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INTERACTIONS BETWEEN PROTEIN FINING AGENTS AND SEVERAL WINE PROANTHOCYANIDINS

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RESUMO

As características sensoriais dos vinhos bem como a sua estabilidade são factores de grande importância para a sua competitividade e consequentemente para a sua comercialização nos mercados internacionais e nacionais. Uma das operações tecnológicas mais utilizada na elaboração dos vinhos é a colagem proteica, pois influência as características sensoriais (amargor e adstringência) bem como a estabilidade do vinho. A eficiência e a actuação das colas proteicas dependem por um lado da composição em proantocianidinas existentes nos vinhos que tem por base a casta e o processo de vinificação utilizado, por outro das características físico-químicas das diversas colas proteicas.

Um dos objectivos do presente trabalho foi procurar conhecer os perfis tânicos de algumas variedades de castas *Vitis vinifera* bem como dos vinhos monovarietais delas resultantes. Pretendeu-se também caracterizar as principiais colas proteicas comercializadas no mercado português bem como avaliar a sua eficácia em relação às diferentes fracções de proantocianidinas dos vinhos tintos e brancos. Por fim, foram adicionadas as colas proteicas anteriormente caracterizadas a soluções modelo semelhantes ao vinho, em que cada uma das soluções era constituída por proantocianidinas com diferentes graus médios de polimerização com o intuito de aprofundar o conhecimento sobre a influência da estrutura das proantocianidinas, da concentração de proantocianidinas, do pH e da temperatura no processo de colagem. Com este estudo pretende-se disponibilizar informação de suporte à escolha do tipo de cola a usar em função do tipo de produto final a obter, e consequentemente optimizar a operação de colagem.

Os resultados mostraram que a quantidade e as características estruturais das proantocianidinas presentes nas grainhas e nas películas são diferentes entre as castas estudadas. Os vinhos monovarietais obtidos a partir dessas castas apresentavam proantocianidinas com um grau médio de polimerização que oscila entre 2,1 e 9,6. Nos vinhos monovarietais obtidos em dois anos consecutivos, verificou-se haver uma variação da concentração em proantocianidinas, no entanto o grau médio de polimerização das proantocianidinas manteve-se inalterado para cada casta. Os vinhos monovarietais analisados após seis meses mostraram uma redução de 39-59 % na quantidade de proantocianidinas e também se constatou uma modificação quanto à distribuição das diferentes proantocianidinas com distintos graus médios de polimerização. Parece ter ocorrido em simultâneo uma polimerização das proantocianidinas com um grau médio de polimerização

mais baixo e uma perda das proantocianidinas com um grau médio de polimerização mais elevado.

As colas proteicas comerciais caracterizadas mostraram diferentes características físico-químicas, quanto à distribuição da sua massa molecular, ponto isoeléctrico e quanto à densidade de carga de superfície. Essas variações verificam-se não só entre colas de diferentes tipos, como seria de esperar, mas também entre cada tipo de cola. Assim, o caseinato de potássio, a caseína, a albumina de ovo e a ictiocola sólida (obtida da bexiga natatória de peixes) são caracterizados por bandas individualizadas, respectivamente nos 30,0 kDa, na vizinhança dos 43,0 kDa e por várias bandas bem definidas superior a 94,0 kDa, entre 94,0 e 43,0 kDa e nos 20,1 kDa, enquanto que o perfil electroforético das gelatinas e ictiocolas líquidas, de uma gelatina sólida e da ictiocola sólida (obtida da hidrólise da pele dos peixes) são caracterizados por uma polidispersão na distribuição das suas massas moleculares. Em duas outras gelatinas sólidas não foram detectadas bandas entre as massas moleculares de 14,4 a 94,0 kDa. A densidade de carga de superfície também apresenta valores diferentes entre as colas estudadas. Assim, a albumina de ovo, a ictiocola e a gelatina (em soluções a 1%) mostraram densidade de carga de superfície superior quando estas se apresentavam sobre a forma sólida.

A adição de colas proteicas com diferentes características físico-químicas a vinhos tintos e brancos mostrou que estas actuam diferenciadamente sobre as fracções de proantocianidinas de diferentes graus médios de polimerização. Foi também mostrado que o decréscimo depende da cola proteica mas também do grau médio de polimerização da fracção de proantocianidina. As duas ictiocolas estudadas decresceram as fracções de grau médio de polimerização 1,5 e de 3,4 do vinho tinto, no entanto a ictiocola obtida da bexiga natatória do peixe reduziu o dobro essas fracções do que a ictiocola caracterizada por uma polidispersão inferior a 20,1 kDa. Os resultados sugerem que a acção das colas depende do tipo de moléculas de proantocianidina com que interage e não tanto se a operação se efectua em vinho tinto ou branco. Assim, a adição de ictiocolas, não induziu uma diminuição notória nas proantocianidinas com um grau médio de polimerização de 3,8 no vinho branco bem como na fracção de proantocianidinas com um grau médio de polimerização de 3,4 no vinho tinto. Por outro lado, foram a caseína e a gelatina caracterizada por uma baixa massa molecular as que mais reduziram a fracção de grau médio de polimerização 1,5 em ambos os vinhos.

Após aplicação de colas proteicas as características estruturais das proantocianidinas que permanecem no vinho colado são diferentes das do vinho inicial. Constatou-se um decréscimo do grau médio de polimerização das proantocianidinas induzido pela albumina de ovo na fracção mais polimerizada de 26 % no vinho branco e de 24 % no vinho tinto, e nos ensaios com as outras proteínas foi registrado um decréscimo de 6 a 14 % no vinho tinto e de 3 a 24 % no vinho branco.

A intensidade corante bem como as moléculas relacionadas com a cor foram menos influenciadas pela colagem proteica comparativamente às proantocianidinas. Pelo método do CIELab verificou-se que em todos os vinhos tintos colados, a luminosidade (L*) aumentou acentuadamente o que parece estar associado a uma redução dos vermelhos (a*), proporcionado pela redução dos pigmentos. Estes dados estão em concordâncias com os resultados obtidos para as antocianinas monoméricas bem como para os pigmentos totais e poliméricos.

No que diz respeito à limpidez, foi constatado, que quanto maior for a densidade de carga de superfície da proteína maior é a capacidade de clarificação do vinho. Foi estabelecida uma correlação linear entre a densidade de carga de superfície total e o decréscimo da turvação.

Nos estudos efectuados em soluções modelo, mostrou-se que as ictiocolas e as gelatinas apresentam uma correlação ($r=0.52$ e $r=-0.49$, respectivamente; $P< 0,05$) estatística significativa entre a percentagem de decréscimo das proantocianidinas e o grau médio de polimerização das fracções de proantocianidinas presentes nas soluções. Foi ainda mostrado que o decréscimo de proantocianidinas era sempre superior à temperatura de 10 ºC do que à temperatura de 20 ºC. Para uma concentração de proantocianidinas superior, verificou-se um maior decréscimo para as fracções de proantocianidinas de grau médio de polimerização mais elevado. Não se verificou influencia do pH, quando se aplicou a ictiocola obtida da bexiga natatória de peixe na fracção de proantocianidinas com um grau médio de polimerização superior e quando se aplicou a ictiocola obtida da hidrólise da pele de peixe na fracção de proantocianidinas com um grau médio de polimerização inferior.

Palavras-chave: vinho, cola, proteína, proantocianidinas, masa molecular, densidade de carga de superfície.

ABSTRACT

Wine sensory characteristics and stability are of great importance for the wine competitivity and consequently for their commercialization on the national and international market. One of the most required technological process in winemaking is protein fining which influenced wine sensory (bitterness and astringency) and stability. The effect of protein fining depends on the wine proanthocyanidin composition, which is influenced by the grape variety and the wine production process employed as well as on the physic-chemical characteristics of the protein fining agents.

The aim of this work was to know the tannic profile of grapes from *Vitis vinifera* varieties and from their monovarietal wines as well as to characterise commercial protein fining agents. The characterised proteins were added to white and red wine in order to better understand their action on the proanthocyanidin fractions and on the sensory characteristics. The characterised proteins were also added to wine-like model solutions containing each one proanthocyanidin fractions with an identified mean degree of polymerisation to enhance the information of the influence of environmental factors (pH and temperature), proanthocyanidin structural characteristics and concentration on the fining process. With these work we want to improve the knowledge of the protein fining agents and consequently allow the optimisation of the fining process.

The results showed that the quantity and the structural characteristics of the proanthocyanidins of grape seeds and skins differed between the *V. vinifera* L. cv grape varieties studied. On the monovarietal wines obtained from these grape varieties, the mean degree of polymerisation ranged from 2.1 to 9.6. In monovarietal wines obtained from two different vintages was observed that the concentration altered but the mean degree of polymerisation remained unchanged. The monovarietal wines analysed after 6 month showed a reduction of 39-59 % on the amount of proanthocyanidins and the distribution of the diverse proanthocyanidin fractions is different.

The different proteins characterised showed distinct physic-chemical characteristics such as molecular weight distribution, isolectric point and surface charge densities. These differences are not only confirmed among the different proteins as it would be accepted, but also in fining agents obtained from the same type of protein.

The addition of proteins with different physic-chemical characteristics to red and white wines showed that they decrease differently the proanthocyanidin fractions with diverse mean degree of polymerisation. The decrease depends on the fining agent but also on the mean degree of polymerisation of the proanthocyanidin fraction. Therefore, the two isinglasses assayed decreased the proanthocyanidin fractions with mean degree of polymerisation 1.5 and 3.4 from red wine, however isinglass obtained from fish swim bladder decreases these fractions more than the twice as effectively as isinglass obtained from fish skin. The results suggested that the proteins acted in function of the mean degree of polymerisation of the proanthocyanidins independently they come from red or white wine. Any of the isinglass diminished the proanthocyanidins with a mean degree of polymerisation of 3.8 in white wine as well as with 3.4 in red wine.

After employ of proteins the structural characteristics of the proanthocyanidin remained in the fined wine were different from that presented on the initial wine. Regarding the mean degree of polymerisation of fined wines, the egg albumin induced a decrease on the mean degree of polymerisation of 24 % in red wine and 26 % in white wine for the more polymerised tannin fraction; although within all assays were observed a decrease ranged from 6 to 14 % in red wine and from 3 to 24 % in white wine.

Colour intensity and molecules related to wine colour were shown to be less influenced by proteins than proanthocyanidins. A linear correlation was found between total surface charge density and decrease of turbidity.

In wine-like model solution was shown that isinglasses and gelatines presented a statistical significant correlation between the decrease of the percentage of proanthocyanidins and the mean degree of polymerisation of the proanthocyanidin fractions presented in the solution. The proanthocyanidin decrease was always higher at 10 ºC than at 20 ºC. At a higher proanthocyanidin concentration, a greater decrease was shown for the proanthocyanidin fractions with higher mean degree of polymerisation. The pH did not influenced the decrease of proanthocyanidin fractions with higher mean degree of polymerisation after fining with swim bladder isinglass, and the proanthocyanidin fractions with lower mean degree of polymerisation after fining with isinglass obtained from fish skin.

Key words: wine, fining, protein, proanthocyanidins, molecular weight, surface charge density.

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1. GENERAL INTRODUCTION

1.1. STATE OF ART SUMMARY

Wine has great importance in the history of the Mediterranean people and in the last years it has been brought great improvements on their production, quality and commercialization. Wine stability is an important quality factor for the final product. The stabilisation process involves frequently modifications on wine composition. The physic-chemical stability depends on some factors such as the type and structure of the molecule involved, for example proanthocyanidins and proteins.

Wine proanthocyanidins are extracted during wine making from the solid parts (seed and skin) of the grape (Bourzeix et al. 1986, Escribano-Bailón et al. 1992, Ricardo-da-Silva et al. 1992, Dallas et al. 1995), as a result, wine proanthocyanidins contained procyanidins and prodelphinidins, oligomers or polymers of catechins or epicatechins and gallocatechins or epigallocatechins, esterified or no with gallic acid (Ricardo-da - Silva et al. 1991, Prieur et al. 1994; Souquet et al. 1996, Sun et al. 1998, Fulcrand et al. 1999, De Pascual-Teresa et al. 1998, 2000, Sun et al. 2001, Monagas et al. 2003, González-Manzano et al. 2004, Cheynier et al. 2006). The composition and the structural characteristics of proanthocyanidins are dependent of the grape localisation (seed or skin) and of the variety, as it was shown in same works (Table 1.1).

Table 1.1 – Mean degree of polymerisation (mDP), percentage of galloylation (% gal) and percentage of prodelphinidins (%prodeph) of grape seeds and skins proanthocyanidins from *Vitis Vinifera* grapes varieties.

Grape *V. vinifera* variety	number of tannic fractions separated	mDP	% gal	% prodeph	Reference
Seed					
Alicante Bouchet	5	2.3-15.1	13.2-30.2	-	Prieur et al. (1994)
Tinta Miúda	Oligomeric and polymeric	9.8 and 31.5	23.0 and 26.2	-	Sun et al. (1998)
Cabernet Franc	8	4.7-15.7	20.0	-	Labarbe et al. (1999)
Syrah	2	2.8 and 8.9	16.2 and 22.5	-	Vidal et al. (2003)
Gamay	10	1.8 to 19.3	-	-	Perret et al. (2003)
Temperanillo	Polymeric	7.1	14.3	-	Monagas et al. (2003)
Graciano	Polymeric	7.3	10.9	-	Monagas et al. (2003)
Cabernet Sauvignon	Polymeric	6.4	12.9	-	Monagas et al. (2003)
Skin					
Merlot	6	3-80	3-6	17 to 31	Souquet et al. (1996)
Cabernet Franc	11	9.3 to 73.8	2.7	-	Labarbe et al. (1999)
Syrah	3	3.0-19.8	4	9.0-16.3	Vidal et al. (2003)
Temperanillo	Polymeric	72.3	2.9	13.3	Monagas et al. (2003)
Graciano	Polymeric	33.8	6.5	10.7	Monagas et al. (2003)
Cabernet Sauvignon	Polymeric	85.7	3.8	31.2	Monagas et al. (2003)

Consequently, grape proanthocyanidins quantity, composition and structural characteristics at harvest plays a decisive role in wine quality.

Proanthocyanidins are particularly important for the sensory characteristics of red wine, since they have the propriety to bind salivary proteins. They are relevant to red wine quality due to their astringent properties (Grawel 1998) and their responsibility in colour stability (Somers 1971). In enology, the proanthocyanidins-salivary protein associations are frequently related with the sensation of astringency (Kallithraka et al. 1998; Saint-Cricq-de-Gaulejac et al. 1999). Nevertheless, Lea and Arnold (1978) had suggested that not all wine phenolic compounds contribute in a similar form for wine astringency, and showed that the sensation of astringency was essentially due to the more polymerised tannins and those esterified with gallic acid. Therefore, it is important to know the proanthocyanidin profile of wine. In the literature there are some works that focused the structural characteristics of wine proanthocyanidins (Table 1.2).

Table 1.2 – Mean degree of polymerisation (mDP), percentage of galloylation (%gal) and percentage of prodelphinidins (% prodeph) of wine proanthocyanidins obtained from different *Vitis Vinifera* grapes varieties.

Grape *V. vinifera* variety	**number of tannic fractions separated**	**mDP**	**% gal**	**% prodeph**	**Reference**
Tinta Miúda	Oligomeric and polymeric	4.8 and 22.1	3.0 and 7.3	-	Sun et al. (1998, 2001)
Temperanillo	Polymeric	13	2.8	11.3	Monagas et al. (2003)
Graciano	Polymeric	6.9	2.8	8.2	Monagas et al. (2003)
Cabernet Sauvignon	Polymeric	9.0	3.4	10.6	Monagas et al. (2003)
Merlot/Carignan (50%/50%)	proanthocyanidins	6.2	3.9	19.2	Sarni-Manchado et al. (1999)
Syrah	proanthocyanidins	10.3	5.0	19.2	Maury et al. (2001)
Merlot	proanthocyanidins	5.8	8.3	17.7	Maury et al. (2001)
Syrah/Gernache (75%/25%)	proanthocyanidins	10.4	5.0	19.9	Maury et al. (2003)
Syrah/Gernache (25%/75%)	proanthocyanidins	12.3	4.8	22.6	Maury et al. (2003)

Frequently the new wines do not have the required final sensory characteristics. In most cases it is necessary to use specific processes to modify wine proanthocyanidins profile and consequently the sensory characteristics. Protein fining is one of the most common technological processes available that is associated with wine clarification and the improving of the sensory characteristics such as reduction of the wine astringency. Sarni-Manchado et al. (1999) in studies with gelatines showed that proanthocyanidins with higher degree of polymerisation are more astringent and other authors also in works with gelatines showed that the wine proanthocyanidins structural characteristics influenced wine fining

process (Ricardo-da-Silva et al. 1991; Sarni-Manchado et al. 1999; Maury et al. 2001; Maury et al. 2003).

At the present time, the most commonly used protein fining agents for wine fining are gelatines, egg albumins, caseins, potassium caseinates or isinglasses. The diverse protein fining agents can behave differently, depending on their composition, their origin and their preparation condition. Consequently, it is essential to know the characteristics of the fining agent and to comprehend the fining mechanisms, to achieve the proposed objectives. Proteins used as wine fining agents have different physic-chemical characteristics mainly molecular weight distribution, isoelectric point and surface charge density (Lagune and Glories 1996 a, b; Lagune-Ammirati et al. 1996; Maury et al. 2003).

Several works are focused on the influence of the fining proteins on wine composition, using in their studies different types of proteins (Ough 1960; Cruess et al. 1963; Amati et al. 1979; Yokotsuka et al. 1983; Yokotsuka and Singleton 1987; Jouve et al. 1989; Castino 1992, Gorinstein et al. 1993, Yokotsuka and Singleton 1995; Sims et al. 1995; Machado-Nunes et al. 1995; 1998, Panero et al. 2001; Fischerleitner et al. 2002, 2003; Stankovic et al. 2004). However, there are few works that shown the relation between the physic-chemical characteristics (molecular weight and surface charge density) of fining protein and their effect on wine composition in especially their interaction with proanthocyanidins. The majority of these studies were performed with gelatines (Ricardo-da-Silva et al. 1991; Lagune and Glories 1996c; Versari et al. 1998; 1999; Lefebvre et al. 1999; Sarni-Manchado et al. 1999; Maury et al. 2001; 2003) or vegetable proteins (Lefebvre et al. 1999; Marchal et al. 2000a, b; 2002; Maury et al. 2003; Bonerz et al. 2004).

Gelatines like salivary proteins are composed by a higher concentration of proline than the majority of the other proteins (Lagune and Glories 1996a). According to Sarni-Manchado et al. (1999) and Maury et al. (2001), the addition of gelatine to the wine leads to a proanthocyanidin reduction, mainly to the more polymerised and esterified with gallic acid. It was also observed that the molecular weight distribution of gelatines influenced the type of proanthocyanidins removed from red wine (Hrazdina et al. 1969; Lefebvre et al. 1999; Sarni-Manchado et al. 1999; Maury et al. 2001; 2003; Bonerz et al. 2004), and that the surface charge densities affect the precipitation of wine components (Versari et al. 1999). So, it is important for the fining process to know the physic-chemical characteristics of the proteins used and to study their interaction with the different proanthocyanidins presented in the different red and white wines.

It is known that the two main types of interactions between proteins and proanthocyanidins are: hydrogen bonds and hydrophobic interactions (Murray et al. 1994). The formation of these complexes is directly related with some factors such as the proanthocyanidin structure, the protein structure, their concentration and the environmental conditions such as pH and temperature (Calderon et al. 1968, Lea and Arnold 1978; Yokotsuka and Singleton 1978). The study of the interactions occurred between wine proantocyanidins with different mean degree of polymerisation and fining proteins are particularly important for the wine industry.

1.2. AIMS OF THE STUDY

In the last years advances on the knowledge of protein fining agents characteristics as well as on their interactions with wine phenolic compounds were done. However, the majority of this works do not perform a detailed and comparative study of the effect of different characterised protein fining agents with the distinct proanthocyanidins fractions existent in the wine. So, the goal of this work was to study both, the protein fining agents characteristics and the structural characteristics of wine proanthocyanidins and consequently the effect of adding protein fining agents on the wine proanthocyanidins final composition, since the proanthocyanidins have an important function on the sensory characteristics of wines, such as colour, bitterness and astringency.

1) Wine proanthocyanidins are extracted during wine making from the solid parts (seed and skin) of the grapes. Therefore, a better knowledge of the structural characteristics and distribution of the grape seeds and skins proanthocyanidins as well as from the monovarietal wines seems to be useful.

The aim of this point was to study the tannic profile of the proanthocyanidins from the grape seeds and skins of varieties grown in Portugal as well as from the respective monovarietal wines. The tannic profile from the monovarietal wines obtained from two vintages and also one of these vintage after 6 month of aging were compared.

2) Given the role that proteic products take in fining processes and in wine quality, it is useful to characterize them. In addition, the knowledge of the physic-chemical

characteristics of protein fining agents is important for optimizing the fining process, which affects wine quality.

Consequently, the main objectives of this part of the work was to describe and compare the characteristics such as molecular weight distribution, surface charge density, isoelectric point, and protein, Pb and Cd contents of several protein fining agents present on the market.

3) In spite of, the maiority of the authors had shown that protein fining agents interact with phenolic compounds presented in the wine, few is known about the specificity and efficiency of each protein in the interaction with each of the different proanthocyanidin fractions of wine.

