoproteins (NS, VP, BM, Mb, B150, BB, NF, B20, PG,V, BA).

A



B



C

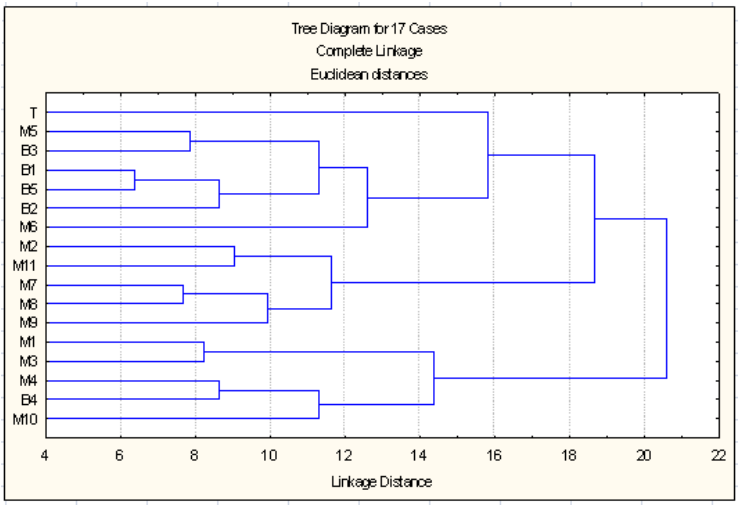
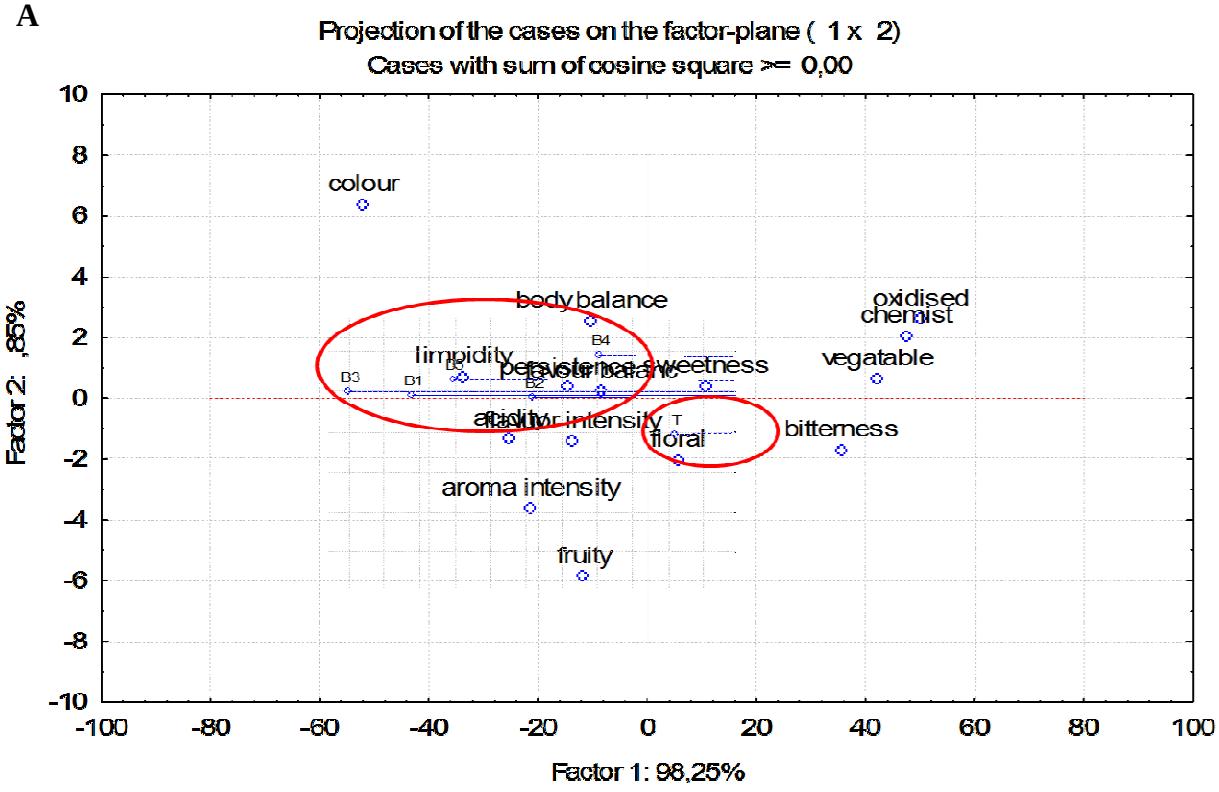


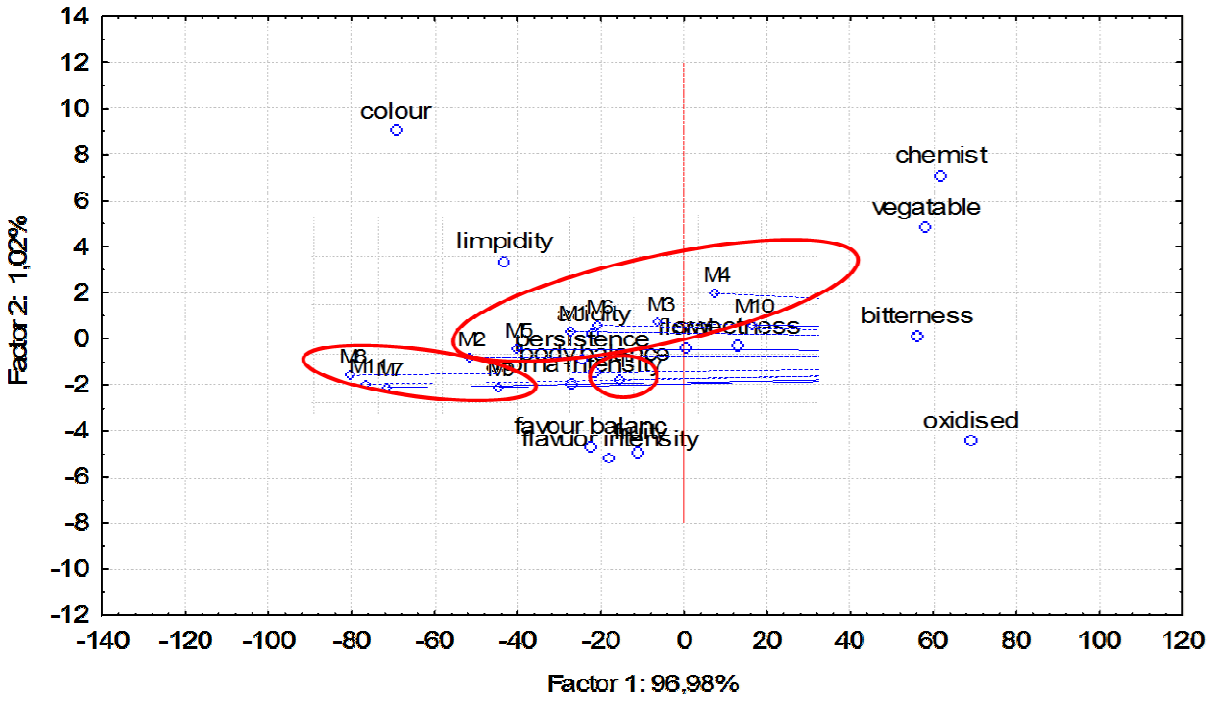
Figure 3.12 – Phenogram obtained by clusters analysis of sensorial data of the wine treated with bentonite (A), mannoprotein (B), bentonite and mannoprotein (C)

Untreated wine (C), sodium and calcium bentonite (P), activated sodium and calcium bentonite (Br), natural sodium bentonite (PN), activated calcium bentonite (M), natural calcium bentonite (Vy), mannoproteins (M1-NS, M2-VP, M3-BM, M4-Mb, M5-B150, M6-BB, M7-NF, M8-B20, M9- PG, M10-V, M11-BA).

A


Projection of the cases on the factor-plane (1 x 2)
Cases with sum of cosine square >= 0,00
colour
body balance
oxidised
chemist
vegatable
limpidity
sweetness
bitterness
floral
aroma intensity
fruity
Factor 2: ,85%
Factor 1: 98,25%

B


colour
chemist
vegatable
limpidity
bitterness
oxidised
Factor 2: 1,02%
Factor 1: 96,98%