The aim of these point was to undertake a comparative study on the effect of protein fining agents (gelatine, egg albumin, casein, potassium caseinate and isinglass) with distinct physic-chemical characteristics (molecular weight distributions, isoelectric points, surface charge densities) on the structural characteristics (mean degree of polymerisation, galloylation and the percentage of prodelphinidins) of oligomeric and polymeric proanthocyanidins remaining in wine after fining as a function of the type of fining protein added to red and white wine.

4) The wine complexity leads to the use of wine-like model solutions to study the extent of protein and proanthocyanidin interaction, mainly to study the influence of physic-chemical factors such as temperature, pH and proanthocyanidin concentration.

The main propose of this point was to assay the characterised fining agents in wine-like model solutions composed by proanthocyanidins with different mean degree of polymerisation. The quantity and structural characteristics (mean degree of polymerisation, percentage of galloylation and percentage of prodelphinidins) of the proanthocyanidins remaining in wine-like model solutions after fining with different type of proteins as well as the influence of environmental factors (pH and temperature) were studied. An enhanced understanding of all the molecules implicated on fining and there behaviour at different environmental conditions could conduct to an improved control of the fining operation and thus to an optimisation of this enological practice.

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2. TANNIC PROFILES OF *VITIS VINIFERA* L. CV. RED GRAPES GROWING IN LISBON AND THEIR MONOVARIETAL WINES PROFILE

Submited to *Food Chemistry*

Tannic profiles of Vitis vinifera L. cv. red grapes growing in Lisbon and from their monovarietal wines

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ABSTRACT

The tannic profiles of five grapes (*Vitis vinifera* L. cv. Touriga Nacional, Trincadeira, Castelão, Syrah and Cabernet Sauvignon) as well as the profile of their red monovarietal wines [vintage 2004 and 2005] were studied. In seeds and skins depending on the variety, the polymeric fraction represented, respectively, 77-85 % and 91-99 % of the total proanthocyanidins. The distribution of the mean degree of polymerisation (mDP) of the proanthocyanidins ranged from 2.8 to 12.8 for seeds and from 3.8 to 81.0 for skins. In monovarietal wines, the distribution of the mDP of the proanthocyanidins ranged from 2.1 to 9.6. The polymeric fraction represented 77-91 % and 82-95 % of the total proanthocyanidins, respectively, in vintage 2004 and 2005. The wine proanthocyanidins of Trincadeira and Cabernet Sauvignon, in the two vintages, showed a similar tannic profile. After 6 month it was measured a noticeably decreases on total proanthocyanidins concentration accompanied by a little decrease of the prodelphinidins percentage but the percentage of galloylation and mDP remained unchangeable.

Keywords: *Vitis vinifera*, grape seed, grape skin, red wine, proanthocyanidins, tannin, thiolysis.

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2.1. INTRODUCTION

Proanthocyanidins (condensed tannins) are found in all grape clusters (skins, seeds, stems and pulps), however, skins contain lower amount of proanthocyanidins (oligomeric and polymeric flavan-3-ols) than seeds and their structural characteristics differ (Bourzeix, Weyland & Heredia, 1986; Ricardo-da-Silva, Rigaud, Cheynier, Cheminat & Moutounet, 1991a, Ricardo-da-Silva, Belchior, Spranger & Bourzeix, 1992a, Ricardo-da-Silva, Rosec, Bourzeix, Mourgues & Moutounet, 1992b, Labarbe, Cheynier, Braussaud, Souquet & Moutounet, 1999, Souquet, Cheynier & Moutounet, 2000, Sun, Spranger, Roque-do-Vale, Leandro & Belchior, 2001, Monagas, Gómez-Cordovés, Bartolomé, Laureano & Ricardo-da-Silva, 2003, Ó-Marques, Reguinga, Laureano & Ricardo-da-Silva, 2005).

Grape seed tannins are composed only by procyanidins (Prieur, Rigaud, Cheynier & Moutounet, 1994, Labarbe et al. 1999, Vidal, Cartalade, Souquet, Fulcrand & Cheynier, 2002) whereas grape skin tannins are composed by prodelphinidins and procyanidins (Souquet, Cheynier, Brossaud & Moutounet, 1996, Cheynier, Prieur, Guyot, Rigaud & Moutounet, 1997, Labarbe et al. 1999, Vidal et al. 2002, Cheynier et al. 2006). Skin proanthocyanidins have a higher average molecular weight and a lower percentage of galloylated subunits than those from seeds (Moutounet, Rigaud, Souquet & Cheynier, 1996, Cheynier et al. 1997, Labarbe et al. 1999, Kennedy, Hayasaka, Vidal, Waters & Jones, 2001, Vidal et al. 2003). However, in both seeds and skins, the polymeric tannins were presented to a greater extent than the monomers and dimers (Cheynier et al. 1997).

According to Prieur et al. (1994) the grape seed proanthocyanidins (*V. vinifera*, var. Alicante Bouchet fractionated into 5 fractions) showed an mDP ranging from 2.3 (fraction 1) to 15.1 (fraction 5) and the proportion of galloylated units increased with the mDP from 13.2 % to 30.2 %. Sun, Leandro, Ricardo-da-Silva and Spranger (1998) determined an mDP of 9.8 and 31.5 and a percentage of galloylation of 23.0 and 26.2, respectively on oligomeric and polymeric proanthocyanidins of seed extracts (*V. vinifera*, var. Tinta Miúda). The mDP of the separated seed proanthocyanidins (*V. vinifera*, var. Cabernet Franc fractionated into eight fractions) characterised by Labarbe et al. (1999) ranged increasingly from 4.7 (fraction 1) to 15.7 (fraction 8). However, these authors showed that the galloylation rate remained constant (20%) in each fraction, which seems indicate that the extension of galloylation is independent from mDP.

Vidal et al. (2003) studied also the structural characteristics of seeds proanthocyanidins from *V. vinifera*, var. Syrah fractionated into two fractions and verified an mDP of 2.8 and 8.9 and a percentage of galloylation of 16.2 and 22.5, respectively. Perret, Pezet and Tabacchi (2003), fractionated grape seed proanthocyanidins from *V. vinifera*, var. Gamay into ten fractions and observed that the mDP varied from 1.8 to 19.3. Kennedy and Taylor (2003) fractionated grape seed proanthocyanidins from *V. vinifera*, var. Pinot noir into five fractions observed that the mDP varied from 2.0 to 24.1.

The mDP and the degree of galloylation (%gal) of the seed polymeric proanthocyanidins from Tempranillo (mDP = 7.1, % gal = 14.3), Graciano (mDP = 7.3, % gal = 10.9) and Cabernet Sauvignon (mDP = 6.4, % gal = 12.9) were determined by Monagas et al. (2003). The mDP measured in seeds from *V. Vinifera* cv. Cabernet Sauvignion at harvest was 5.6 (Kennedy, Matthews & Waterhouse, 2000a) and in seed from Syrah around 5 (Kennedy et al. 2000b, Downey, Harvey & Robinson, 2003).

The mDP of skin proanthocyanidins (*V. vinifera* var. Merlot fractionated into six fractions) determined by Souquet et al. (1996) ranged from 3 (fraction 1) to 80 (fraction 6). Nevertheless, these authors showed that the galloylation rate was low (3-6 %), and seems also to be independent from mDP, and that the percentage of prodelphinidins ranged from 17 to 31 %. Likewise, skins proanthocyanidins (*V. vinifera*, var. Cabernet Franc fractionated into eleven fractions) analysed by Labarbe et al. (1999), presented an mDP that ranged increasingly from 9.3 (fraction 1) to 73.8 on the last fraction (fraction 11). These authors also showed that the galloylation rate (2.7%) was low and independent from mDP and that the percentage of (-) epigallocatechin units (prodelphinidins) increased slightly with mDP.

Vidal et al. (2003) studied the structural characteristics of skin proanthocyanidins from *V. vinifera*, var. Syrah fractionated into three fractions and found that the mDP ranged from 3.0 to 19.8 and the percentage of (-) - epigallocatechin units (prodelphinidins) from 9.0 to 16.3, however the percentage of galloylation was around 4 %. Kennedy and Taylor (2003), fractionated grape skin proanthocyanidins from *V. vinifera*, var. Pinot noir into 7 fractions and observed that the mDP varied from 3.8 to 39.0. Monagas et al. (2003), also determinate the mDP, degree of galloylation (%gal) and percentage of prodelphinidins (% prodelph) of the skin polymeric proanthocyanidin fraction from Tempranillo (mDP = 72.3, % gal = 2.9, % prodelph = 13.3), Graciano (mDP = 33.8, % gal = 6.5, % prodelph = 10.7) and Cabernet Sauvignon (mDP = 85.7, % gal = 3.8, % prodelph = 31.2) grape varieties.

The mDP determined in skins from *V. Vinifera* cv. Syrah at commercial harvest was 27.0 by Kennedy et al. (2001) and 28.5 by Downey et al. (2003).

Wine proanthocyanidins were extracted during wine making from the solid parts of the clusters, mainly from skins and seeds, and stems if they are present (Bourzeix et al. 1986, Escribano-Bailón, Gutiérrez-Fernández, Rivas-Gonzalo & Santos-Buelga, 1992, Ricardo-da-Silva et al. 1992a, Dallas, Ricardo-da-Silva & Laureano, 1995, Fuleki & Ricardo-da-Silva, 1997, Sun, Pinto, Leandro, Ricardo-da-Silva & Spranger, 1999). Consequently, wine proanthocyanidins enclosed procyanidins and prodelphinidins (De Pascual-Teresa, Treutter, Rivas-Gonzalo & Santos-Buelga, 1998, González-Manzano, Rivas-Gonzalo & Santos-Buelga, 2004).

The mDP and percentage of galloylation of oligomeric and polymeric proanthocyanidins from red wine of Tinta Miúda [(mDP 4.8 and 22.1, respectively), (% gal 3.0 and 7.3, respectively)] were determinate by Sun et al. (1998), as well as from red wines obtained by various winemaking technologies [(mDP 3.7-5.0 and 11.1 – 15.6, respectively), (% gal 1.8 -3.3 and 5.9 – 8.3, respectively)] (Sun et al. 2001).

Monagas et al. (2003), measured the mDP, the percentage of galloylation and the percentage of prodelphinidins of the polymeric proanthocyanidins from Tempranillo (mDP = 13.0, % gal =2.8, % prodelph = 11.3), Graciano (mDP = 6.9, % gal =2.8, % prodelph = 8.2) and Cabernet Sauvignon (mDP = 9.0, % gal =3.4, % prodelph = 10.6) wines. Sarni-Manchado, Deleris, Avallone, Cheynier and Moutounet (1999) estimated an mDP of 6.2, percentage of galloylation of 3.9 and percentage of prodelphinidin of 19.2 on the proanthocyanidins of a wine from *V. vinifera* var. Merlot (50%) and var. Carignan (50%).

The mDP, percentage of galloylation and prodelphinidins of wine proanthocyanidins from Syrah [(mDP = 9.5, % gal = 5.0, % prodelph = 19.2), (mDP = 10.3, % gal = 5.1, % prodelph = 19.5)], and Merlot [(mDP = 5.8, % gal = 8.3, % prodelph = 17.7), (mDP = 5.8, % gal = 8.3, % prodelph = 12.8)] were determined by Maury, Sarni-Manchado, Lefebvre, Cheynier and Moutounet (2001) and by Maury, Sarni-Manchado, Lefebvre, Cheynier and Moutounet (2003), respectively.

A wine made from 75% Syrah and 25 % Grenache presented mDP = 10.4, percentage of galloylation and percentage of prodelphinidins of 5.0 % and 19.9%, respectively. A wine made from 25% Syrah and 75 % Grenache showed mDP = 12.3, percentage of galloylation and percentage of prodelphinidins of 4.8 % and 22.6 %, respectively (Maury et al. 2001).

Cheynier et al. (1997) observed that a red wine after four months aging showed a decrease in total proanthocyanidins, particularly on the prodelphinidins and also on the galloylated compounds, but in a lesser extend. These authors also verified that the mDP diminished which could be related to easier degradation of the proanthocyanidins with higher molecular weight. Vidal et al. (2002) also attributed the decrease of mDP to cleavage reaction that occurred in acidic medium like wine, which in this case probably dominate in relation to the polymerisation reaction of proanthocyanidins that also could occur (Haslam 1974).

According to several studies, proanthocyanidins are concerned an important function on the sensory characteristics of red wines, such as colour, bitterness and astringency. It was shown, that astringency depends on the proanthocyanidin structural characteristics such as mDP and degree of galloylation (Peleg, Gacon, Schlich & Noble, 1999, Vidal et al. 2003). Therefore, the knowledge of the wine proanthocyanidin structural composition could be essential for the definition of the sensory characteristics of the final wine. It was also evidenced by some authors that the mDP and galloylation of wine proanthocyanidins are essential structural characteristics affecting wine fining agents action (Ricardo-da-Silva, Cheynier, Souquet, Moutounet, Cabanis & Bourzeix, 1991c; Sarni-Manchado et al. 1999; Maury et al. 2001, 2003; Cosme, Ricardo-da-Silva & Laureano, 2007, 2008).

The aim of this work was to study the tannic profile from the grape seed and skin from *Vitis vinifera* L. cv. Touriga Nacional, Tricadeira, Cabernet Sauvignon, Castelão and Syrah growing in Lisbon, Portugal, as well as from the monovarietal wines produced from these grapes since there is no information about this subject. The tannic profile from the monovarietal wines from two vintages (2004 and 2005) and the evolution of same wine aged six month were compared.

2.2. MATERIALS AND METHODS

Reagents

All solvents and acids were of HPLC grade. Toluene-α-thiol was purchased from Fluka (Buchs, Switzerland).

Grapes

Vitis vinifera L. cv. Touriga Nacional, Trincadeira, Castelão, Syrah and Cabernet Sauvignon berries grown during the 2005 harvest season on the vineyards of the Tapada da Ajuda at the Instituto Superior de Agronomia located in Lisbon were used in this study. Approximately 250 berries at their technological maturity were randomly selected. The solid parts of the grape, skins and seeds, were manually separated for subsequent analysis.

Preparation of phenolic extracts from grape seeds and skins

Grape seeds were ground to a fine powder using a coffee-bean miller. The phenolic compounds from grape seeds (≈ 9 g) and skins (≈ 50 g) were extracted following the method described by Bourzeix et al. (1986).

Monovarietal Wines

Monovarietal red wines were made from grapes from *Vitis vinifera* L. cv. Touriga Nacional, Trincadeira, Cabernet Sauvignon, Castelão and Syrah grown in the same geographical area (Instituto Superior de Agronomia vineyard, Lisbon) and harvested at their technological maturity (vintage 2004 and 2005, respectively) to produced the wine for these study. The wines were elaborated at the Instituto Superior de Agronomia experimental cellar located in Lisbon, by classic vinification with maceration during approximately 12 days. The 2004 and 2005 wines were analysed around 5 month after vinification (the malolactic fermentation was already achieved). The 2004 wine was also analyses after six month storage. The chemical characteristics of wines from vintage 2004 and 2005 are: Touriga Nacional 2004 (11.8 % v/v, 7.1 g/L tartaric acid, pH 3.51), Touriga Nacional 2005 (11.6 % v/v, 5.1 g/L tartaric acid, pH 3.84), Trincadeira 2004 (11.0 % v/v, 7.5 g/L tartaric acid, pH 3.43), Trincadeira 2005 (12.4 % v/v, 6.8 g/L tartaric acid, pH 3.62), Cabernet Sauvignon 2004 (13.0 % v/v, 7.5 g/L tartaric acid, pH 3.39), Cabernet Sauvignon 2005 (13.0 % v/v, 7.1 g/L tartaric acid, pH 3.62), Castelão 2004 (11.8 % v/v, 8.0 g/L tartaric acid, pH 3.20), Castelão 2005 (11.9 % v/v, 6.9 g/L tartaric acid, pH 3.50), Syrah 2005 (14.7 % v/v, 6.8 g/L tartaric acid, pH 3.53), Syrah 2005 (14.4 % v/v, 6.6 g/L tartaric acid, pH 3.75).

Separation of proanthocyanidins by C_{18} Sep-Pak cartridges and determination of the flavan-3-ol content by the vanillin assay

The separation of flavanols was performed in a C_{18} Sep-Pak cartridge (Waters, Milford, Ireland) according to their degree of polymerisation in three fractions monomeric, oligomeric and polymeric, in agreement with the method described by Sun et al. (1998). The total flavan-3-ol of each fraction was performed by the vanillin assay according to the method described by Sun et al. (1998). Quantification was carried out by means of standards curves prepared from monomers, oligomers, and polymers of flavan-3-ol isolated from grape seeds, as described earlier (Sun et al. 1998, 2001).

Fractionation of proanthocyanidins (wines, seeds and skins) according to their degree of polymerisation using a sequential dissolving procedure on an inert glass powder column

Proanthocyanidins (oligomeric and polymeric) extracted from seeds, skins and wines were separated from phenolic monomers, by fractionation in a C_{18} Sep-Pak cartridge (Waters, Milford, Ireland), and in agreement with the method described by Sun et al. (2001). The proanthocyanidin extract from seeds (Touriga Nacional, Trincadeira, Cabernet Sauvignon, Castelão and Syrah), skins (Touriga Nacional, Cabernet Sauvignon, and Castelão) or wines (Touriga Nacional, Trincadeira, Cabernet Sauvignon, Castelão and Syrah), were separated according to their degree of polymerisation following the method described by Labarbe et al. (1999). The elution gradient (methanol/chloroform) applied for wines and seeds was the following FI-25:75 (v/v); FII-30:70 (v/v); FIII-35:65 (v/v); FIV-40:60 (v/v); FV-45:55 (v/v); FVI-50:50 (v/v); FVII-55:45 (v/v); FVIII 100:0 (v/v) and for skins was used the subsequent gradient FI-25:75 (v/v); FII-30:70 (v/v); FIII-35:65 (v/v); FIV-40:60 (v/v); FV-45:55 (v/v); FVI-50:50 (v/v); FVII-55:45 (v/v); FVIII 60:40 (v/v); FIX 65:35 (v/v); FX 70:30 (v/v); FXI 100:0 (v/v).

Those tannin fractions were analysed by HPLC after thiolysis, to estimate their structural characteristics (mDP, % gal and % prodelph) and to determine their concentration.

Characterisation of wines, seeds and skins proanthocyanidins by acid-catalysed depolymerisation in the presence of toluene– α-thiol followed by reversed-phase HPLC analysis

The acid-catalysed degradation was carried out according to Monagas et al. (2003) and the thiolysed sample were then analysed by reversed-phase HPLC. The equipment and elution conditions employed for analytical HPLC were the same used by Cosme et al. (2008). The amounts of monomers (terminal units) and toluene-α-thiol adducts (extension units) released from the depolymerisation reaction in the presence of toluene-α-thiol, were calculated from the areas of the chromatographic peaks at 280 nm by comparison with calibration curves (Rigaud, Perez-Ilzarbe, Ricardo-da-Silva & Cheynier, 1991; Prieur et al. 1994, Kennedy et al. 2000a).

2.3. RESULTS AND DISCUSSION

2.3.1. Grape tannic profile

Concentration and structural composition of the proanthocyanidins from grape seeds and skins greatly differed among the V. V*inifera* L. cv grape varieties studied, which agrees with previous studies performed by other authors. On an mg/g basis, the grape seed proanthocyanidin concentration was always higher than in skins (Table 2.1).

Structural characterisation and quantification of grape seed proanthocyanidin fractions

The flavan-3-ols, of seed fractions (monomeric, oligomeric and polymeric) determined by the vanillin reaction, are shown in Table 2.1. The grape seeds of Cabernet Sauvignon presented higher level of oligomeric plus polymeric flavan-3-ols when compared with the other V. V*inifera* L. cv grape seed proanthocyanidin analysed (Table 2.1). The lowest values of monomeric, oligomeric and polymeric flavan-3-ols were measured for Touriga Nacional grape seed. The highest mDP for the polymeric grape seed fraction was verified for Castelão (8.8 mDP) followed by Syrah (7.8 mDP). The mDP values for Syrah grown in Portugal are in the range of already published data concerning Syrah seed proanthocyanidins (Vidal et al. 2002, 2003).

Proanthocyanidins extracted from grape seeds were also fractionated according to their degree of polymerisation on an inert glass powder column eluted with a gradient of methanol/chloroform. The data concerning the structural characteristics of all the fraction of seed proanthocyanidins after toluene-α-thiolyse are summarized in Table 2.2. The percentage of galloylation ranged from 9.4 to 32.2 %, and it was observed that the degree of galloylation of the proanthocyanidins increased with an increase of the mDP, as it was previously observed for grape seeds from Alicante Bouchet (Prieur et al. 1994), but not in grape seeds from Cabernet Franc (Labarbe et al. 1999).

The proanthocyanidins of grape seeds showed an mDP ranging from 2.8 to 12.8 (Table 2.2). Among the varieties analysed different tannic profiles were observed (Fig. 2.1). Touriga Nacional measured the lowest concentration of total proanthocyanidins, and showed a distribution of tannin fractions as follow: 36 % for 2-4 mDP, 44 % for 5-8 mDP and 17% for 12-13 mDP. Trincadeira and Syrah presented the major quantity of proanthocyanidins (85% and 76 %, respectively) on the mDP 4-7 and on the mDP 3-6, respectively, and a lower amount at a higher mDP (13% at 10-11 mDP and 23 % at 12-13 mDP, respectively). As already noticed Cabernet Sauvignon measured the highest concentration of total proanthocyanidins. This variety showed the major quantity of tannins on the mDP 3-5 (59 %) and at 6-7 mDP (30%) and a fewer quantity of proanthocyanidins on higher mDP (10 % at 11-12 mDP). Castelão presented 40 % of the proanthocyanidins with an mDP of 3-6, 24 % at mDP 8-9 and 33 % at mDP 11-12.

Table 2.1 - Concentration (mg/g) and mean degree of polymerisation (mDP) of seeds and skins of the monomeric flavanols, oligomeric proanthocyanidins and polymeric proanthocyanidins of *Vitis vinifera* L. cv. Touriga Nacional, Trincadeira, Cabernet Sauvignon, Castelão and Syrah grape seeds and skins (mean±SD).

	Monomeric flavanols	Oligomeric proanthocyanidins	Polymeric proanthocyanidins	Total proanthocyanidins*
Touriga Nacional				
Seed (mg/g)	0.3±0.0	7.7±0.1	27.1±3.6	34.8±0.1
Seed mDP	-	3.8±0.2	6.2±0.5	
Skin (mg/g)	0.02±0.00	0.01±0.00	2.36±0.39	2.36±0.56
Skin mDP	-	7.5±0.7	26.4±2.9	
Trincadeira				
Seed (mg/g)	1.1±0.3	18.0±0.6	64.1±0.9	82.1±2.2
Seed mDP	-	4.3±0.4	6.6±0.7	
Skin (mg/g)	0.03±0.01	0.23±0.11	2.95±0.35	3.17±0.65
Skin mDP	-	6.1±0.5	33.4±3.7	
Cabernet Sauvignon				
Seed (mg/g)	1.8±0.2	17.7±2.5	74.3±0.6	91.9±2.7
Seed mDP	-	2.3±0.1	5.1±0.7	
Skin (mg/g)	0.02±0.00	0.04±0.01	1.05±0.03	1.09±0.04
Skin mDP	-	9.0±0.8	43.9±3.9	
Castelão				
Seed (mg/g)	0.3±0.0	7.8±0.8	49.7±1.6	57.5±1.1
Seed mDP	-	5.2±0.6	8.8±0.4	
Skin (mg/g)	0.01±0.00	0.08±0.02	5.76±0.54	5.84±0.74
Skin mDP	-	9.0±0.5	22.5±2.7	
Syrah				
Seed (mg/g)	2.0±0.4	15.1±0.4	57.9±1.6	72.9±1.7
Seed mDP	-	3.3±0.2	7.8±0.4	
Skin (mg/g)	0.01±0.00	0.09±0.02	2.43±1.33	2.52±1.91
Skin mDP	-	7.6±1.4	45.1±2.6	

*Sum of oligomeric and polymeric proanthocyanidins

Structural characterisation and quantification of skins proanthocyanidin fractions

In relation to the grape skins, the quantification by the vanillin assay revealed that the monomeric and oligomeric flavan-3-ol concentration was similar for all the five varieties studied; with exception of the content of the skin oligomeric fraction in Trincadeira which was the highest one when compared to the other grape skins oligomeric proanthocyanidins (Table 2.1). The polymeric proanthocyanidin fraction represented the highest proportion of total flavan-3-ols content in the different grape varieties studied, but the skins from Castelão measured the highest concentrations of polymeric proanthocyanidins compared to the other grape varieties. The highest mDP for the

polymeric grape skin fraction was verified for Syrah (45 mDP) followed by Cabernet Sauvignon (44 mDP) and the lowest values for Castelão grape skins (22.5 mDP) (Table 2.1). The mDP values for the polymeric fraction of Syrah skin proanthocyanidins are in agreement with previous results (Moutounet et al. 1996, Vidal et al. 2002).

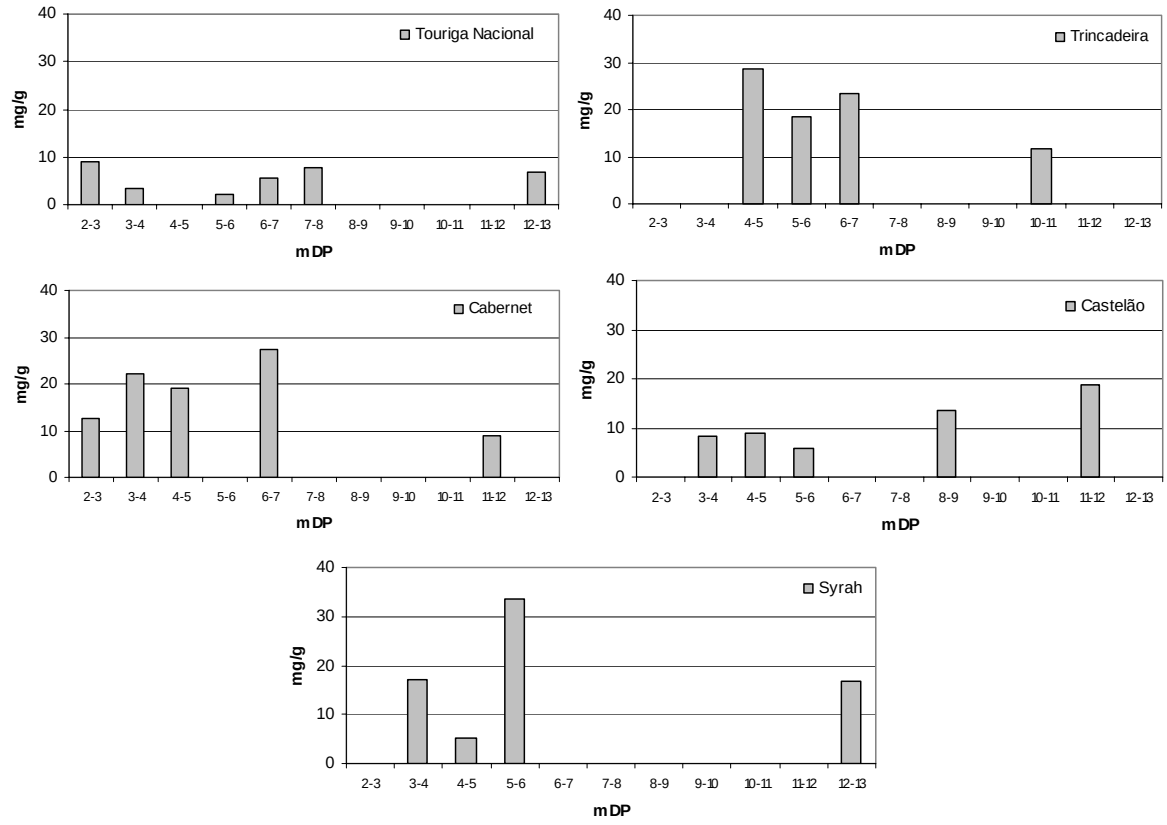

Fig. 2.1 - Tannic profile of *Vitis vinifera* L. cv. Touriga Nacional, Trincadeira, Cabernet Sauvignon, Castelão and Syrah grape seeds.

The tannic profile of grape skins and their structural characteristics were performed in three grape vine varieties, Touriga Nacional, Cabernet Sauvignon and Castelão (Table 2.3). In grape skin proanthocyanidins as expected from other studies (Souquet et al. 1996, Labarbe et al. 1999) was also verified the existence of (-) epigallocatechin units (prodelphinidin), therefore the skin proanthocyanidins contained both procyanidin and prodelphinidin units (Souquet et al. 1996, Souquet et al. 2000, Labarbe et al. 1999, Kennedy et al. 2000a, Fulcrand, Remy, Souquet, Cheynier & Moutounet, 1999). In addition, skin proanthocyanidins diverge from seed proanthocyanidins by their lower percentage of galloylation and higher mDP, which agrees with other woks (Labarbe et al.

1999, Souquet et al. 2000, Sun et al. 2001, Monagas et al. 2003). The percentage of galloylation of the skin proanthocyanidins ranged from 2.3 to 7.3 %, and it seem to be not a relation between the mDP and the percentage of galloylation, as it was previously observed by grape skins from Merlot (Souquet et al. 1996), Cabernet Franc (Labarbe et al. 1999) and Syrah (Vidal et al. 2003). The percentage of prodelphinidins in the skins ranged from 12.9 to 42.1 % and it is observed the tendency of proanthocyanidins with a higher mDP also showed a higher percentage of epigalhocatechins units. This tendency was also observed in grape varieties Merlot, Cabernet Franc and Syrah (Souquet et al. 1996, Labarbe et al 1999, Vidal et al. 2003).

The proanthocyanidins of grape skins of the three studied varieties showed an mDP ranging from 3.8 to 81.0 (Table 2.3). It was also observed, for grape skins proanthocyanidins that the tannic profile differed among the varieties analysed. Castelão shows the lowest mDP (3.8 to 49.3) values and Cabernet Sauvignon the highest mDP (6.0 to 81.0). Cabernet Sauvignon measured the lowest concentration of total proanthocyanidins on the skins and the proanthocyanidins distribution was mainly (84 %) at the higher mDP (mDP >30), with only 23 % of the proanthocyanidins with mDP 6-12. The tannic profile of Touriga Nacional was composed by 51 % of the proanthocyanidins with mDP 3-18, 20 % with mDP 24 and 27 % with mDP 65. The tannic profile of Castelão skins presented 19 % of the proanthocyanidins with an mDP of 3-6, 57 % at mDP 12 - 18 and 20 % at mDP 44.

Table 2.2 - Structural characteristics (mDP – mean degree of polymerisation, %gal – percentage of galloylation) and concentration (mg/g) of the proanthocyanidin fractions from Touriga Nacional, Trincadeira, Castelão, Syrah and Cabernet Sauvignon grape seeds (mean±SD).

Proanthocyanidin fractions	Touriga Nacional			Trincadeira			Cabernet Sauvignon			Castelão			Syrah		
	mg/g	mDP	% gal	mg/g	mDP	% gal	mg/g	MDP	% gal	mg/g	mDP	% gal	mg/g	mDP	% gal
FI	6.5±0.2	2.8±0.1	10.9±0.6	12.6±0.3	4.1±0.9	9.4±0.1	12.6±0.2	2.9±0.2	10.4±0.5	8.3±0.2	3.3±0.5	11.6±0.6	9.2±0.2	3.1±0.4	11.5±0.3
FII	2.6±0.1	2.9±0.6	11.8±0.2	12.4±0.5	4.2±0.7	13.0±0.9	9.4±0.2	3.0±0.1	12.3±0.8	2.9±0.2	4.0±0.3	12.2±0.7	6.3±0.3	3.5±0.4	13.5±0.5
FIII	1.9±0.1	3.1±0.6	12.7±0.4	3.5±0.2	4.9±0.3	14.4±0.3	3.5±0.2	3.1±0.3	14.6±0.5	2.7±0.7	4.2±0.2	12.3±0.4	1.5±0.4	3.9±0.6	14.2±0.3
FIV	1.6±0.3	3.4±0.3	13.6±0.2	4.5±0.3	5.0±0.9	15.2±0.7	4.5±0.6	3.3±0.2	15.4±0.8	3.4±0.3	4.5±0.7	12.6±0.4	3.1±0.6	4.3±0.6	16.7±0.4
FV	2.1±0.2	5.1±0.5	14.2±0.9	4.9±0.6	5.5±0.9	16.1±0.1	4.9±0.4	3.4±0.2	16.6±0.5	2.9±0.4	5.2±0.6	14.6±0.2	2.1±0.8	4.9±0.9	19.1±0.2
FVI	5.5±0.3	6.6±0.7	15.3±0.4	9.2±0.6	5.9±0.7	20.7±0.6	19.2±0.8	4.9±0.4	23.6±0.5	2.9±0.5	5.7±0.9	15.0±0.7	15.0±0.5	5.3±0.8	20.3±0.3
FVII	7.7±0.4	7.0±0.8	17.5±0.3	23.4±2.0	6.5±0.3	21.5±0.7	27.4±0.6	6.3±0.9	24.1±0.6	13.6±0.6	8.9±0.8	20.7±0.6	18.6±0.2	5.9±0.9	20.5±0.2
FVIII	6.1±0.5	12.8±0.4	28.7±0.2	10.8±0.8	11.7±0.4	32.2±0.5	8.8±0.2	11.1±0.7	28.9±0.9	18.8±0.9	11.6±0.6	26.1±1.7	16.9±0.6	12.4±0.7	22.5±0.3
Total seed extract	34.0	6.2	16.4	81.3	6.1	18.5	90.3	5.1	19.7	55.6	7.6	18.6	72.6	6.5	18.7

Table 2.3 - Structural characteristics (mDP – mean degree of polymerization, %gal – percentage of galloylation, % prodelph – percentage of prodelphinidins) and concentration (mg/g) of the proanthocyanidin fractions from Touriga Nacional, Castelão and Cabernet Sauvignon grape skins (mean±SD).

Proanthocyanidin fractions	Touriga Nacional				Cabernet Sauvignon				Castelão			
	mg/g	mDP	% gal	% prodelph	mg/g	mDP	% gal	% prodelph	mg/g	mDP	% gal	% prodelph
FI	0.08±0.02	4.6±0.2	4.9±0.6	17.9±0.6	0.02±0.01	6.0±0.9	2.9±0.2	22.6±0.7	0.09±0.03	3.8±0.6	4.5±0.8	12.9±0.7
FII	0.03±0.01	5.4±0.6	5.8±0.8	19.8±0.7	0.01±0.00	6.2±0.3	3.8±0.4	24.4±0.9	0.05±0.01	4.1±0.6	4.8±1.0	15.8±0.5
FIII	0.04±0.01	7.7±0.4	5.7±0.4	22.7±0.4	0.01±0.00	7.1±0.8	4.1±0.6	26.2±1.1	0.06±0.01	4.6±0.4	5.2±0.4	18.7±0.9
FIV	0.09±0.03	8.7±0.6	2.6±0.2	23.6±0.6	0.01±0.00	7.3±0.8	5.6±0.6	25.9±0.6	0.07±0.01	5.8±0.8	3.6±0.2	22.6±0.7
FV	0.05±0.02	10.5±0.7	3.2±0.9	24.2±0.9	0.02±0.01	7.7±0.9	6.2±1.2	27.5±1.2	0.06±0.01	5.9±0.9	4.2±0.9	26.2±1.1
FVI	0.16±0.03	11.7±0.9	3.3±0.4	25.3±0.4	0.04±0.01	7.9±0.8	7.3±0.7	29.7±0.9	0.19±0.01	6.1±0.3	2.3±0.4	25.9±0.6
FVII	0.19±0.02	12.9±0.8	3.5±0.5	27.5±0.3	0.03±0.01	9.1±1.0	4.5±0.7	34.5±0.4	0.22±0.03	6.9±0.9	3.5±0.5	29.5±1.2
FVIII	0.25±0.02	13.6±0.9	3.7±0.2	28.7±0.9	0.09±0.02	11.7±1.1	3.7±0.3	42.1±1.3	0.39±0.09	8.3±0.7	5.7±0.3	27.7±1.1
FIX	0.28±0.08	15.2±0.9	3.3±0.4	32.3±0.8	0.33±0.01	26.9±1.2	3.3±0.5	40.3±0.7	1.78±0.02	12.9±1.1	4.3±0.5	32.9±0.7
FX	0.47±0.04	23.8±1.9	3.5±0.3	27.5±0.7	0.27±0.09	38.9±1.1	3.5±0.6	41.1±1.1	1.47±0.09	16.6±2.0	4.5±0.5	29.1±2.0
FXI	0.62±0.07	64.5±2.8	3.7±0.3	33.7±1.1	0.26±0.08	81.0±4.4	4.7±0.6	40.1±1.3	1.18±0.08	49.3±3.2	4.7±0.4	37.1±1.1
Total skin extract	2.3	28.4	3.6	28.5	1.1	41.7	4.3	41.8	5.6	19.8	4.3	30.5

2.3.2. Wine tannic profile

Table 2.4 depicts the flavan-3-ols, of wine fractions (monomeric, oligomeric and polymeric) measured by the vanillin reaction. The data showed that the concentration of the total proanthocyanidins of all the five monovarietal wines elaborated from grapes cultivated in the same geographical area and under the same winemaking conditions was lower in vintage 2004 than in vintage 2005. However, the highest concentration of oligomeric plus polymeric proanthocyanidins in vintage 2004 was measured in wines from Touriga Nacional and in vintage 2005 in wines from Syrah. The polymeric fraction from the five monovarietal wines ranged from 77-91 % and 82-95 % of the total proanthocyanidins in vintage 2004 and 2005, respectively (Table 2.4).

The mDP values of the total proanthocyanidins ranged from 4.3 to 5.9 in vintage 2004 and from 4.4 to 6.2 in vintage 2005 (Table 2.5). These data informed that the higher concentrations of proanthocyanidins measured in vintage 2005 seems to be not associated with a higher mDP of the total proanthocyanidins. It could also be observed in Fig. 2.2, that the distribution of the proanthocyanidin fractions with different mDP in the wines from Trincadeira and Cabernet Sauvignon was similar in vintage 2004 and 2005. It is also to point out, that for the two vintages, wines from Castelão do not show proanthocyanidin fractions with mDP among 2 and 3 and that the wines from Cabernet Sauvignon do not show proanthocyanidins fraction with mDP above 7.

The structural characteristics presented in Table 2.5 showed that the percentage of galloylation and the percentage of prodelphinidins were very close in the two vintages. The values measured for the percentage of galloylation are in agreement with other studies done in wine from Tinta Miúda (Sun et al. 1998), Syrah and blends from Syrah (Maury et al. 2001, 2003). Also, the values obtained for the percentage of prodelphinidins were similar to that measured in wines from Syrah and blends from Syrah (Maury et al. 2001, 2003).

Table 2.4 - Concentration (mg/L) of the monomeric flavanols, oligomeric proanthocyanidins, polymeric proanthocyanidins, total proanthocyanidins and the mean degree of polymerisation (mDP) of the total proanthocyanidins of *Vitis Vinfera* L. cv Touriga Nacional, Trincadeira, Cabernet Sauvignon, Castelão and Syrah monovarietal wines of the vintage 2004 and 2005 (mean±SD).

Wine	Monomeric flavanols	Oligomeric proanthocyanidins	Polymeric proanthocyanidins	Total proanthocyanidins*	mDP
Touriga Nacional					
2004	14.9±1.2	152.5±2.2	507.0±6.0	659.5±22.1	4.5±0.6
2004 (S)	7.7±0.3	62.3±1.0	288.3±8.2	350.6±10.7	4.6±0.2
2005	11.5±1.0	73.9±6.9	670.0±8.0	743.6±16.1	5.0±0.3
Trincadeira					
2004	3.5±1.2	22.1±0.5	171.2±3.9	193.3±21.3	4.8±0.9
2004 (S)	2.7±0.2	21.2±5.9	72.1±9.6	93.3±7.9	5.1±0.6
2005	16.3±1.6	61.4±2.3	816.4±9.8	877.8±11.1	4.6±0.8
Cabernet Sauvignon					
2004	5.5±0.1	27.8±1.5	261.3±9.1	289.1±4.7	4.3±0.5
2004 (S)	2.2±0.7	12.1±0.3	103.6±12.7	115.7±13.5	4.7±0.7
2005	30.4±3.7	87.5±3.0	689.2±9.3	776.7±17.1	4.4±0.3
Castelão					
2004	5.6±0.6	40.6±1.8	405.4±8.2	446.0±16.4	5.9±0.2
2004 (S)	4.3±1.0	12.9±3.3	253.4±5.7	266.3±8.1	5.5±0.3
2005	8.5±0.7	42.4±0.5	792.8±12.7	835.2±19.2	6.2±0.5
Syrah					
2004	12.7±0.8	65.9±8.7	427.9±7.8	493.8±18.5	5.2±0.4
2004 (S)	6.0±0.1	10.9±5.6	244.4.±6.1	255.3.±10.5	4.5±0.6
2005	28.8±1.1	228.3±5.1	1002.3±15.2	1230.6±25.1	5.3±0.5

2004 (S) – analysis performed after 6 month storage. * Sum of oligomeric and polymeric proanthocyanidins

Data concerning the proanthocyanidin content of the five monovarietal wines of the vintage 2004 analysed showed that the concentration of proanthocyanidins in wines during six month decreased 39-59 %. On Fig. 2.2 we could also observed that the changes were not only on the proanthocyanidin concentration by also on the distribution of the different proanthocyanidin fractions. It seems that simultaneously occurs a polymerisation of the lower mDP fraction and a loss of the higher mDP fraction. However, no changes on the mDP and percentage of galloylation (exception for Castelão) of the total proanthocyanidins were observed (the small differences found are within experimental error). The percentage of prodelphinidins showed small decrease during storage. The modifications during wine

aging (six month) leads to structural diversity of proanthocyanidins but not to larger polymer, as shown in Table 2.5 and Fig. 2.2. Analogous results were observed for the percentage of galloylation and of prodelphinidins of a bended wine of Merlot and Carignan, aged during three month (Sarni-Manchado et al. 1999).