C

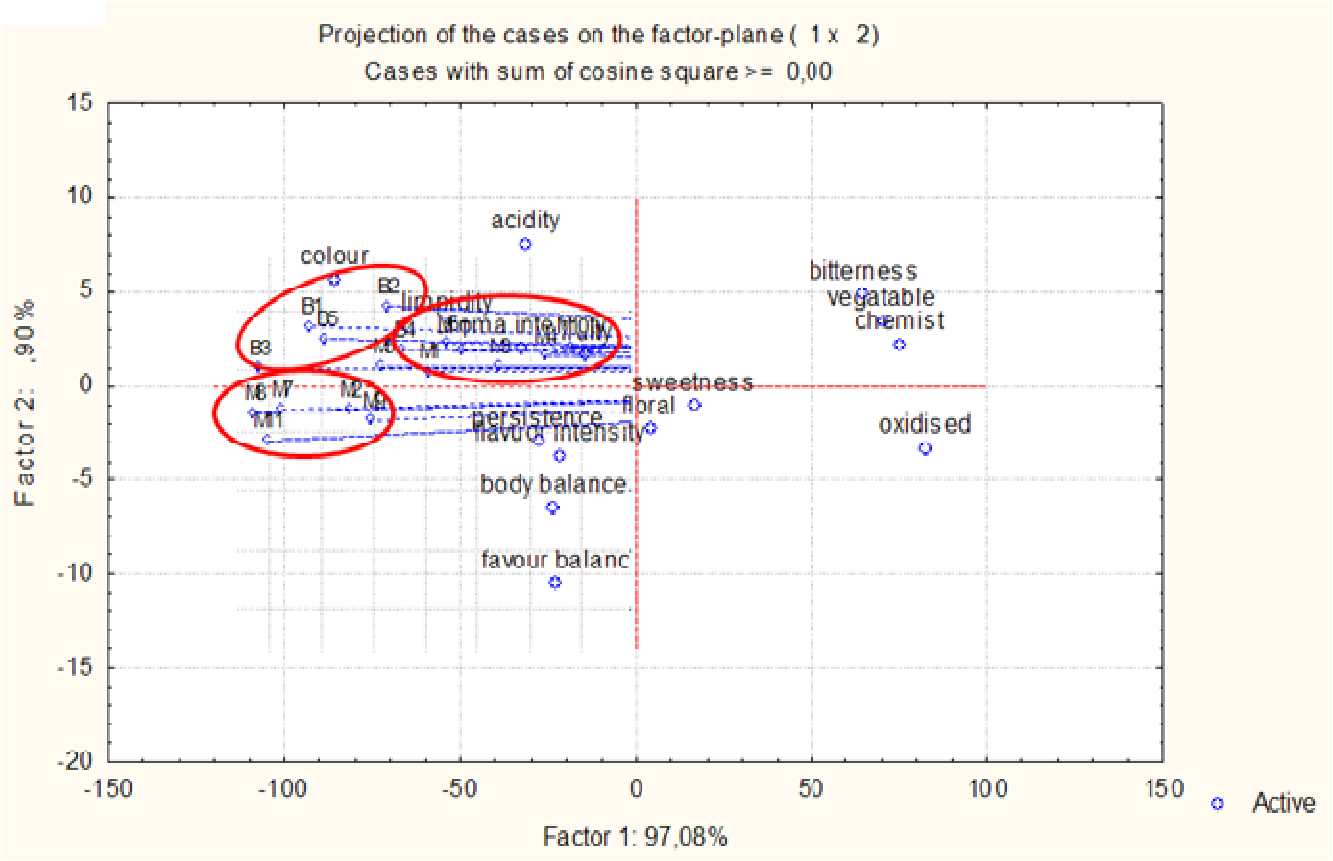


Figure 3.13 - PCA analysis projection of sensorial data of wines treated with bentonite (A), mannoprotein (B), bentonite and mannoprotein (C)

Untreated wine (C), sodium and calcium bentonite (P), activated sodium and calcium bentonite (Br), natural sodium bentonite (PN), activated calcium bentonite (M), natural calcium bentonite (Vy), mannoproteins (M1-NS, M2-VP, M3-BM, M4-Mb, M5-B150, M6-BB, M7-NF, M8-B20, M9- PG, M10-V, M11-BA).

3.5 Conclusions

Results of this work confirm the relative good efficiency of bentonites to remove unstable white wine proteins. Interesting results were obtained with mannoproteins, because high thermal stability of white wine proteins was achieved.