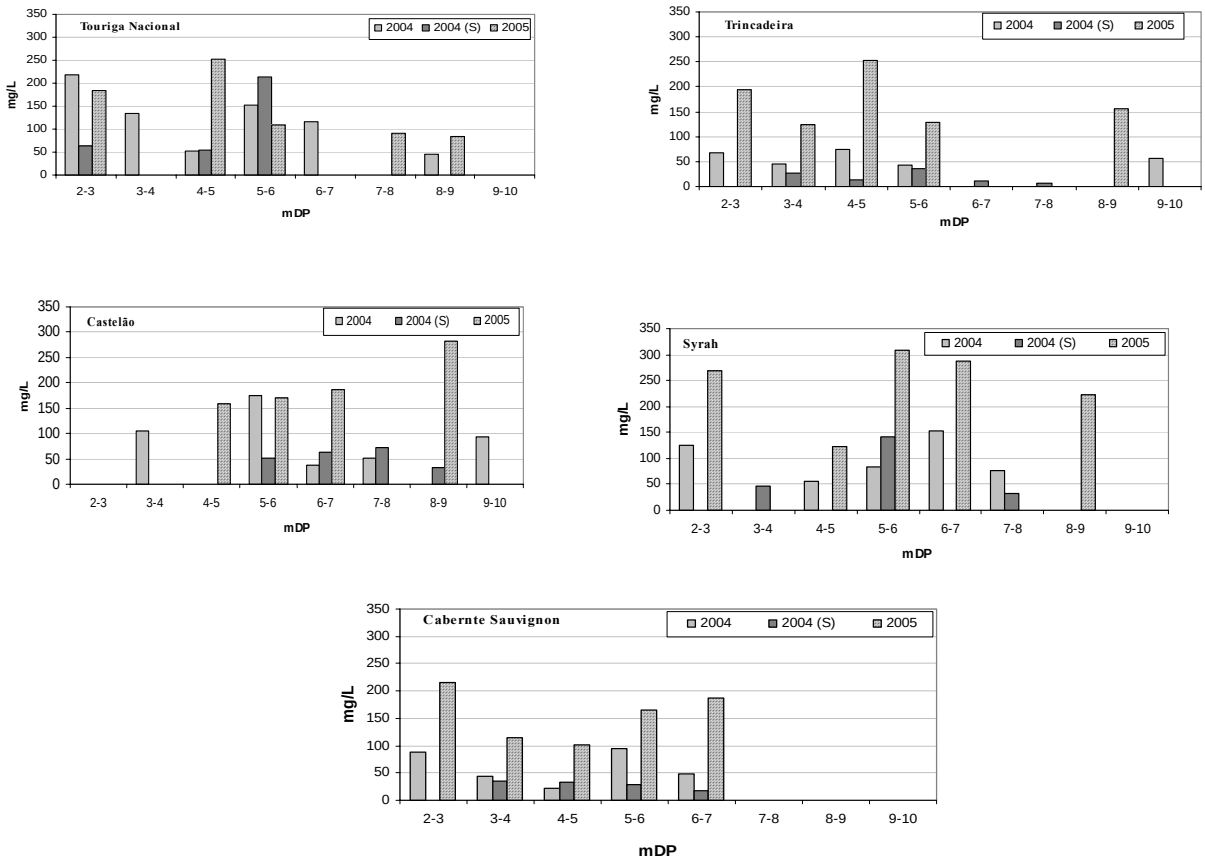

Fig. 2.2 - Tannic profile of *Vitis vinifera* L. cv. Touriga Nacional, Trincadeira, Castelão, Syrah and Cabernet Sauvignon monovarietal wine (vintage 2004 and 2005).

Table 2.5 - Structural characteristics (mDP – mean degree of polymerisation, %gal – percentage of galloylation, % prodelph – percentage of prodelphinidins) and concentration (mg/L) of the proanthocyanidin fractions from Touriga Nacional, Trincadeira, Cabernet Sauvignon, Castelão and Syrah monovarietal wines of the vintage 2004, 2004 (S) and 2005 (mean±SD).

Proanthocyanidin	2004				2004 (S)				2005			
Fractions	mg/L	mDP	% gal	% prodeph	mg/L	mDP	% gal	% prodeph	mg/L	mDP	% gal	% prodeph
Touriga Nacional												
F1	218.3	2.4	3.9	20.8	64.5	2.8	3.3	16.2	184.1	2.9	4.2	24.8
F2	134.4	3.8	9.9	20.4	53.9	4.9	9.4	18.4	124.7	4.2	10.9	23.3
F3	52.9	4.5	3.6	16.2	53.6	5.0	3.4	14.1	39.0	4.5	4.6	18.1
F4	107.4	5.4	5.3	18.2	35.8	5.4	4.9	16.1	87.7	4.9	5.9	18.9
F5	45.0	5.7	6.3	8.2	33.0	5.5	5.8	6.2	42.1	5.2	6.8	9.5
F6	67.8	6.4	1.8	8.5	33.8	5.5	1.5	7.9	65.9	5.8	2.8	8.7
F7	48.1	6.9	2.2	22.3	33.0	5.6	2.1	19.7	90.8	7.6	2.7	23.1
F8	45.9	8.1	2.1	13.7	24.6	5.9	2.0	12.1	83.7	8.9	2.9	12
TOTAL	719.8	4.5	4.9	17.5	332.2	4.6	4.1	13.7	718.0	5.0	5.2	19.0
Trincadeira												
F1	67.6	2.1	5.4	18.4	26.7	3.3	4.7	17.1	194.1	2.9	5.6	20.1
F2	44.9	3.3	7.3	33.2	12.6	5.0	6.8	29.3	123.3	3.4	7.5	33.4
F3	24.2	4.3	7	29.4	13.0	5.4	6.5	23.2	80.1	4.1	7.3	29.6
F4	25.0	4.6	7.2	16.8	10.0	5.9	6.4	13.9	78.4	4.6	7.5	15.9
F5	26.4	4.8	6.4	16.9	12.5	5.9	6	12.9	94.7	4.7	6.5	16.7
F6	21.6	5.2	2.9	16.6	5.4	6.5	2.3	12.7	60.9	5.2	2.8	16.8
F7	21.4	5.4	5.4	15.9	5.5	6.9	5	12.7	67.7	5.4	5.5	16.1
F8	57.0	9.6	4.3	19.1	6.8	7.0	3.5	16.3	156.5	8.1	4.4	19.6
TOTAL	288.1	4.8	5.5	20.6	92.5	5.1	5.4	18.0	855.6	4.5	5.7	21.0
Cabernet Sauvignon												
F1	87.7	2.7	7.1	27.6	35.83	3.7	6.6	24.6	216.6	2.4	7.6	29.1
F2	44.9	3.4	6.5	29.5	12.4	4.4	5.6	25.2	115.4	3.9	6.7	29.2
F3	22.0	4.9	8.7	19.9	10.9	4.6	8.2	17.4	45.5	4.4	8.9	20.1
F4	24.5	5.1	8.7	20.8	9.8	4.8	7.7	17.4	55.6	4.7	8.8	20.9
F5	29.6	5.3	5.6	15.7	11.3	5.1	7.0	13.3	61.1	5.2	7.9	15.9
F6	18.9	5.5	4.6	20.2	8.9	5.5	4.6	16.5	49.3	5.3	5.1	20.7
F7	22.3	5.9	6.2	17.2	9.1	5.7	6.0	15.9	54.8	5.7	6.7	17.8
F8	49.3	6.6	4.3	18.2	16.8	6.2	4.1	14.9	186.1	6.6	4.5	18.9
TOTAL	299.2	4.3	6.2	22.1	114.9	4.7	6.2	19.4	784.4	4.4	6.5	22.6
Castelão												
F1	104.3	3.2	9.7	32.9	18.5	5.0	9.6	30.9	159.1	4.5	9.9	32.9
F2	54.1	5.1	4.5	15.9	17.9	5.2	4.4	13.8	91.5	5.2	5.2	17.9
F3	43.4	5.7	4.8	19.1	15.9	5.9	4.6	18.1	78.7	5.8	5.3	21.8
F4	38.7	5.9	4.9	17.2	19.9	6.1	4.6	16.6	69.7	6.2	5.3	19.6
F5	39.9	5.98	6.1	17.8	21.5	6.2	5.9	17.0	59.5	6.4	6.7	19.4
F6	38.0	6.1	6.4	15.0	21.2	6.4	6.1	14.9	58.1	6.6	6.9	18.1
F7	52.3	7.1	2.4	18.5	71.9	7.3	2.3	16.9	95.3	8.1	2.8	19.2
F8	94.3	9.3	3.2	19.2	32.9	8.1	3.1	18.3	186.0	8.5	3.9	20
TOTAL	465.0	5.9	5.4	20.8	219.8	5.5	3.6	14.8	797.9	6.2	5.5	21.0
Syrah												
F1	125.3	2.2	8.7	19.8	47.3	3.2	8.5	16.8	267.8	2.9	9.5	22.6
F2	56.0	4.9	6.4	18.6	19.5	5.1	6.3	17.2	121.9	4.1	7.1	21.4
F3	41.7	5.4	8.1	18.4	42.4	5.2	7.8	18.2	91.5	5.1	8.7	19.8
F4	40.9	5.8	3.0	17.5	19.0	5.2	2.8	16.3	112.3	5.3	3.3	18.2
F5	44.6	6.0	4.7	19.1	20.7	5.6	4.5	18.7	103.8	5.8	4.9	21.7
F6	48.4	6.2	1.4	17.6	19.5	5.7	1.3	17.0	131.3	6.3	1.9	19.2
F7	59.5	6.6	4.7	16.4	21.1	5.8	4.4	15.9	156.5	6.7	5.2	18.9
F8	75.6	7.8	3.3	18.7	32.8	7.2	2.9	17.7	223.3	8.1	3.9	20.9
TOTAL	492.0	5.2	5.5	18.4	222.3	4.5	4.76	15.1	1208.4	5.3	5.6	19.8

2004 (S) – analysis performed after 6 month of storage

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3. PROTEIN FINING AGENTS: CHARACTERIZATION AND RED WINE FINING ASSAYS

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PROTEIN FINING AGENTS: CHARACTERIZATION AND RED WINE FINING ASSAYS

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ABSTRACT

The physico-chemical characteristics of protein fining agents are important for optimizing the fining treatment which affects wine quality. The aim of this study was to characterize nineteen commercial fining products. Furthermore, a fining trial was done to evaluate the influence of different fining proteins on some phenolic characteristics of red wine. The results show that the molecular weight (MW) distribution of caseins and potassium caseinate products are characterized by a band at 30.0 kDa and egg albumins by a band close to 43.0 kDa. Isinglass (swim bladder) has bands at 20.1, between 94.0 – 43.0 and above 94.0 kDa. In addition, two of the gelatins studied do not have any band in the MW range studied. The other fining agents displayed polydispersion. The isoelectric point (IEP) of the proteins ranged from 4.20 to 6.48. The effects of egg albumin (AS_1), isinglass (IL_1 and IS_4), potassium caseinate (CKS_1), casein (CS_4) and gelatin (GS_2, GS_4 and GL_1) on red wine phenolic compounds are discussed.

Key words: fining agents, isoelectric point, phenolic compounds, protein, SDS-PAGE, surface charge density, wine.

RIASSUNTO

Agenti Proteici chiarificanti: Caratterizzazione e prova di chiarificazione del vino rosso

Le caratteristiche chimico-fisiche degli agenti chiarificanti proteici sono importanti per l'ottimizzazione del trattamento di chiarificazione che influenza la qualità del vino. Lo scopo di questo studio era la caratterizzazione di diciannove chiarificanti commerciali. È stata inoltre effettuata una prova di chiarificazione allo scopo di valutare l'influenza di differenti proteine chiarificanti su alcuni composti fenolici caratterizzanti il vino rosso. I risultati hanno mostrato che la distribuzione del peso molecolare (PM) della caseina e del caseinato di potassio è caratterizzata da una banda di 30,0 kDa e quella di albumina di uova da una banda di 43,0 kDa. La colla di pesce (vescica natatoria) presenta bande a 20,1, comprese tra 94,0 – 43,0 e sopra i 94,0 kDa. Inoltre, due delle gelatine esaminate non presentano alcuna banda nell'intervallo di peso PM considerato. Gli altri agenti di chiarificazione hanno rivelato polidispersione. Il punto isoelettrico della proteine studiate variava tra 4,20 a 6,48. Sono stati discussi gli effetti dell' lbumina d'uova (AS_1), della colla di pesce (IL_1 e IS_4), del caseinato di potassio (CKS_1), della caseina (CS_4) e della gelatina (GS_2, GS_4, e GL_1) sui composti fenolici del vino rosso.

3.1. INTRODUCTION

The oenological fining agents are very diverse and complex. They are usually made from non-modified animal proteins or from protein extracts obtained after adequate treatment of animal tissue (AMATI and MINGUZZI, 1976). Recently, other protein sources, such as cereals and legumes, have been studied as wine fining agents (MARCHAL *et al.,* 2000a; b; 2002; PANERO *et al.,* 2001; MAURY *et al.,* 2003). Gelatin, isinglass, casein, potassium caseinate and egg albumin are the most commonly used proteins in wine fining. They can be used separately or with mineral fining agents (MACHADO-NUNES *et al.,* 1998), such as bentonite or silica gel.

Proteins used as wine fining agents have different physico-chemical characteristics mainly molecular weight (MW) distribution, isoelectric point (IEP) and surface charge density. Several authors have shown that these characteristics influence the properties of fining agents (HRAZDINA *et al.,* 1969; PAETZOLD and GLORIES, 1990; LAGUNE and GLORIES, 1996a; b; VERSARI *et al.,* 1998). It has been pointed out that the molecular weight of gelatin influences the amount and type of phenolic compounds removed from red wine (HRAZDINA *et al.,* 1969; YOKOTSUKA and SINGLETON, 1987; RICARDO-DA-SILVA *et al.,* 1991; LAGUNE and GLORIES, 1996b; SCOTTI and POINSAUT, 1997; VERSARI *et al.,* 1998; LEFEBVRE *et al.,* 1999; SARNI-MANCHADO *et al.,* 1999; MAURY *et al.,* 2001). For example, MAURY *et al.* (2001) showed that more hydrolysed gelatins eliminate more polymerized tannins than less hydrolysed ones.

Gelatins have been the most studied fining agents (PAETZOLD and GLORIES, 1990; MARCHAL *et al.,* 1993; 2002; LAGUNE and GLORIES, 1996a; b; VERSARI *et al.,* 1998; 1999). These products, obtained by enzymatic hydrolysis, showed that most of the protein fractions had MWs lower than 13.7 kDa (PAETZOLD and GLORIES, 1990); several authors have also verified that liquid and hot soluble gelatins show polydispersion in the MW distribution (PAETZOLD and GLORIES, 1990; MARCHAL *et al.,* 1993; 2000a, b; 2002; VERSARI *et al.,* 1998; 1999). The IEP of gelatin depends on the technological processes. When the insoluble collagen is transformed into soluble gelatin by either an acid or basic process, a gelatin of Type A or B, respectively, is obtained (KAUFMANN, 1988; LAGUNE and GLORIES, 1996c): The IEP of Type A gelatin ranges from 7.5 to 9.5 and of Type B from 4.7 to 5.0 (PAETZOLD and GLORIES, 1990). MARCHAL *et al.* (2000a) reported that the electrophoretic pattern of solid isinglass presented individualized bands

with a MW between 17 and 80 kDa, and another isinglass revealed bands with MWs between 110 and 220 kDa. The casein fining agent showed a band close to 30 kDa (MARCHAL *et al.,* 2000a; b), with some other bands with lower MWs (10-23 kDa), as well as some with higher MWs (50-80 kDa). Milk casein is a heterogeneous group of four principal phosphoproteins and phosphoglycoproteins (α_{s1}-casein, α_{s2} -casein, κ-casein and β-casein) whose MW ranges from 11.6 to 24.1 kDa with an average isoelectric point of 4.6 (EVANS, 1982; FOX *et al.,* 1982). Similarly, egg white is a mixture of different proteins, where ovalbumin (phosphoglycoprotein) makes up about 54 % of the total proteins, with a MW of 45 kDa and an isoelectric point of 4.6 (CHEFTEL *et al.,* 1985; FRONING, 1988). Other proteins in egg white showed antimicrobial factors such as conalbumin (MW 76 kDa; pI 6.1), lysozyme (MW 14.3 kDa; pI 10.7) and avidin (MW 68.3 kDa; pI 10.0) or enzyme inhibitors including ovomucoid (MW 28 kDa; pI 4.1), ovoinhibitor (MW 49 kDa; pI 5.1) and ficin (MW 12.7 kDa; pI 5.1) (FRONING, 1988). The electrophoretic pattern of solid egg albumin fining agent had a band close to 43-45 kDa, along with two other bands at 15 and 90 kDa, as well as several minor bands between 25 and 100 kDa (MARCHAL *et al.,* 2002).

Protein fining agents exhibit different surface charge densities when evaluated in a model solution like wine. Depending on the type of gelatin and the pH of the medium, the surface charge density ranged from 0.02 to 1.2 meq/g (PAETZOLD and GLORIES, 1990; LAGUNE and GLORIES, 1996a; b; LAMADON *et al.,* 1997). Surface charge density for different isinglasses, evaluated at a pH between 2.8 and 3.8 ranged from 0.32 to 0.83 meq/g, and for egg albumin (solid and fresh) at a pH between 3.0 and 4.0 ranged from 0.22 to 0.96 meq/g. The surface charge density of potassium caseinates estimated at pH 7 was close to 0.5 meq/g (LAMADON *et al.,* 1997).

Wine fining agents are added exogenous products that should not contribute compounds such as lead (Pb) and cadmium (Cd) to the wine (OIV, 2006a; b). The technology of making high-quality wines includes an accurate quantitative knowledge of the presence of these elements and their continuous monitoring (BRAININA *et al.,* 2004). These elements need to be quantified due to their high toxicity and potentially by adverse health effects. In order to protect consumer health, Pb and Cd levels are limited by regulations (for fining agents and wine) (MENA *et al.,* 1996; LEMOS *et al.,* 2002).

Given the important role that protein fining agents play in wine quality and safety, it is important to characterize them. To our knowledge, there are no data in the literature concerning the electrophoretic patterns for MW distribution of potassium caseinate, liquid

isinglass and liquid egg albumin. To our knowledge, the surface charge densities of casein, liquid isinglass and liquid egg albumin have not been published, nor have the isoelectric points of solid and liquid isinglass or of solid and liquid egg albumin. Consequently, the main objectives of this study were: 1) to describe and compare the characteristics such as molecular weight distribution, surface charge density, isoelectric point, and protein, Pb and Cd contents of several protein fining agents present on the market and 2) to increase the understanding of the action of these proteins on wine limpidity, monomeric anthocyanins and flavonoid and non-flavonoid compounds during the wine fining process.

3.2. MATERIALS AND METHODS

3.2.1. Fining agent characterization

Protein fining agents: Two potassium caseinates, two caseins, four egg albumins, four isinglasses and seven gelatins from different companies were characterized (Table 3.1).

Table 3.1 - Protein fining agents characterized and used in this study.

Product	Code	Concentration[a] (g of commercial fining agent [wet weight])	Producer information
Egg albumin solid	AS_1	12.5 g/hL	-
Egg albumin solid	AS'_1	-	With lysozyme.
Egg albumin solid	AS_4	-	-
Egg albumin liquid	AL_4	-	-
Isinglass solid	IS_1	-	Collagen hydrolysis contained in fish skin.
Isinglass solid	IS_4	2.25 g/hL	Obtained from swim bladder.
Isinglass liquid	IL_1	50 ml/hL	Collagen hydrolysis contained in fish skin.
Isinglass liquid	IL_4	-	Collagen hydrolysis contained in fish skin.
Potassium caseinate solid	CKS_3	-	-
Potassium caseinate solid	CKS_1	40 g/hL	-
Casein solid	CS_2	-	-
Casein solid	CS_4	40 g/hL	-
Gelatin solid	GS_3	-	Cold soluble.
Gelatin solid	GS_2	8 g/hL	Hot soluble.
Gelatin solid	GS_4	8 g/hL	Cold soluble. High hydrolysis degree.
Gelatin liquid	GL_1	50 mL/hL	High concentrated, obtained by chemical.
Gelatin liquid	GL_2	-	-
Gelatin liquid	GL_5	-	-
Gelatin liquid	GL_4		Pig source.

A-Egg albumin, C-Casein, CK-Potassium caseinate, I-Isinglass, G-Gelatin, S-Solid, L-Liquid.

1, 2, 3, 4 and 5 different fining agent suppliers. a-used in the wine fining trials.

Protein quantification: Total nitrogen was determined by the Kjeldahl method based on mineralization, distillation and titration with 0.1 N HCl (MANFREDINI, 1989; OIV, 2006b). Total protein content was estimated as Kjeldahl nitrogen multiplied by the following factors: 6.38 (OIV, 2006b), for casein and potassium caseinate, 5.55 (Lees, 1971) for gelatin, 6.68 (Lees, 1971) for egg albumin and 6.25 (Mackie, 1983) for isinglass.

Protein concentration was also determined by the Bradford method modified by READ and NORTHCOTE (1981) to reduce the variation in the response of different proteins. The assay was performed by adding different proteins [protein fining agents and standard protein (bovine serum albumin)] to a dye reagent [Coomassie brilliant blue G-250 (Acros Organics, New Jersey, NJ, USA), ethanol, phosphoric acid and deionized water], which resulted in an increased absorbance at 595 nm, due to the formation of a protein-dye complex (READ and NORTHCOTE, 1981).

Protein molecular weight distribution characterized by SDS-PAGE: Molecular weight distributions of oenological protein fining agents were studied by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method as suggested by LAEMMLI (1970) and adapted for protein fining agents by MARCHAL *et al.* (2000a; b; 2002). Standard proteins covering a 14.4 to 94.0 kDa range were used to evaluate the molecular weight [Low Molecular Weight (LMW) Amersham Biotech, London, U.K.]. Samples and standard proteins were treated with buffer [(0.125 M Tris-Cl, 4 % SDS, 20 % glycerol, 2% 2-mercaptoetanol, pH 6.8)] (v/v) and denatured at 100 ºC for 5 minutes. A 5 µL sample was loaded in each electrophoresis well, which corresponds to a protein content (determined by the modified Bradford method) of 2.7 – 2.8 µg for potassium caseinates, 1.5 – 3.0 µg for caseins, 1.3 – 6.0 µg for isinglasses and 1.0 – 5.8 µg for gelatins. The gel with 0.75 mm thickness was run in a mini-vertical gel electrophoresis unit (Mighty-Small II SE 250, Hoefer, San Francisco, CA, USA) at a constant voltage (75 V) at 20 ºC until the bromophenol blue reached the bottom of the gel. After migration, proteins were stained in a solution made up of one part Coomassie blue R-350 (Amersham Bioscience, Uppsala, Sweden) and nine parts of a solution with methanol: acetic acid: water (3:1:6) and destained in a mixture of acetic acid: methanol: water (1:2:7) (MARCHAL *et al.,* 2000a; b; 2002).

pH: The pH was measured on a 1 % solution of initial product (w/v) of solid gelatin, solid isinglass and solid egg albumin. The pH was measured on a 5 % solution of initial

product (w/v), of solid potassium caseinate and on a 10 % solution of initial product (w/v) of solid casein. The pH determination was based on the International Codex of Oenology (OIV, 2006b). The pH was measured directly in the colloidal solution of the liquid fining agents (gelatin, isinglass and egg albumin).

Weight loss on drying: The weight loss was determined according to the International Codex of Oenology (OIV, 2006b) at 100-105 ºC on a 2 g sample of the following proteins: casein, potassium caseinate, egg albumin, solid gelatin and solid isinglass. In the case of a colloidal solution of gelatin, egg albumin or isinglass, a 10 g sample was used, which was dried over water at 100 ºC for four hours, and then dried in an oven at 100-105 ºC for three hours.

Ash: Ash was evaluated by progressive incineration at 500-550 ºC of the residue that remained after the determination of loss during drying, according to the International Codex of Oenology (OIV, 2006b).

Lead and Cadmium: Lead and cadmium were determined by graphite furnace atomic absorption spectrometry using Zeeman background correction according to CATARINO and CURVELO-GARCIA (1999). These analyses were performed at the "Estação Vitivinicola Nacional" laboratory, Dois Portos, Portugal.

Surface charge density: Surface charge density of protein fining agents was measured with a particle charge detector – produced by MÜTEK (Herrsching, Germany) model PCD 03 pH – by titration with a charge compensating polyelectrolyte 0.001 N electropositive-polydiallyldimethylammonium [polyDADMAC (Herrsching, Germany)] or 0.001 N electronegative-sodium polyethylensulfate [PES-Na (Herrsching, Germany)] (PAETZOLD and GLORIES, 1990; DIETRICH and SCHÄFER, 1991) until the streaming potential was 0 mV, which corresponds to the point where all charges are neutralized. The volume of polyelectrolyte needed for the neutralisation allowed the surface charge density of the product, to be evaluated; it is expressed in milliequivalents of polyelectrolyte per gram of fining agent (meq/g). All determinations were done at 20 ºC.

The fining agents - gelatin, isinglass and egg albumin - were dispersed in a model solution similar to wine but lacking ethanol (VERNHET *et al.,* 1996).

Caseins and potassium caseinates were first dissolved in 0.1 N KOH and then dispersed in the model solution. The surface charge density of these fining agents was measured at the pH of dissolution and at pH 3.4 (adjusted with 50 % HCl and centrifuged at 4000 rpm for 15 min).

Isoelectric point: The isoelectric point from the protein fining agents dispersed in distilled water was evaluated with a model PCD 03 pH particle charge detector (MÜTEK, Herrsching, Germany) by titration with an acid or basic solution until the streaming potential was 0 mV. The pH measured corresponds to the isoelectric point.

3.2.2. Wine fining trials

Protein fining agents: One egg albumin (AS_1), two isinglasses (IL_1, IS_4), one potassium caseinate (CKS_1), one casein (CS_4) and three gelatins (GL_1, GS_2 and GS_4) were added to a young red wine. All these protein fining agents were previously characterized.

Red wine: The young red wine (vintage 2003) used in this study was produced from different grapevine varieties from the Estremadura Region (North of Lisbon) and had the following chemical characteristics: alcohol content 8.7 % (v/v), density 0.9972, titratable acidity 7.6 g/L expressed as tartaric acid, volatile acidity 0.76 g/L expressed as acetic acid, pH 3.31, free sulphur dioxide 10 mg/L and total sulphur dioxide 46 mg/L.

Fining trials: Fining experiments were carried out by adding protein fining agents (isinglass, egg albumin, casein, potassium caseinate and gelatin) at the average levels and prepared as recommended by the producers (Table 3.1) to 250 mL of wine. An untreated sample was used as a control. The fining agents were thoroughly mixed and allowed to remain in contact with the wine for 7 days at 20 ºC. All experiments were done in duplicate.

Limpidity: Limpidity was evaluated by measuring the optical density at 650 nm of the centrifuged and non-centrifuged wine as described by FEUILLAT and BERGERET (1966).

Monomeric anthocyanins: Monomeric anthocyanin analysis was performed by High Performance Liquid Chromatograph (HPLC) according to Dallas and Laureano (1994).

The equipment used for the HPLC analysis was a Perkin-Elmer (Norwalk, CT, USA) system, equipped with a model L-7100 Lachrom Merck Hitachi-High-Technologies pump (Tokyo, Japan), a model LC-95 UV-Vis detector set at 520 nm coupled to a version 6.2 Konikrom data chromatography treatment system (Konik Instruments, Konik-Tech, Barcelona, Spain). The column was a reversed-phase C_{18} Lichrosphere 100 (5 μm packing, 250mm x 4.6 mm i.d.) (Merck, Darmstadt, Germany) protected with a guard column of the same material. The separation was performed at room temperature. The elution conditions for monomeric anthocyanins was as followed: 0.7 mL/min., flow rate, solvent A was 40 % formic acid, solvent B was CH_3CN and solvent C was double distilled water. The initial conditions were 25 % of A, 6 % of B and 69 % of C for 15 min followed by a linear gradient to 25 % of A, 25.5 % of B 49.5 % of C during 70 min, and 20 min of 25 % A, 25.5 % of B and 49.5 % of C. Wine samples were analysed in duplicate after filtration.

Quantification of monomeric anthocyanins in wine was carried out by means of standard curves prepared by using different concentrations of malvidin 3-glucoside chloride in methanol 0.1 % HCl. The peak area was converted to mg/L of malvidin 3-glucoside equivalent. Twenty μL of each concentration were injected in triplicate.

Chromatic characterization: The absorption spectra of the wine samples were recorded with a Unicam UV-vis UV4 spectrophotometer (Unicam, Cambridge, U.K.), scanned over the range 380 to 770 nm using quartz cells of 1-mm path length. Data were collected at 10 nm intervals, and referred to a 1-cm path length, in order to calculate L* (lightness), a* (measure of redness) and b* (measure of yellowness) coordinates using the CIELab method (OIV, 1990). The spectrophotometer has the required software to calculate the CIELab parameters directly (Chroma version 2.0 Unicam, Cambridge, U. K.). To differentiate the colour more precisely, the colour difference was obtained using the following expression: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2\ ]^{1/2}$, in CIELab units. It quantifies the overall colour difference of a given sample when compared to a reference sample (non-treated sample). The mean visual perception of colour difference between two solutions will be assumed as a value of $\Delta E^* = 1$ (GONNET, 1998). All samples had been clarified by centrifugation and were analysed in duplicate.

Quantitative estimation of flavonoid phenols and non-flavonoid phenols: Determination of the phenol content before and after precipitation of the flavonoids through

reaction with formaldehyde was done according to KRAMLING and SINGLETON (1969). All samples were analysed in duplicate.

3.2.3. Statistical analysis

The data are presented as the mean±SD. Analysis of variance and comparison of treatment means (LSD, 5% level) were performed using ANOVA Statistica 5.1 software (StatSoft, Tulsa, OK, USA) in that it compared the effect of the protein fining agents.

3.3. RESULTS AND CONCLUSIONS

3.3.1. Characterization of fining agents

Protein content

Total nitrogen values of the protein fining agents ranged from 11.1 to 22.8 % (w/w) expressed in dry weight (Table 3.2). Regarding the protein content estimated by the total nitrogen, the liquid fining agents in general had the highest values when expressed in dry weight. In the case of solid gelatins, the values [88 - 98 % (w/w)] agree with previously published data (VERSARI *et al.,* 1998). The protein content obtained by a modified Bradford method (READ and NORTHCOTE, 1981) were lower than those estimated by converting total nitrogen to protein. This can probably be explained by the fact that Coomassie Blue G Dye reacts poorly with proteins whose MW ranges from 3 to 10 kDa (BOULTON *et al.,* 1995; MARCHAL *et al.,* 1997). The fining agents that are intensely hydrolyzed during production include many low MW protein fractions, which have a reduced response to this method, as can be seen for gelatin. Due to the large range in protein concentrations observed, different quantities of fining agent were added in order to obtain the same final concentration in the wine.

Table 3.2 - Total nitrogen, surface charge density, isoelectric point, protein, lead and cadmium content of the fining agents.

Product	Total Nitrogen [a] (% N) (% w/w, dry weight)	Protein content [a] as % Nxk (% w/w, dry weight)	Protein content [a] by Bradford method (% w/w, dry weight)	Surface charge density [a] (meq /g product at pH 3.4)	Isoelectric point [a]	Pb [a] (mg/kg dry weight)	Cd [a] (mg/kg dry weight)
AS_1	11.9 ± 0.2	78 ± 1	52.6 ± 2.0	0.73 ± 0.01	5.00 ± 0.02	0.25±0.003	n.d.
AS'_1	14.1 ± 0.2	94 ± 2	54.1 ± 0.8	0.72.±. 0.02	4.96.±. 0.01	0.22±0.003	n.d.
AS_4	14.5 ± 0.2	97 ± 1	65.7 ± 0.7	0.79 ± 0.01	5.01 ± 0.03	0.16±0.002	n.d.
AL_4	15.9 ± 0.3	106 ± 2	55.8 ± 0.8	0.07 ± 0.01	5.14 ± 0.05	0.64±0.027	0.01±0.0003
IS_1	17.0 ± 0.2	106 ± 1	2.0 ± 0.2	0.20 ± 0.02	4.21 ± 0.05	0.44±0.016	n.d.
IS_4	11.6 ± 0.4	73 ± 3	26.4 ± 1.0	0.41 ± 0.01	6.48 ± 0.03	0.82±0.003	0.01±0.0003
IL_1	17.9 ± 0.6	112 ± 4	1.8 ± 0.3	0.04 ± 0.00	4.55 ± 0.02	0.38±0.018	n.q.
IL_4	19.1 ± 0.7	119 ± 4	6.2 ± 0.1	0.09 ± 0.00	5.24 ± 0.04	0.16±0.007	n.q.
CKS_3	14.5 ± 0.2	93 ± 2	58.8 ± 1.6	0.09 ± 0.01	4.53 ± 0.02	0.78±0.008	n.q.
CKS_1	13.3 ± 0.2	85 ± 2	59.5 ± 5.5	0.04 ± 0.00	4.51 ± 0.04	0.62±0.002	0.01±0.0003
CS_2	14.2 ± 0.2	91 ± 1	65.9 ± 5.3	0.25 ± 0.03	4.65 ± 0.01	0.35±0.006	0.01±0.0006
CS_4	11.1 ± 0.2	71 ± 1	33.4 ± 1.2	0.09 ± 0.01	4.64 ± 0.06	0.47±0.032	n.d.
GS_3	15.9 ± 0.3	88 ± 2	4.0 ± 0.1	0.28 ± 0.00	4.65 ± 0.06	0.22±0.003	0.01±0.0012
GS_2	17.7 ± 0.2	98 ± 1	15.0 ± 4.1	0.74 ± 0.02	4.74 ± 0.00	0.40±0.019	n.q.
GS_4	16.3 ± 0.7	91 ± 4	4.8 ± 0.1	0.26 ± 0.00	4.50 ± 0.00	0.28±0.031	n.d.
GL_1	16.5 ± 0.4	92 ± 2	4.8 ± 0.3	0.11 ± 0.00	4.20 ± 0.01	1.10±0.057	n.d.
GL_2	18.1 ± 0.1	100 ± 1	2.8 ± 0.3	0.07 ± 0.00	4.41 ± 0.03	0.55±0.007	0.09±0.0014
GL_5	20.0 ± 0.7	111 ± 4	7.6 ± 0.1	0.07 ± 0.00	5.46 ± 0.01	0.22±0.025	n.d.
GL_4	22.8 ± 0.5	126 ± 3	14.1 ± 0.2	0.10 ± 0.00	5.31 ± 0.00	0.18±0.006	n.q.

A-Egg albumin, C-Casein, CK-Potassium caseinate, I-Isinglass, G-Gelatin, S-Solid, L-Liquid.
1, 2, 3, 4 and 5 different fining agent suppliers.
k – Multiplication factor, which was 6.68 for egg albumin; 6.25 for isinglass; 6.38 for casein and potassium caseinate; 5.55 for gelatin.
a – mean values of triplicate determinations ± Standard Deviation (SD).
nd-not detected (values below the limit of detection), nq-not quantified (values below the limit of quantification).

Protein molecular weight distribution

The MW distributions of potassium caseinate (CKS_1 and CKS_3) and casein (CS_2 and CS_4) observed in the SDS-PAGE electrophoretic patterns (Fig. 3.1), differed among these fining agents, but were similar within each group (potassium caseinate or casein). The potassium caseinates (CKS_1 and CKS_3) and caseins (CS_2 and CS_4) both presented a major band at 30.0 kDa with other bands at lower and higher MWs. The potassium caseinates however had more bands with MWs less than 30.0 kDa, particularly CKS_3.

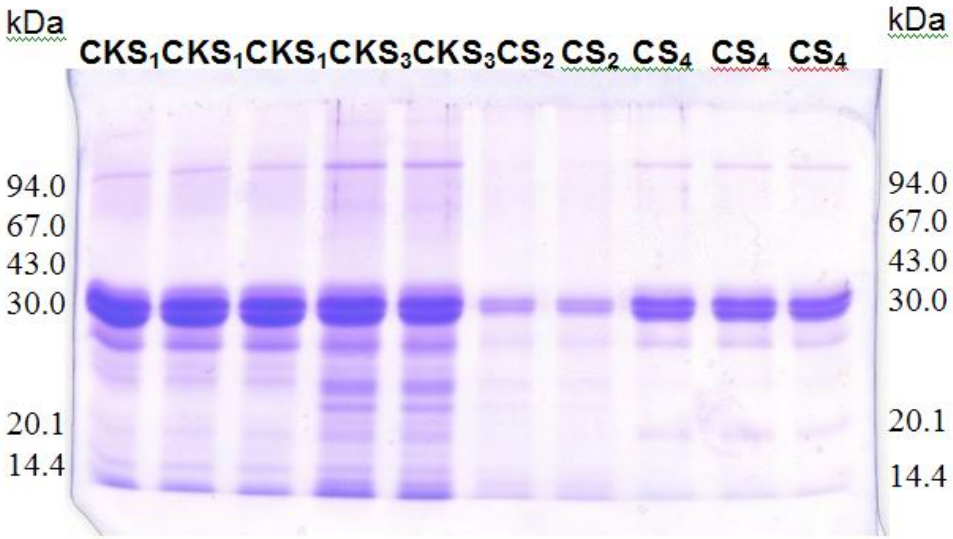

Fig. 3.1 - Electrophoretic patterns of potassium caseinates – CKS_1, CKS_3 and caseins – CS_2, CS_4.

No relevant differences were detected in the MW distribution among the egg albumins (AS_1, AS'_1 , AS_4 and AL_4) (Fig. 3.2). They were characterized by bands at 43.0 kDa and at 14.4 kDa, with other bands between 67.0 and 94.0 kDa and between 20.1 and 43.0 kDa, as reported by MARCHAL *et al.* (2002) for solid egg albumin.

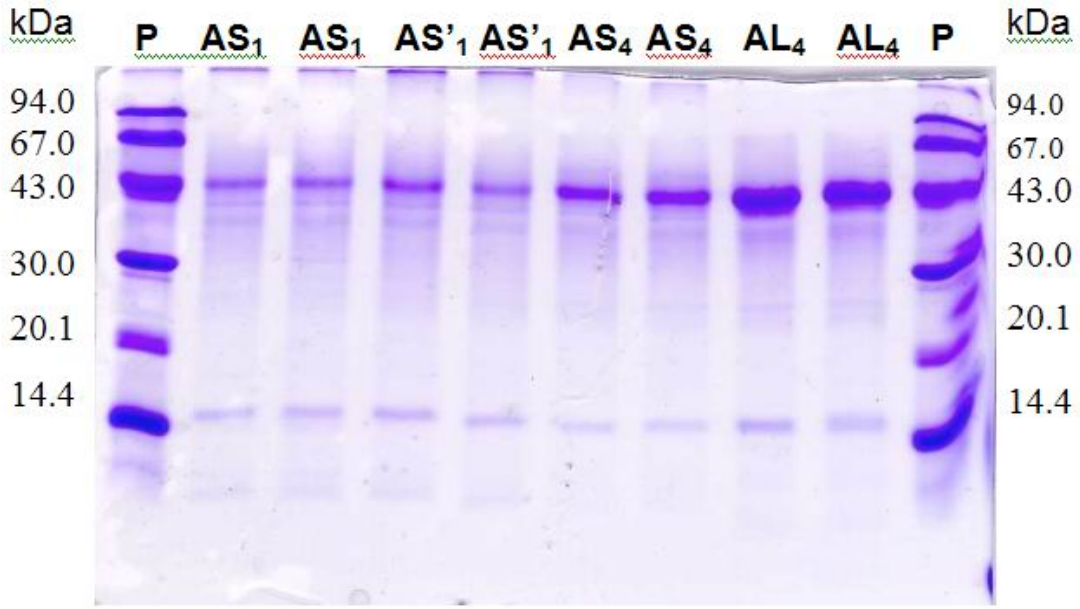

Fig. 3.2 - Electrophoretic patterns of egg albumins – AS_1, AS'_1, AS_4 and AL_4. MW standard – P, are given on the left and right side.

However, in the case of several isinglasses (IS_1, IL_1, IS_4 and IL_4) the electrophoretic patterns were not similar (Fig. 3.3). IL_1 and IS_1 showed a polydispersion in the low MW range (20.1-14.4 kDa), as did, IL_4 (94.0 - 14.4 kDa). In contrast, IS_4 presented several individual bands, namely: one at 20.1, several above 94.0 and some between 94.0 and 43.0 kDa. To our knowledge, the literature has only reported electrophoretic patterns of isinglasses with individualized bands (MARCHAL *et al.*, 2000a; BONERZ *et al.*, 2004).

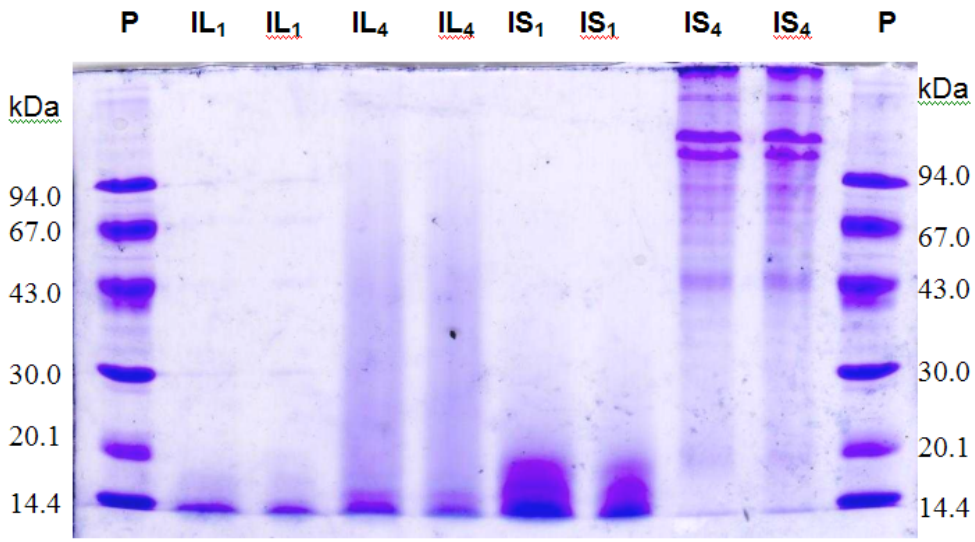

Fig. 3.3 - Electrophoretic patterns of isinglasses – IL_1, IL_4, IS_1 and IS_4. MW standard – P, are given on the left and right side.

The electrophoretic patterns of gelatins (GS_3, GS_4, GS_2, GL_1, GL_2, GL_4 and GL_5) are illustrated in Figs. 3.4 and 3.5. No bands were detected in the MW range (94.0-14.4 kDa) for gelatins GS_3 and GS_4 (Fig. 3.4). These results are in accordance with PAETZOLD and GLORIES (1990) who indicated that gelatins obtained by enzymatic hydrolysis present several polypeptides with MWs lower than 13.7 kDa. The gelatins GS_2, GL_1, GL_2, GL_4 and GL_5 showed polydispersion in the MW distribution, which has also been confirmed by other authors (MARCHAL *et al.*, 1993; 2000a; b; 2002).