Almost mannoproteins decreased total phenols, flavonoids and non-flavonoid concentration. Mannoprotein addition generally improved lightness in all wines. Furthermore, mannoprotein seems to improved sensorial characteristics of wine. The obtained results suggests that effectiveness of mannoproteins in wine protein stabilization is related with amount of mannose, being more effective the ones with higher percentage.

These results suggest that, to stabilize white wine proteins, the use of mannoprotein could be an effective alternative to bentonite.

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4. Considerações finais e perspetivas futuras

Com base nos resultados obtidos e tendo em conta os métodos experimentais utilizados para realização deste trabalho, apresentam-se de forma sucinta os objetivos cumpridos e as conclusões mais relevantes.

Recorrendo aos testes de estabilidade proteica e com base nos resultados obtidos pôde verificar-se a eficácia da bentonite na estabilização proteica dos vinhos brancos, já referida por diversos autores. Ainda através dos testes referidos, foi possível verificar a capacidade das manoproteínas na estabilização das proteínas do vinho branco, podendo tornar-se um aditivo alternativo ou complementar aos métodos recorrentes.

Considerando os resultados obtidos na estabilização proteica do vinho, foram selecionadas e caracterizadas onze manoproteinas para estudo. Cada uma destas manoproteínas apresentou diferentes tipos de açúcar, nomeadamente fucose, arabinose, galactosamina, galactose, glucose e manose, sendo os níveis de cada variável entre elas, contudo a glucose e a manose, são os açúcares que se encontram em percentagem mais elevada. Através desta caracterização foi possível estabelecer uma ligação entre a percentagem de manose e capacidade das manoproteínas estabilizarem o vinho, sendo o efeito estabilizante mais elevado quanto maior a percentagem de manose presente nas manoproteínas. Porém a concentração de proteínas presente na manoproteínas também tem influência na estabilidade, sendo que concentrações mais elevadas podem induzir turvação.

No que respeita às características físico-químicas do vinho, foram avaliados parâmetros como concentração de fenóis totais, flavonóides e não-flavonóides, verificando-se que o tratamento com bentonite não teve efeitos significativos, o mesmo não se verificou no tratamento com manoproteinas, pois estas provocaram um decréscimo na composição fenólica, à excepção de três manoproteínas.

O método CIELab, evidenciou que todos os vinhos após o tratamento com bentonite tiveram um aumento na luminosidade (L*), sugerindo uma ação clarificante. Os valores correspondentes à coordenada da cromaticidade (b*), que neste trabalho definiram a cor amarela, por apresentarem valores positivos, apresentam uma diminuição quando o vinho é tratado com bentonite. Estes resultados estão de acordo com valores obtidos para a cor do vinho branco (expressa para uma absorvância de 420 nm), que igualmente mostraram diminuição após a aplicação da bentonite. Também os valores da croma (C*) diminuíram após adição de bentonite. Nas manoproteínas também se verificou um aumento da luminosidade (L*). Porém os valores correspondentes à coordenada da

cromaticidade (b*), apresentaram apenas um decréscimo para quatro das manoproteínas testadas. Estes resultados sugerem que poderá ocorrer cedência de pigmentos amarelos por parte de algumas das manoproteínas, o que está de acordo com o aumento obtido para a cor dos vinhos brancos (absorvância a 420 nm). Apesar disso é apenas no vinho tratado com uma das manoproteínas, utilizadas no estudo que a sua cor é capaz de ser distinguida pelo olho humano.

A avaliação sensorial mostrou que não existem diferenças significativas entre os vinhos tratados com bentonite e manoproteinas; porém, após uma análise de componentes principais, verificou-se a formação de três grupos, sendo o grupo II o mais pontuado, do qual fazem parte apenas vinhos tratados com manoproteínas. Estes resultados vão de encontro à caracterização dos açúcares das manoproteínas, podendo justificar-se a pontuação deste grupo com a elevada percentagem em glucose, isto é, os vinhos melhor pontuados, correspondem aos vinhos tratados com manoproteínas contendo elevada percentagem de glucose, o que demonstra capacidade em apurar as características sensoriais dos vinhos, melhorando a sua qualidade.

Dos resultados globais obtidos neste trabalho, pode-se concluir que as manoproteínas podem ser uma alternativa válida à estabilização proteica dos vinhos, porém, muito permanece ainda por estudar nesta área. Uma vez que neste trabalho foi apenas estudado um tipo de vinho, seria importante testar estes mesmos produtos enológicos em outros vinhos, com vista a verificar a reprodutibilidade e a adequação dos resultados.