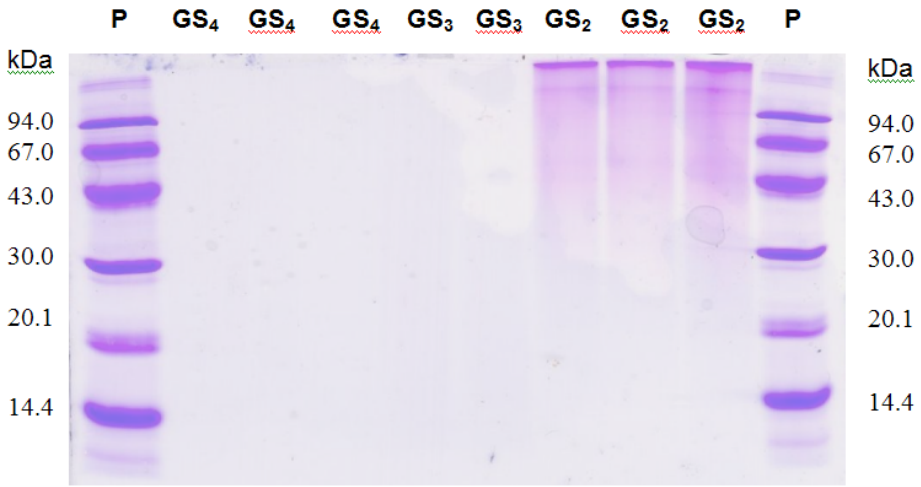

Fig. 3.4 - Electrophoretic patterns of gelatins – GS_4, GS_3 and GS_2. MW standard – P, are given on the left and right side.

The polydispersion with respect to molecular weight is a result of the breakdown of intact collagen to produce commercial gelatins (SIMS *et al.* 1997). While, GS_2 showed more protein fraction with high MW (> 43 kDa) (Fig. 4), GL_1, GL_2, GL_4 and GL_5 presented very similar electrophoretic profiles, with MWs lower than 43.0 kDa (Fig. 3.5). According to the literature (LAGUNE and GLORIES, 1996a; VERSARI *et al.,* 1999), the electrophoretic profiles are directly related to the particular elaboration processes.

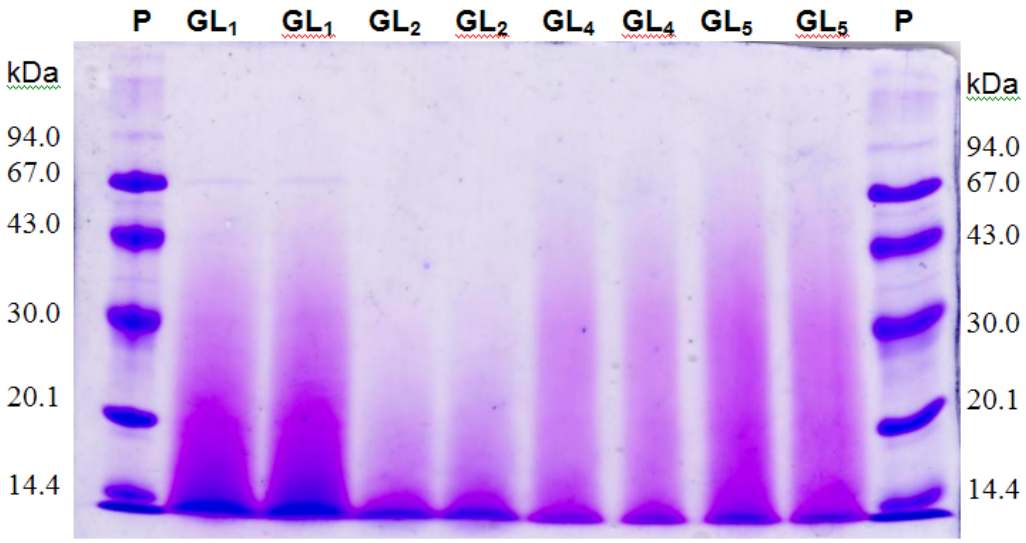

Fig. 3.5 - Electrophoretic patterns of gelatins – GL_1, GL_2, GL_4 and GL_5. MW standard – P, are given on the left and right side.

Surface charge density

The highest surface charge densities were found in solid egg albumin (AS_1, AS'_1 and AS_4) and gelatin GS_2, which had the highest value within the solid gelatins studied (Table 3.2). These results can be associated with the lower degree of hydrolysis of these proteins (SCOTTI and POINSAUT, 1997; LAMADON *et al.,* 1997) as shown by the electrophoretic profiles.

As previously described, casein and potassium caseinate were first dissolved in KOH and then dispersed in a model solution without ethanol. The surface charge densities of these fining agents were measured at the pH of dissolution (CS_2 – pH 5.30, CS_4 – pH 6.80, CKS_1 – pH 5.48 and CKS_3 – pH 5.30) and afterwards, at pH adjusted to 3.4. The surface charge density of CKS_1, CKS_3 and CS_4 decreased after the pH adjustment (from 0.32 to 0.04; 0.33 to 0.09 and 0.61 to 0.09 meq/g of product, respectively), but the surface charge density of CS_2 remained constant (0.25 meq/g of product).

It was also observed that the shape of the titration curve was related to the electrophoretic pattern. If the fining agent showed an electrophoretic pattern with individualized bands, the titration curve was constant during a certain volume of titration and then presented a sudden decrease. In contrast, for the fining agents that had an electrophoretic pattern that was a polydispersion, the titration curve showed a continuous decrease. DIETRICH and SCHÄFER (1991) suggested that the sample conductivity may influence the shape of the titration curve.

Isoelectric point

The IEP of the studied fining agents ranged from 4.20 to 6.48 (Table 3.2). Potassium caseinate (CKS_3 and CKS_1) and casein (CS_2 and CS_4) had very similar IEP values that are in accord with the data reported by MANFREDINI (1989) and STOCKÉ and ORTMANN (1999) for potassium caseinate. The egg albumin IEP values (AS_1, AS'_1, AS_4 and AL_4) were 4.96 - 5.14 and the isinglass IEP values varied from 4.21 to 6.48 in solid or liquid state. The gelatins had IEP values ranging from 4.20 (GL_1) to 5.46 (GL_5), which suggests that the gelatins studied are of Type B - basic hydrolysis (PAETZOLD and GLORIES, 1990).

Lead and Cadmium

The level of Pb was below 0.5 mg/kg in 68 % of the fining agents studied. The average Pb content was about 0.43 mg/kg, ranging from 0.16 to 1.10 mg/kg (Table 3.2).

In thirteen samples the Cd levels were below the quantification and detection limits (QL= 0.15 μg/L, DL= 0.05 μg/L). In the other six samples the Cd content was less than 0.01 mg/kg, except gelatin GL_2, which had 0.09 mg/kg (Table 3.2).

All of the Pb and Cd values measured in the protein fining agents were below the limits recommended by the International Organization of Vine and Wine [(fining agents: Pb < 5 mg/kg for casein, potassium caseinate, gelatin, isinglass and egg albumin, Cd < 1 mg/kg and < 0, mg/kg for casein and gelatin, respectively); (wine: Pb < 200 μg/L; Cd < 10 μg/L)] (OIV, 2006a; b) which guarantees wine production without metal enrichment.

Loss during drying, ash and pH

The loss of solid fining agents during drying was 6.6 to 12.0 % (w/w); as expected the values were lower than those for the liquid agents in which water loss was 52.1 to 89.5

% (w/w) (Table 3.3). The lowest ash content was observed for gelatin GS_4 and isinglass IS_1, respectively, 0.3 and 0.8 % (w/w) (Table 3.3). An unexpected value of 22.1 % (w/w) was found for casein CS_4. According to MANFREDINI (1989) the ash content of casein could indicate the manner of production in that the low values indicated that casein was coagulated by mineral acid.

All of the fining agents studied had acidic or almost neutral pH (Table 3.3). Potassium caseinate with higher pH values had a better solubilization but a lower flocculation capacity (MANFREDINI, 1989).

Table 3.3 - Weight loss on drying, ash and pH of fining agents.

Product	Weight loss [a] (%w/w)	Ash [a] (% w/w, dry weight)	pH [a]
AS_1	7.3 ± 0.0	3.6 ± 0.3	6.15 ± 0.01
AS'_1	8.2 ± 0.1	2.6 ± 0.3	5.94 ± 0.01
AS_4	8.4 ± 0.1	4.1 ± 0.2	6.23 ± 0.02
AL_4	88.0 ± 0.0	6.3 ± 0.1	6.64 ± 0.04
IS_1	6.8 ± 0.2	0.8 ± 0.1	5.06 ± 0.03
IS_4	9.9 ± 0.1	2.0 ± 0.3	3.58 ± 0.09
IL_1	71.6 ± 0.1	1.7 ± 0.0	5.32 ± 0.01
IL_4	83.7 ± 0.1	1.9 ± 0.1	4.47 ± 0.02
CKS_3	7.7 ± 0.0	3.3 ± 1.1	6.04 ± 0.02
CKS_1	6.6 ± 0.0	5.1 ± 0.2	7.83 ± 0.03
CS_2	8.7 ± 0.2	3.3 ± 0.0	5.72 ± 0.03
CS_4	9.7 ± 0.0	22.1 ± 0.0	5.86 ± 0.01
GS_3	8.2 ± 0.1	1.1 ± 0.1	5.47 ± 0.03
GS_2	12.0 ± 0.9	2.3 ± 0.1	4.68 ± 0.03
GS_4	10.1 ± 0.0	0.3 ± 0.0	5.51 ± 0.02
GL_1	52.1 ± 1.4	2.3 ± 0.6	6.08 ± 0.01
GL_2	53.5 ± .0.7	2.1 ± 0.1	5.66 ± 0.02
GL_5	89.5 ± 0.0	2.9 ± 0.7	3.87 ± 0.01
GL_4	85.1 ± 0.2	4.6 ± 0.8	4.22 ± 0.01

A-Egg albumin, C-Casein, CK-Potassium caseinate, I-Isinglass, G-Gelatin, S-Solid, L-Liquid.
1, 2, 3, 4 and 5 different fining agent suppliers.
a – mean values of triplicate determinations ± Standard Deviation (SD).

3.3.2. Wine fining trials

Limpidity

With respect to limpidity, it was shown that proteins with higher surface charge density increased wine limpidity. A linear correlation was found between total surface charge density and decrease of turbidity (Fig. 3.6).

So, among the fining proteins assayed, the best results were obtained with gelatin GS_2 (MW > 43.0 kDa) and egg albumin AS_1 (band close to 43.0 kDa) and the worst with isinglass IL_1 (MW < 20.1 kDa). These results are in accord with SCOTTI and POINSAUT

(1997) and VERSARI *et al.* (1998; 1999), in which assays made with gelatin, showed an increase in precipitation due to the increase of the surface charge density and therefore greater limpidity in the wine.

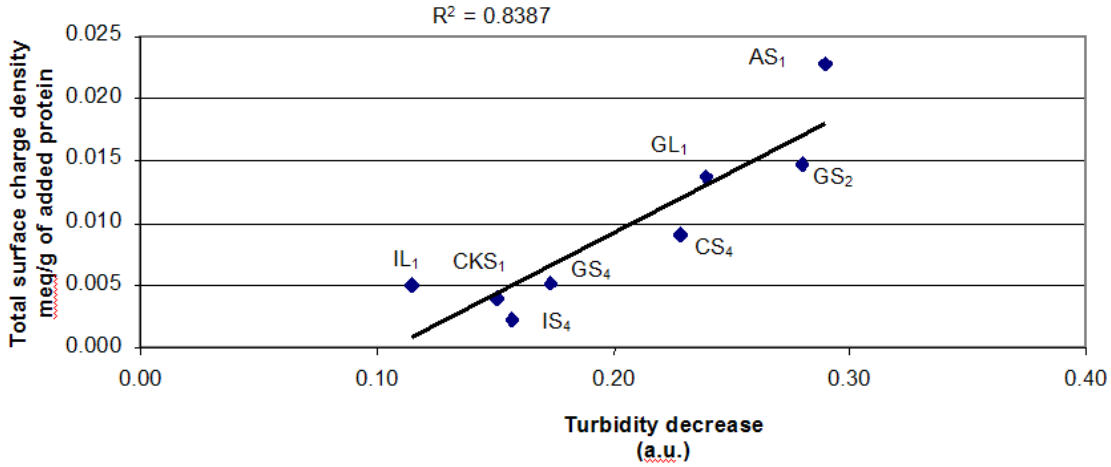

Fig. 3.6 - Total surface charge density of protein fining agents versus turbidity decrease. Isinglass (IL_1), isinglass (IS_4), casein (CS_4), potassium caseinate (CKS_1), egg albumin (AS_1), gelatin (GL_1), gelatin (GS_2), gelatin(GS_4). The turbidity of the unfined wine (T) was 0.421.

Influence of the fining protein on chromatic characteristics and monomeric anthocyanins

The results obtained with the CIELab method for determining the chromatic characteristics of the unfined and fined wine with different proteins showed that there were significant changes after fining (Table 3.4). In all fined wines, the lightness (L*) increased significantly, therefore the unfined wines were darker. The increase in lightness (L*) of the fined wine seemed to be correlated with less redness a* (NEGUERUELA *et al.,* 1995), due to the removal of pigments as previously observed by GIL-MUÑOZ *et al.* (1997). These data are in agreement with the results obtained for monomeric anthocyanins (Table 3.5) and for total and polymeric pigments (molecules that result from the condensation of anthocyanins with tannins) (COSME *et al.,* 2006). This confirms that the different protein fining agents promote a decrease of these compounds. With regard to the values obtained for the colour difference (ΔE), between each wine and the unfined wine (Table 3.4), with exception of wine fined with IL_1 all the others had values higher than one cielab unit, indicating that the colour differences can be detected visually (GONNET, 1998).

The isinglasses had the least effect on the total monomeric anthocyanin content (<5% decrease); similar results were reported by BONERZ *et al.* (2004). However, isinglass IS_4 obtained from swim bladder (with bands at MW > 94.0, 94.0-43.0 and at 20.1 kDa) had a different effect when compared with isinglass IL_1 (MW < 20.1 kDa). This fining agent induced a minor decrease in peonidin-3-glucoside, peonidin-3-p-coumarylglucoside, malvidin-3-glucoside, malvidin-3-acetylglucoside and malvidin-3-p-coumarylglucoside. Gelatin GS_4, casein and potassium caseinate removed the total monomeric anthocyanins to the greatest extent (28.2, 20.2 and 11.9 %, respectively). These findings agree with LOVINO *et al.* (1999) who found that fining red wine with casein decreased the monomeric anthocyanin levels and with RICARDO-DA-SILVA *et al.* (1991) who observed lower concentrations of total anthocyanins in the treated wine. Our results show that the more hydrolysed gelatin GS_4 (MW < 14.4 kDa) always decreased the monomeric anthocyanins more than GL_1 (MW < 43.0 kDa) and GS_2 (MW > 43.0 kDa). Those effects were not statistically different. While potassium caseinate decreased the concentration of total monomeric anthocyanins (66 mg/L) and casein (116 mg/L), these two fining agents had identical electrophoretic profiles and isoelectric points. It should be noted that the reduction observed for casein was mainly due to a decrease in peonidin-3-glucoside and malvidin-3-glucoside.

Influence of the fining protein on flavonoid and non-flavonoid phenols

Regarding the total phenolic content, fining agents induced a reduction from 1.1 to 7.8 %; the greatest decrease was observed on gelatin GS_4 (7.8 %) followed by casein (6.6 %). Fining with higher molecular weight proteins and agents with higher surface charge density such as gelatin GS_2 (MW > 43.0 kDa) and egg albumin (MW close to 45.0 kDa) resulted in a decrease in total phenolic compounds (1.1 and 1.6 %, respectively) (Table 3.4). These results were not statistically significant.

In contrast, significantly different results were observed among the three gelatins (GL_1, GS_2 and GS_4). The gelatin with the lowest MW [GS_4 (MW < 14.4 kDa)] removed significantly more total phenolic compounds than the gelatin with the highest MW [GS_2 (MW > 43.0 kDa)]. With respect to the non-flavonoid and flavonoid compounds, gelatin GL_1 (MW < 43.0 kDa) mainly reduced the non-flavonoid compounds, while gelatin GS_4 (MW < 14.4 kDa) mainly reduced the flavonoids which was probably due to the decrease in anthocyanins (Table 3.5).

The results for isinglass were similar to those for gelatin. Isinglass IS_4 (with bands at MW > 94.0, 94.0-43.0 and at 20.1 kDa) exerted a greater effect on the total phenolic compounds than IL_1 (MW < 20.1 kDa), by removing a significant amount of non-flavonoid compounds.

While casein and potassium caseinate have analogous protein MW distributions and isoelectric points, the results regarding the reduction of flavonoids and non-flavonoids differed. The casein mainly removed non-flavonoid compounds (decrease of 17.8 %), while the potassium caseinate only induced a 3.6 % decrease of these compounds.

Table 3.4 - Flavonoids, non-flavonoids, total phenols and chromatic characteristics of both fined and unfined red wine (means ± SD).

Fining Treatment	Flavonoid phenols (mg/L gallic acid)	Non-flavonoid phenols (mg/L gallic acid)	Total phenols (mg/L gallic acid)	L* (%)	a*	b*	Δ E*
T	3816±56 a	361±4 a	4177±63 a	46.3±0.2 a	59.40±0.31 a	5.80±0.06 a	
IL_1	3705±2 b	361±5 a	4066±7 bc	46.8±0.1 b	58.19±0.02 c	4.72±0.07 de	1.72±0.10 b
IS_4	3649±35 bc	319±9 c	3969±45 de	46.7±0.1 bc	58.89±0.28 b	5.50±0.04 b	0.97±0.17 a
CS_4	3605±4 c	297±5 d	3901±0 ef	47.9±0.3 d	56.00±0.13 f	4.66±0.19 e	3.94±0.24 f
CKS_1	3658±14 bc	348±5 b	4005±8 cd	48.5±0.1 e	56.90±0.07 e	4.89±0.06 d	3.46±0.11 e
AS_1	3787±60 a	323±7 c	4110±69 ab	47.0±0.1 bc	58.14±0.04 c	5.07±0.01 c	1.69±0.11 b
GL_1	3710±9 b	306±1 d	4017±9 cd	47.2±0.1 c	57.86±0.08 c	4.65±0.01 e	2.13±0.08 c
GS_2	3810±32 a	322±5 c	4132±27 ab	47.7±0.2 d	57.40±0.28 d	5.75±0.00 a	2.46±0.15 d
GS_4	3489±32 d	361±5 a	3850±27 f	48.8±0.1 e	55.42±0.16 g	5.74±0.05 a	4.70±0.17 g

Unfined (T), isinglass (IL_1), isinglass (IS_4), casein (CS_4), potassium caseinate (CKS_1), egg albumin (AS_1), gelatin (GL_1), gelatin (GS_2), gelatin (GS_4) ; L* - lightness, a* - redness, b*- yellowness, ΔE – total colour difference. The values corresponding to ΔE were obtained taking as a reference the unfined wine (T). Means (n=2) within a column followed by the same letter are not significantly different (LSD, 5%).

Table 3.5 - Monomeric anthocyanins (mg/L malvidin-3-glucoside) for both fined and unfined red wine (means ± SD).

	T	**IL_1**	**IS_4**	**CS_4**	**CSK_1**	**AS_1**	**GL_1**	**GS_2**	**GS_4**
Delphinidin-3-glucoside	16.7±0.7 a	14.5±2.1 abc	14.5±0.2 abc	11.3±0.7 cd	11.8±0.7 bcd	15.4±2.0 ab	14.4±0.1abc	14.1±0.1 abc	8.9±1.6d
Cyanidin-3-glucoside	1.8±0.0 a	1.8±0.4 a	1.8±0.5 a	1.3±0.1 a	1.4±0.1 a	1.6±0.2 a	1.5±0.1 a	1.5±0.1 a	1.3±0.1 a
Petunidin-3-glucoside	19.8±0.2 a	18.9±2.9 a	18.9±2.6 a	16.1±0.7 ab	16.3±0.5 ab	18.1±0.2 a	17.6±0.1 ab	17.5±0.1 ab	13.6±0.0 b
Peonidin-3-glucoside	131.3±2.4 a	124.4±14.2 a	128.9±15.2 a	94.1±1.4 b	109.1±5.4 ab	124.5±4.5 a	121.7±5.5 a	119.7±0.4 a	93.5±1.1 b
Malvidin-3-glucoside	351.7±6.2 a	342.9±37.9 a	347.2±38.9 a	298.6±16.5 ab	326.8±8.5 ab	337.9±9.3 a	327.6±1.7 ab	321.3±1.6 ab	266.5±3.8 b
Malvidin-3-acetylglucoside	11.6±0.3 a	10.9±1.9 a	11.5±1.7 a	8.3±0.2 bc	9.6±0.1 abc	11.2±0.5 a	10.2±0.0 ab	9.9±0.0 ab	7.6±0.1 c
Peonidin-3-p-coumarylgucoside	10.3±0.0 a	9.3±1.5 ab	9.8±1.5 a	6.6±0.4 d	7.4±0.2 bc	8.7±0.1 abc	9.2±0.0 ab	8.6±0.0 abc	5.0±0.0 d
Malvidin-3-p-coumarylgucoside	31.9±0.3 a	28.8±6.0 a	29.9±6.1 a	22.7±1.2 ab	23.8±0.1 ab	26.3±1.6 ab	26.7±0.0 ab	25.1±0.0 ab	17.9±0.2 b
Σ monomeric anthocyanins	575.5±10.2 a	551.5±18.1 abc	562.5±14.6 ab	459.2±16.0 de	509.2±18.4 cd	543.2±18.0 abc	528.5±17.7 abc	519.7±11.0 bc	414.1±13.4 e

Unfined (T), isinglass (IL_1), isinglass (IS_4), casein (CS_4), potassium caseinate (CKS_1), egg albumin (AS_1), gelatin (GL_1), gelatin (GS_2), gelatin (GS_4). Means (n=2) within a line followed by the same letter are not significantly different (LSD, 5%).

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4. BEHAVIOR OF VARIOUS PROTEINS ON WINE FINING: EFFECT ON DIFFERENT MOLECULAR WEIGHT CONDENSED TANNIN FRACTIONS OF RED WINE

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BEHAVIOUR OF VARIOUS PROTEINS ON WINE FINING: EFFECT ON DIFFERENT MOLECULAR WEIGHT CONDENSED TANNIN FRACTIONS OF RED WINE

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ABSTRACT

The effect of several proteins on the three main wine tannic fractions, with the mean degree of polymerisation (mDP) of 1.5 (FI), 3.4 (FII) and 4.9 (FIII) was studied. In spite of casein and potassium caseinate showing similar molecular weight (MW) distribution, casein decreases the FI fraction more than the twice as effectively as potassium caseinate. The gelatine with a medium MW polydispersion induced a quite similar decrease, of around 20%, in all tannin fractions. The gelatine having low MW removed mainly the tannin fractions of lower mDP (FI and FII), while the gelatine having a higher MW had a small effect (5%) on the fraction of higher mDP (FIII). Any of the two studied isinglasses showed influence on the reduction of FII fraction. The tannins of FI and FIII were removed by swim bladder isinglass twice as effectively as fish skin isinglass. Regarding the mDP of fined wines, the egg albumin induced a decrease on mDP of 24% for the more polymerised tannin fraction (FIII); although within all assays were observed a decrease ranged from 6 to 14%

KEY WORDS: Wine, protein, fining agents, proanthocyanidins, pigments, thiolysis.

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4.1. INTRODUCTION

The oenological protein fining agents are mainly used in red wine for clarification and also for reduction of the wine's phenolic compounds. The main protein fining agents use in wine are animal proteins such as gelatine, egg albumin, casein, potassium caseinate and isinglass. However, in recent years certain proteins of vegetable origin (cereals and legumes) have also been investigated as possible wine fining agents (Fischerleitner et al. 2003, Marchal et al. 2000a, 2000b, 2002, Maury et al. 2003, Panero et al. 2001). Proteins employed as wine fining agents have distinct physic-chemical characteristics such as molecular weight distribution, isoelectric point and surface charge density (Cosme et al. 2007, Iturmendi et al. 2005, Lagune and Glories, 1996a, Lagune-Ammirati and Glories, 2001, Lamandon et al. 1997, Marchal et al. 2000a, 2000b, 2002, Maury et al. 2003, Paetzold and Glories, 1990, Versati at al. 1998).

Studies on wine fining have used two different approaches. In the first, the authors concentrate their interest on the influence of the fining proteins on wine composition, not on characterising the protein fining agents. In the second, the relation between the physic-chemical characteristics (molecular weight and surface charge density) of the fining protein on the effect of wine composition are specified.

Thus, several authors have studied the influence of protein fining agents on wine composition (Bravo-Haro et al. 1991, Castellari et al. 1998, 2001, Fischerleitner et al. 2003, Flak et al. 1990, Lovino et al. 1999, Machado-Nunes et al. 1995, Ough, 1960, Panero et al. 2001, Ricardo-da-Silva et al. 1991a, Sims et al. 1995, Stankovic et al. 2004, Yokotsuka et al. 1983). It has been observed that fining a young red wine (Mourvèdre) with gelatine and casein promotes a reduction on the concentration of total anthocyanins and on the absorbance at 420, 520 and 620 nm whereas the concentrations of flavanol monomers and several procyanidins dimers and trimers, esterified or not with gallic acid, were not affected (Ricardo-da-Silva et al. 1991a). It has been supposed that proteins interact more intensely with the more polymerised proanthocyanidins and also those esterified with gallic acid. In this study, the authors accept that other, more active phenolic compounds, namely high MW proanthocyanidins and anthocyanin-proanthocyanidin complexes (polymeric pigments) protect the small oligomeric procyanidins. It is also evidenced by other authors that gelatine selectively decreases the polymerised phenolic compounds (Sims et al. 1995, Yokotsuka et al. 1983, Yokotsuka and Singleton, 1995, 1987). The addition of casein to red wine

influences its low molecular weight flavonoid composition (Machado-Nunes et al. 1995). These authors established the importance of the wine's initial phenolic composition, mainly anthocyanins and condensed tannins, on the fining process. It was also shown that casein significantly reduced the absorbance at 520 nm, total and polymeric phenolic compounds (Sims et al. 1995). The effect of this protein was attributed to its alternative polar and apolar zones, as well as, hydrophobic and hydrophilic amino acid distribution (Stocké and Ortmann 1999).

However, the relation between the physic-chemical characteristics of protein fining agents and their interaction with wine phenolic compounds has been specified in relatively few studies. It is also noted that most studies have been carried out on gelatines (Bonerz et al. 2004, Hrazdina et al. 1969, Kaufmann, 1988, Lagune and Glories, 1996b, Lefebvre et al. 1999, Marchal et al. 1993, Maury et al. 2001, 2003, Sarni-Manchado et al. 1999, Versari et al. 1998, 1999) and vegetable proteins (Lefebvre et al. 1999, Marchal et al. 2000a, 2002, Maury et al. 2003).

Some studies have shown that the protein MW distribution of gelatines influences the protein-tannin interaction (Bonerz et al. 2004, Hrazdina et al. 1969, Lefebvre et al. 1999, Maury et al. 2001, 2003, Sarni-Manchado et al. 1999). Thus, gelatines with a high MW distribution preferentially remove proanthocyanidins rich in epigallocatechin units while gelatines with low MW distribution selectively deplete the high polymerised proanthocyanidins (Lefebvre et al. 1999, Maury et al. 2001, Sarni-Manchado et al. 1999). It has also been shown that gelatines with low surface charge densities precipitate fewer wine components compared with gelatines with higher surface charge densities (Versari et al. 1999). In addition, it has been confirmed that gelatines selectively remove proanthocyanidins with high degrees of polymerisation (about 12 mDP) and, also galloylated procyanidins (Sarni-Manchado et al. 1999).

An enhanced knowledge of the quantity and type of the phenolic compounds remaining in red wine after fining with proteins having distinct physic-chemical characteristics should lead to an optimisation of the fining process. To our knowledge there are no detailed studies available on the structural composition of flavonoids remaining in the wine, nor on the effects on the three main tannin fraction after the addition of protein fining agents such as swim bladder isinglass, egg albumin, casein and potassium caseinate.

Therefore, the main objectives of this work was to carry out a comparative study on the effect of oenological protein fining agents (gelatine, egg albumin, casein, potassium

caseinate and isinglass) with distinct physic-chemical characteristics (molecular weight distributions, isoelectric points, surface charge densities) on the structural characteristics of proanthocyanidins, as well as on the three main tannin fractions (monomeric, oligomeric and polymeric flavan-3-ols), and also on colour and pigments of red wine after fining.

4.2. MATERIALS AND METHODS

Chemicals

Vanillin was purchased from Merck (Darmstadt, Germany) and toluene-α-thiol from Fluka (Buchs, Switzerland). Solvents and acids used were of HPLC grade.

Protein fining agents

The fining agents previously characterised (Cosme et al. 2007) were used in this study: one egg albumin (AS_1), two isinglasses (IL_1, IS_4), one potassium caseinate (CKS_1), one casein (CS_4) and three gelatines (GL_1, GS_2 and GS_4) (Table 4.1).

Table 4.1 - Fining agents employed in this study.

Fining agent	Code	Concentration	Producer information
Isinglass	IL_1	50 mL hL^{-1}	Obtained from fish skin
Isinglass	IS_4	2.25 g hL^{-1}	Obtained from swim bladder
Casein	CS_4	40 g hL^{-1}	
Potassium caseinate	CKS_1	40 g hL^{-1}	
Egg albumin	AS_1	12.5 g hL^{-1}	
Gelatine	GL_1	50 mL hL^{-1}	High concentrated, obtained by chemical hydrolysis
Gelatine	GS_2	8 g hL^{-1}	
Gelatine	GS_4	8 g hL^{-1}	High hydrolysis degree

Fining experiments

A young blended red wine (vintage 2003) of Castelão, Tinta Roriz and Caladoc grape varieties from the Estremadura Region (North of Lisbon) was used on fining experiments.

Experiments were carried out by addition of standard quantities of the protein fining agents (isinglass, casein, potassium caseinate and gelatine) prepared as recommended by the

manufacturers (Table 4.1) to 250 mL of wine. Untreated wine was used as control. The fining agents were thoroughly mixed and allowed to remain in contact with the wines for 7 days at 20 ºC, the samples were then centrifuged at 4,000 rpm for 15 min before analysis. All fining experiments were done in duplicate.

Phenolic compounds analysis

Separation of proanthocyanidins according to degree of polymerisation by C_{18} Sep-Pak cartridges and determination of the flavan-3-ol content by the vanillin assay

The separation of flavanols was performed in a C_{18} Sep-Pak cartridge (Waters, Milford, Ireland) according to their degree of polymerisation in three fractions FI (monomeric), FII (oligomeric) and FIII (polymeric) (Sun et al. 1998a). Quantification of the total flavan-3-ol in each fraction was performed using the vanillin assay (Sun et al. 1998a, 1998b). For the FI fraction, the absorbance at 500 nm was read after a reacting at 30ºC for 15 min using a Unicam UV-vis UV4 spectrophotometer (Unicam, Cambridge, U.K.). For the FII and FIII fractions the reaction was at room temperature and left until the maximum absorbance value at 500 nm was achieved. Quantification was carried out by means of standards curves prepared from monomers (FI), oligomers (FII), and polymers of flavan-3-ol (FIII) isolated from grape seeds, as described previously (Sun et al., 1998a, 1998b, 2001).

Characterisation of wine proanthocyanidins (fractionated by C_{18} Sep-Pak cartridges) by acid-catalysed depolymerisation in the presence of toluene–α-thiol followed by reversed-phase HPLC analysis

The proanthocyanidins were depolymerised in the presence of a nucleophilic agent (toluene–α-thiol) in an acid medium. Depolymerisation allows the distinction between terminal units, which are released as flavan-3-ols, and extension units released as their benzyl thioethers (Maury et al. 2001; Souquet et al. 2000). Reversed-phase HPLC analysis of the products formed allows determination of the structural composition of proanthocyanidins, which are characterised by the nature of their constitutive extension units (released as their benzylthioethers) and terminal units (released as flavan-3-ols). It also allows calculation of their structural characteristics such as the mean degree of polymerisation (mDP), the average molecular mass (mM), the cis: trans ratio, the percentage

of prodelphinidins (% prodelph) and the percentage of galloylation (% gal) (Kennedy et al. 2000, Prieur et al. 1994, Ricardo-da-Silva et al. 1991b, Rigaud et al. 1991).

To perform the acid-catalysed depolymerisation, 100 μL of sample were introduced in a glass tube with a hermetic seal together with 100 μL of a solution of toluene-α-thiol in methanol containing HCl (0.2M). After closure, the mixture was mixed gently and incubated at 55ºC for 7 min by which time the despolymerisation yield was around 70% (Monagas et al. 2003) The thiolysed sample was then analysed directly by HPLC. The HPLC system used includes a Konik Instruments (Konik Instruments, Konik-Tech, Barcelona, Spain) UV-vis detector (Uvis 200) set at 280 nm, and a Merck Hitachi Intelligent pump model L-6200A (Tokyo, Japan), coupled to a Konikrom data chromatography treatment system version 6.2 (Konik Instruments, Konik-Tech, Barcelona, Spain). The column was a reversed-phase C_{18}Lichrosphere 100 (250 mm x 4.6 mm, 5 μm) (Merck, Darmstadt, Germany), and the separation was performed at room temperature. The elution condition were as follows: 1.0 mL/min., flow rate, solvent A; (water/formic acid, 98/2, v/v), solvent B; (acetonitrile/formic acid/water 80/2/18, v/v/v), 5-30 % B linear from 0 to 40 min., 30-50% B linear from 40 to 60 min., 50–80 % B linear from 60 to 70 min., followed by washing (solvent B) and reconditioning of the column from 75 to 97 min. The amounts of monomers (terminal units) and toluene-α-thiol adducts (extension units) released from the depolymerisation reaction in the presence of toluene-α-thiol, were calculated from the areas below the chromatographic peaks at 280 nm by comparison with calibration curves (Kennedy et al. 2000, Prieur et al. 1994, Sun et al. 2001).

Separation of monomeric and small oligomeric flavan-3-ols (dimers and trimers) by polyamide column chromatography and quantification by HPLC analysis

Procyanidin separation was performed using a 3 mL red wine volume (Ricardo-da-Silva et al. 1990). The HPLC system used was the same as employed for the HPLC analysis of the products released by acid-catalysed depolimerisation in the presence of toluene-α-thiol. The elution conditions for monomeric flavan-3-ols were as follows: 0.9 mL/min., flow rate, solvent A; (water/acetic acid, 97.5/2.5, v/v), solvent B; (acetonitrile/solvent A 80/20, v/v), 7-25 % B linear from 0 to 31 min. followed by washing (methanol/water, 50/50, v/v) from 32 to 50 min and reconditioning of the column from 51 to 65 under initial gradient conditions. The elution conditions for oligomeric procyanidins (dimeric and trimeric) were

as follows: 1.0 mL/min., flow rate, solvent A, (distilled water), solvent B, (water/acetic acid, 90/10, v/v), 10-70% B linear from 0 to 45 min., 70 – 90 % B linear from 45 to 70 min., 90 % B isocratic from 70 to 82 min., 90-100% B linear from 82 to 85 min., 100 % B isocratic from 85 to 90 min., followed by washing (methanol/ water, 50/50, v/v) from 91 to 100 min. and reconditioning of the column from 101 to 120 min. under initial gradient conditions. Identification (Ricardo-da-Silva et al. 1991b; Rigaud et al. 1991) and quantification (Dallas et al. 1995; 1996, Ricardo-da-Silva et al. 1990;) of monomeric flavan-3-ols and oligomeric procyanidins (some dimers and trimers) was performed.

Colour and Pigments

Colour was determined by measuring absorbance at 620, 520 and 420 nm (1-mm cell) using a Unicam UV-vis UV4 spectrophotometer (Unicam, Cambridge, U.K.) (OIV, 2006). The content of total and coloured anthocyanins and total and polymeric pigments were determined (Somers and Evans, 1977).

4.3. RESULTS AND DISCUSSION

The physic-chemical characteristics of the fining agents used in this work are summarised in Table 4.2, and the structural characteristics of the unfined wine proanthocyanidins are presented on the first lines of Table 4.3 and Table 4.4.

The mDP of the FI fraction, the monomeric fraction, from the different samples presented values ranging from 1.47 to 1.58. The mDP of the monomeric fraction should be 1, however, FI fraction also includes two unknown compounds (Sun et al. 1998a). It is probable, that very few oligomeric proanthocyanidins pass through the C_{18} Sep-Pak during separation.

Table 4.2 - Physic-chemical characteristics of the protein fining agents employed on the fining trial (Cosme et al. 2007).

Fining agents	Molecular weight distribution (kDa)	Surface charge density [a] ($meqg^{-1}$ product at pH 3.4)	Protein content [a] as % Nxk (% w/w, dry weight)	Isoelectric point [a]
IL_1	Polydispersion below 20.1	0.04±0.00	112±4	4.55±0.02
IS_4	Bands above 94.0 between 94.0-43.0and at 20.1	0.41±0.01	73±3	6.48±0.03
CS_4	Band close to 30.0	0.09±0.01	71±1	4.64±0.06
CKS_1	Band close to 30.0	0.04±0.00	85±2	4.51±0.04
AS_1	Band close to 43.0	0.73±0.01	78±1	5.00±0.02
GL_1	Polydispersion below 43.0	0.11±0.00	92±2	4.20±0.01
GS_2	Polydispersion above 43.0	0.74±0.02	98±1	4.74±0.00
GS_4	No bands between 94.4 and 14.4	0.26±0.00	91±4	4.50±0.00

Isinglass (**IL_1**), isinglass (**IS_4**), casein (**CS_4**), potassium caseinate (**CKS_1**), egg albumin (**AS_1**), gelatin (**GL_1**), gelatin (**GS_2**), gelatin (**GS_4**).
k – Multiplication factor, which was 6.68 for egg albumin; 6.25 for isinglass; 6.38 for casein and potassium caseinate; 5.55 for gelatin.
a – mean values of triplicate determinations ± Standard Deviation (SD)

4.3.1. Quantification of flavan-3-ol fractions affected by fining

Condensed tannins with mean polymerisation degree of 4.9 (fraction FIII), probably associated with astringency were mainly removed (a 20 to 28 % reduction) by swim bladder isinglass, egg albumin and by the two types of gelatines characterised by a polydispersion on the low MW (Fig. 4.1). The two isinglasses [IL_1 (MW < 20.1kDa) and IS_4 (with bands at MW > 94.0, 94.0-43.0 and at 20.1 kDa), removed 13 % and 28 %, respectively)] showed distinct behaviours in relation to the polymeric flavanols. Swim bladder isinglass decreases the tannin fraction with mDP 4.9 more than the twice as effectively as fish skin isinglass.

The oligomeric flavanols (fraction FII, mDP=3.4) were considerably decreased by egg albumin, casein and by the three gelatines studied. With the gelatines, GS_4 (MW <14.4 kDa) brought about a greater decrease in oligomeric proanthocyanidins (reduction about 55%) compared with the other two gelatines (GS_2 - polydispersion above 43.0 kDa and GL_1 - polydispersion below 43.0 kDa). The isinglasses did not lower the concentration of these compounds greatly. With both casein and potassium caseinate, the oligomeric flavanols tended to be removed but this effect was to a greater extent for casein.

The monomeric flavanols (fraction FI, mDP ≈ 1.5), generally associated with bitterness, were mainly removed by casein, swim bladder isinglass, and the low MW distribution gelatines (Fig. 4.1). Casein and potassium caseinate showed an electrophoretic profile with similar MW distribution (MW ≈ 30.0 kDa) (Cosme et al. 2007). However, their

affinity for monomeric flavanols (fraction FI) was different. Casein decreased these compounds to a greater extent than potassium caseinate. Notably, swim bladder isinglass, having a high MW distribution decreased these compounds more than isinglass with a MW distribution below 20.1 kDa. Egg albumin (7% reduction) did not lower the monomeric flavanols considerably.

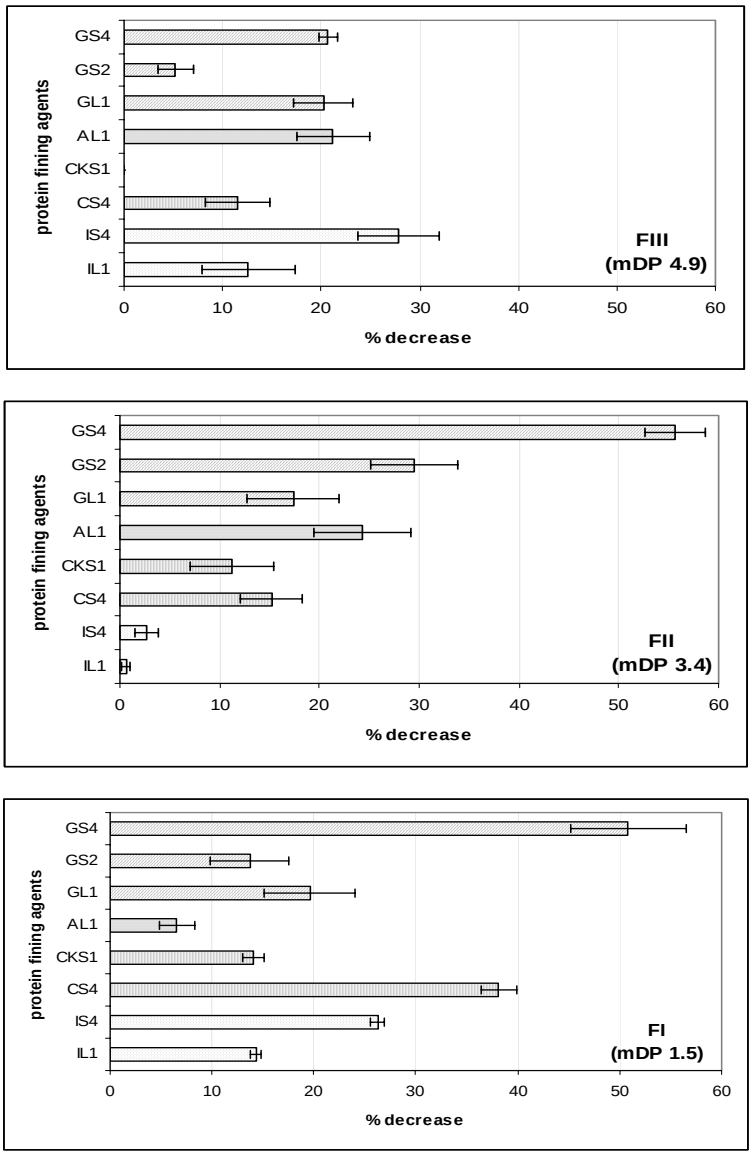

Fig. 4.1 – Decrease of the tannic fractions (%) FI, FII and FIII, with the mean degree of polymerisation (mDP) of 1.5, 3.4 and 4.9, respectively, after fining treatment with distinct proteins. Isinglass (IL_1), isinglass (IS_4), casein (CS_4), potassium caseinate (CKS_1), egg albumin (AS_1), gelatin (GL_1), gelatin (GS_2), gelatin (GS_4). The error bars indicated in the fig. represented the standard deviations.

4.3.2. Structural characterisation of proanthocyanidin fractions affected by fining

The results relating to the structural characteristics of wine proanthocyanidins obtained by reversed phase HPLC of the depolymerisation products released by thiolysis are presented in Table 4.3.

The mDP of the oligomeric and polymeric proanthocyanidins remaining in the fined red wine decreased in all trials (6 to 24%) compared to the unfined wine, which agrees with other studies done with gelatines of different MW (Maury et al. 2001). These results are in agreement with earlier reports, which propose that the largest molecules are precipitated first (Ricardo-da-Silva et al. 1991a). This effect could be due to the higher number of phenolic rings present in the more polymerised proanthocyanidins with an increase in hydrophobicity and therefore the complexes formed with proteins are more effectively removed (Baxter et al. 1997). However, only wine fined with egg albumin and isinglass obtained from swim bladder leads to a major decrease in the mDP of proanthocyanidins remaining in the fined wine (Table 4.3). This allows us to predict that these fining agents should selectively remove proanthocyanidins with higher mDP.

The fining treatment with gelatines and egg albumin promotes a greater decrease on the percentage of galloylation (% gal) in the polymeric proanthocyanidins as it is shown in Table 4.3.

The percentage of prodelphinidins (containing epigallocatechin units) within the polymeric proanthocyanidins fraction was notably lower for all the treatments. These finings suggested that these proteins interacted selectively with epigallocatechin units. However, when gelatine GS_4 (MW < 14.4 kDa) was employed, the decrease was lesser (Table 4.3). This is in accordance with the results obtained by other authors in similar experiments (Sarni-Manchado et al. 1999).

The cis:trans ratio was more reduced by isinglasses and potassium caseinate of the oligomeric proanthocyanidins fraction and increased by gelatines for the polymeric proanthocyanidins fraction.

Table 4.3 - Structural characterization of proanthocyanidins (oligomeric and polymeric), mean degree of polymerization (mDP), percentage of galloylation (% gal), percentage of prodelphinidins (% prodelph), average molecular mass (mM) and the cis/trans (cis:trans) ratio for both unfined red wine and red wine after different fining treatments (mean±SD).

Fining	Oligomeric proanthocyanidins (FII)					Polymeric proanthocyanidins (FIII)				
treatment	mDP	%gal	% prodelph	Mm	cis:trans	mDP	%gal	% prodelph	Mm	cis:trans
T	3.4±0.1	13.6±1.8	43.5±5.2	1066±29	3.9±0.5	4.9±0.0	19.3±0.6	30.2±2.1	1587±1	2.8±0.3
IL_1	3.1±0.1	15.2±0.7	28.1±3.7	995±24	2.0±0.3	4.4±0.6	19.8±1.1	18.1±0.6	1430±19	2.4±0.1
IS_4	3.0±0.0	13.2±0.7	33.1±2.6	936±14	2.5±0.2	4.2±0.3	20.2±1.3	19.3±2.0	1367±10	2.2±0.0
CS_4	3.1±0.0	15.1±1.3	29.0±4.1	983±1	3.1±0.6	4.5±0.6	19.5±0.2	20.1±2.5	1430±20	2.5±0.3
CKS_1	3.1±0.3	14.7±0.9	33.0±1.9	990±19	2.7±0.1	4.4±0.4	19.5±1.6	18.6±3.7	1421±12	2.4±0.2
AS_1	3.2±0.1	12.6±1.4	31.9±2.9	1044±36	3.5±0.8	3.7±0.7	13.3±0.6	12.2±2.1	1148±6	2.4±0.1
GL_1	3.0±0.3	12.2±0.3	41.1±4.2	946±21	3.3±0.3	4.5±0.1	16.0±0.1	26.0±0.5	1397±3	4.3±0.1
GS_2	3.0±0.2	10.9±1.0	42.2±4.2	935±21	3.1±0.9	4.4±0.0	11.7±0.4	25.9±0.5	1389±16	3.8±0.3
GS_4	3.2±0.1	11.7±0.5	40.3±2.3	1002±30	3.0±0.4	4.6±0.0	15.4±0.3	26.5±0.6	1455±17	4.3±0.4

Unfined (T), isinglass (**IL_1**), isinglass (**IS_4**), casein (**CS_4**), potassium caseinate (**CKS_1**), egg albumin (**AS_1**), gelatin (**GL_1**), gelatin (**GS_2**) and gelatin (**GS_4**).

4.3.3. Quantification of some monomeric, dimeric and trimeric flavan-3-ols molecules affected by fining

A detailed HPLC analysis of the most important oligomeric proanthocyanidins, such as procyanidin dimers (B1, B2, B3 and B4), trimers (trimer 2 and C1) and dimer gallates (B2-3-*O*-gallate, B2-3'-*O*-gallate and B1-3-*O*-gallate) that are included in the FII fraction (Table 4.4) was also carried out.

It was observed that the three gelatines, depressed all of the individual dimeric procyanidins (B1, B2, B3 and B4) considerably. In contrast, none of the individual dimeric procyanidins (B1, B2, B3 and B4), was lowered noticeably by the addition of egg albumin. Of the individual trimeric procyanidins (trimer 2 and C1), only gelatine characterised by a polydispersion above 43.0 kDa caused a main decrease in trimer C1. An important decrease of the three dimeric procyanidins esterified by gallic acid (B2-3-*O*-gallate, B2-3'-*O*-gallate and B1-3-*O*-gallate) was only shown with gelatine having a polydispersion above 43.0 kDa (Table 4.4).

In general, it was found that gelatines were the fining agents that most decreased the amount of total dimeric (22-35 %) and trimeric procyanidins (25-38 %), which is in agreement with the results obtained for the oligomeric flavanols (fraction FII). The effects of casein and potassium caseinate on the amount of total trimeric procyanidins were also important. These concentrations were decreased by 48% and 33%, respectively (Table 4.4).

Some other studies have shown that tannins esterified by gallic acid seem to complex more easily with proteins (Maury et al. 2001; Sarni-Manchado et al. 1999). The isinglass obtained from swim bladder and egg albumin resulted in a greater decrease in the amount of total dimeric procyanidins esterified by gallic acid (21% and 14%, respectively) compared with the corresponding nongalloylated procyanidins (dimeric 7% and 3%, respectively and trimeric 15% and 1%, respectively). The gelatine with a polydispersion above 43.0 kDa also showed a greater effect on this type of molecule, producing a decrease of about 48%, while the reduction in the amount of total dimeric and total trimeric procyanidins was of 34%. Nevertheless, this tendency was not observed for all protein fining agents assayed (Table 4.4).

Table 4.4 - (+) – catechin, (-) – epicatechin, sum of dimeric, trimeric and dimeric procyanidins esterified by gallic acid (mg L^{-1}) analysed by HPLC for both the unfined red wine and the red wine after different fining treatments (mean±SD).

Fining treatment	Monomers		Dimers					Trimers			Dimer gallates			
	(+) – catechin	(-) – epicatechin	B3	B1	B4	B2	∑dimeric	T2	C1	∑trimeric	B2-3-*O* gallate	B2-3'-*O*-gallate	B1-3-*O*-gallate	∑gallates
T	14.4±1.7	2.7±0.7	5.4±0.5	31.4±04	11.7±1.1	11.8±0.6	60.5±2.8	5.7±0.0	1.6±0.3	7.3±0.3	6.8±0.4	1.7±0.2	1.2±0.1	9.6±0.1
IL_1	13.9±0.5	2.3±0.6	5.5±0.0	28.6±0.3	7.9±0.2	10.4±0.3	52.4±0.7	3.7±0.4	1.6±0.1	5.3±0.3	6.4±0.1	0.9±0.1	0.7±0.1	8.0±0.2
IS_4	12.6±0.1	2.0±0.0	4.7±0.1	31.5±0.4	9.2±0.8	11.1±0.5	56.5±1.7	4.7±0.1	1.5±0.0	6.2±0.1	6.0±0.7	1.0±0.5	0.5±0.0	7.6±0.3
CS_4	7.3±0.2	1.7±0.1	3.5±0.0	28.3±0.6	5.7±0.4	10.8±0.3	48.2±1.2	2.3±0.3	1.5±0.2	3.8±0.5	5.2±0.3	1.5±0.5	0.5±0.1	7.2±0.7
CKS_1	13.0±1.0	2.7±0.1	3.8±0.1	30.2±0.3	6.0±0.1	10.7±0.9	50.7±1.3	3.3±0.2	1.7±0.6	4.9±0.8	6.4±0.4	1.3±0.2	0.7±0.3	8.3±0.9
AS_1	14.4±0.2	2.4±0.1	5.3±0.2	31.6±0.4	10.5±0.3	11.6±0.4	58.9±0.5	5.7±0.6	1.5±0.2	7.2±0.4	6.5±0.2	1.0±0.0	0.7±0.0	8.3±1.2
GL_1	8.6±0.2c	2.3±0.2	3.5±0.1	28.2±0.6	5.3±0.4	10.0±0.3	47.0±3.4	4.3±0.8	1.3±0.1	5.5±1.9	5.7±1.0	1.6±0.2	0.3±0.1	7.6±0.8
GS_2	10.9±0.1	2.5±0.3	4.4±0.1	22.5±0.4	4.9±0.7	7.8±0.0	39.5±0.0	3.9±0.1	1.0±0.0	4.8±0.2	4.0±0.2	0.7±0.4	0.3±0.0	5.0±0.2
GS_4	5.9±1.1	2.3±0.2	2.5±0.3	25.5±0.1	5.4±0.4	9.8±0.1	43.2±0.7	3.3±0.2	1.2±0.3	4.5±0.5	5.8±0.7	1.4±0.1	0.2±0.0	7.5±1.1

Unfined (T), isinglass (**IL_1**), isinglass (**IS_4**), casein (**CS_4**), potassium caseinate (**CKS_1**), egg albumin (**AS_1**), gelatin (**GL_1**), gelatin (**GS_2**) and gelatin (**GS_4**).

For the monomeric [(+) – catechin and (-) – epicatechin] flavan – 3 – ols it was observed that the protein fining agents promoted a greater decrease in (+) - catechin than in (-) - epicatechin. Except for casein, none of the other fining agents decreased (-) – epicatechin considerably. The (+) – catechin was greatly lowered by casein and by the three gelatines tested. Although casein and potassium caseinate presented similar electrophoretic patterns, the affinity to these isomers was different. Casein induced a major decrease in both isomers but this was not observed with potassium caseinate.

4.3.4. Colour and Pigments

Colour intensity and molecules related to wine colour (mainly coloured anthocyanins, total and polymeric pigments) are less affected by fining with protein agents than are the tannins. However, the addition of casein and gelatines characterised by a polydispersion below 43.0 kDa notably decreased colour intensity whereas the hue remained unchanged after the addition of all the protein fining agents, with the exception for egg albumin. These results are in line with the findings of others (Lovino et al. 1999, Panero et al. 2001; Versari et al. 1998) (Table 4.5).

The gelatine with polydispertion on the low MW was the only fining agent that promoted a considerable decrease in coloured anthocyanins. The three gelatines tested showed different effects on the polymeric pigments. The results reveal that gelatine GL_1 (MW < 43 kDa) induced a major decrease in the polymeric pigments, but this was not observed for gelatine GS_4 (MW < 14.4 kDa) and GS_2 (MW > 43 kDa) (Table 4.5).

Table 4.5 - Total Pigments (TP), colour intensity (CI), hue (H), coloured anthocyanins (CA), polymerized pigments (PP) and total anthocyanins (TA) for both unfined red wine and red wine after different fining treatments (mean±SD).

Fining treatment	TP*	CI*	H*	CA*	PP*	TA (mg L^{-1})
T	39.76±0.09	25.74±0.11	0.442±0.01	13.03±0.09	3.21±0.04	701±3
IL_1	39.49±0.04	24.80±1.02	0.436±0.01	12.62±0.92	3.09±0.08	699±1
IS_4	39.44±0.07	24.64±0.43	0.435±0.00	12.56±0.26	3.07±0.03	699±2
CS_4	38.78±0.08	23.34±1.31	0.442±0.00	11.81±0.81	2.95±0.10	677±2
CKS_1	38.83±0.07	24.07±0.71	0.438±0.01	12.22±0.38	2.96±0.07	681±2
AS_1	39.44±0.07	25.60±0.02	0.424±0.00	13.43±0.03	2.96±0.01	699±1
GL_1	38.53±0.07	23.40±1.43	0.442±0.00	11.81±1.00	2.90±0.04	698±2
GS_2	39.44±0.07	24.77±1.01	0.442±0.00	12.20±1.08	3.21±0.06	697±1
GS_4	38.80±0.04	23.51±0.31	0.442±0.00	11.49±0.28	3.11±0.04	638±0

Unfined (T), isinglass (**IL_1**), isinglass (**IS_4**), casein (**CS_4**), potassium caseinate (**CKS_1**), egg albumin (**AS_1**), gelatin (**GL_1**), gelatin (**GS_2**) and gelatin (**GS_4**).; * - absorbance units.

4.4. CONCLUSIONS

The analysis of the fined red wine shows that each protein fining agent presents a distinct interaction and precipitation capacity in respect to the different condensed tannin fractions. The results show that even proteins of the same general type can have quite different effects on the various tannic fractions. Gelatine characterised by a polydispersion below 43.0 kDa brought about a similar decrease in all the three flavan-3-ol fractions. However, gelatine characterised by a polydispersion above 43.0 kDa did not remove the polymeric proanthocyanidins and monomeric flavanols considerably, while gelatine GS_4 (MW < 14.4 kDa) did remove the various tannic fractions (monomers, oligomers and polymers) notably. Isinglass obtained from fish swim bladder showed an affinity for polymeric (mDP = 4.9) and monomeric (mDP ≈ 1.5) proanthocyanidins, while egg albumin (MW close to 43.0 kDa) showed an affinity for polymeric (mDP = 4.9) and oligomeric (mDP = 3.4) proanthocyanidins. Casein (MW ≈ 30.0 kDa) selectively removes monomeric flavanols (mDP ≈ 1.5). This work indicates that the use of a particular fining protein can

lead to the reduction of a condensed tannin fraction with a specific mean degree of polymerisation.

Gelatine GS_4 (MW < 14.4 kDa) decreased greatly colour intensity and coloured anthocyanins. Also, colour intensity and molecules related to wine colour can be selectively decreased by specific fining proteins.

These results suggest that the oenologist's choice of protein fining agent for clarification, and for the reduction of particular phenolic compounds is important and should be very carefully considered.

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5. INTERACTIONS BETWEEN PROTEIN FINING AGENTS AND PROANTHOCYANIDINS IN WHITE WINE

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INTERACTIONS BETWEEN PROTEIN FINING AGENTS AND PROANTHOCYANIDINS IN WHITE WINE

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ABSTRACT

A comparative fining trial was conducted in a laboratory scale to study the influence of protein fining agents on proanthocyanidins, colour and browning potential of white wine. The monomeric flavanols were significantly depleted by casein, and gelatine with low molecular weight (MW) distribution, and isinglass obtained from fish swim bladder (MW >94.0, containing some bands in the range 94.0-43.0 and at 20.1 kDa). However, the other gelatines and isinglass with a MW polydispersion below 20.1 kDa did not interact significantly ($P < 0.05$) with these compounds. In contrast, the oligomeric compounds were not decreased by swim bladder isinglass. It was also observed that neither of the isinglasses decreased the polymeric flavanols significantly ($P < 0.05$). Although casein and potassium caseinate had similar MW distributions and isoelectric points, potassium caseinate decreased the polymeric flavanols, whereas casein did decrease monomeric, oligomeric and polymeric flavanols significantly ($P < 0.05$). The degree of polymerisation of polymeric proanthocyanidins that remained in the fined wine decreased significantly ($P < 0.05$) after addition of protein fining agents except when potassium caseinate was used. Casein, potassium caseinate and swim bladder isinglass induced a significant ($P < 0.05$) decrease in wine colour ($A_{420\ nm}$), a decrease in browning potential and a decrease in turbidity.

Keywords: White wine, fining, protein, fining agents, polyphenols, proanthocyanidins, thiolysis, turbidity, colour, browning.

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5.1. INTRODUCTION

Proteins have been used in white wine as fining agents for a long time. The various protein fining agents can behave differently, depending on their composition, their origin and their preparation condition. Nowadays, a wide range of protein fining agents are used, including: gelatine, casein, potassium caseinate, egg albumin or isinglass and, more recently some proteins of vegetable origin. In white wine, fining is frequently employed for clarification and/or for improved stabilisation.

The browning of white wine, is a process related to oxidation and represents an important stability problem in white wine. The presence of large quantities of phenolic compounds enhances susceptibility to oxidation, leading to a decrease of the wine's visual and sensory qualities. This is due primarily to the oxidation of phenolic compounds including catechins, proanthocyanidins and hydroxycinnamic acids present in the wine. Barroso, López-Sánchez, Otero, Cela, and Pérez-Bustamente (1989) established a link between susceptibility to browning and the quantity of phenolic compounds present. Spagna, Barbagallo, and Pifferi (2000), therefore recommended the removal of polyphenols to stabilise white wines and reduce the potential for browning. Browning in white wines is usually minimised by the addition of potassium caseinate, which is a very effective fining agent for polyphenols (Amati, Galassi, &Spinabelli, 1979, Manfredini 1989).

The comparative effects of other fining agents such as gelatine, isinglass, potassium caseinate and casein, on the phenolic compounds of white wine has been studied by several authors (Amati et al., 1979; Castino, 1992; Fischerleitner, Wendelin, & Eder, 2002; Fischerleitner, Wendelin, & Eder, 2003; Gorinstein et al., 1993; Jouve et al., 1989; Machado-Nunes, Laureano, & Ricardo-da-Silva, 1998; Sims, Eastridge, & Bates, 1995). All these studies have focussed attention on the wine phenolic composition, but not on characterising the protein fining agents. Furthermore, as far as we can determine there is a lack of information on the structural characteristics (mean degree of polymerisation, galloylation, cis/trans ratio and the percentage of prodelphinidins) of oligomeric and polymeric proanthocyanidins remaining in white wine after fining as a function of the type of fining protein added. A better knowledge of all the molecules involved in fining could lead to an enhanced control and thus to an optimisation of this treatment.

The main goal of this study was, therefore to undertake a comparative study on the effect of eight commercial protein fining agents [gelatine (x3), isinglass (x2), casein (x1),

potassium caseinate (x1) and egg albumin (x1)] on the structural characteristics of proanthocyanidins, as well as on the monomeric flavan-3-ol, and also on flavonoid and non-flavonoid phenolic compounds, chromatic characteristics, turbidity and browning potential of white wine after fining.

5.2. MATERIALS AND METHODS

Reagents

Vanillin was purchased from Merck (Darmstadt, Germany) and toluene-α-thiol from Fluka (Buchs, Switzerland). Solvents and acids used were of HPLC grade.

Protein fining agents

The fining agents previously characterised by Cosme, Ricardo-da-Silva, and Laureano (2007) were used in this work: one egg albumin (AS_1), two isinglasses (IL_1, IS_4), one potassium caseinate (CKS_1), one casein (CS_4) and three gelatines (GL_1, GS_2 and GS_4) (Table 5.1).

Table 5.1 - Fining agents employed in this study.

Fining agents	Code	Concentration	Producer information
Isinglass	IL_1	50 mL/hL	Collagen hydrolysis from fish skin.
Isinglass	IS_4	2.25 g/hL	From fish swim bladder.
Casein	CS_4	40 g/hL	-
Potassium caseinate	CKS_1	40 g/hl	-
Egg albumin	AS_1	12.5 g/hL	-
Gelatine	GL_1	50 mL/hL	High concentration.
Gelatine	GS_2	8 g/hL	-
Gelatine	GS_4	8 g/hL	High degree of hydrolysis.

Fining experiments

Young white wine of vintage 2004 was used in this study made from various white grapevine varieties (all *Vitis vinifera*, L.) from the Estremadura Region, Portugal. It presented the following characteristics: alcohol content 12.0 % (v/v), density (ρ_{20}) 0.9961 g/cm^3, titratable acidity 6.8 g/L (expressed as tartaric acid), volatile acidity 0.36 g/L (expressed as acetic acid), pH 3.41, free sulphur dioxide 9 mg/L and total sulphur dioxide 48 mg/L.

Experiments involved the addition of standard quantities of the protein fining agents (isinglass, casein, potassium caseinate and gelatine) prepared as suggested by the manufacturers (Table 5.1). The trials were conducted at laboratory scale in 250 mL volumes of wine. Untreated wine was used as control. The fining agents were thoroughly mixed and allowed to remain in contact with the wines for 7 days at 20ºC, the samples were then centrifuged at 537.6 g for 15 min before analysis. All experiments were duplicated.

Phenolic compounds analysis

Separation of proanthocyanidins according to degree of polymerisation by C_{18} Sep-Pak cartridges and determination of the flavan-3-ol content by the vanillin assay

The separation of flavanols was performed using a C_{18} Sep-Pak cartridge (Waters, Milford, Ireland) according to the degree of polymerisation in three fractions FI (monomeric), FII (oligomeric) and FIII (polymeric) in line with the method described by Sun, Leandro, Ricardo-da-Silva, and Spranger (1998a). Quantification of the total flavan-3-ol in each fraction was carried out using the vanillin assay according to the method described by Sun et al. (1998a) and by Sun, Ricardo-da-Silva, and Spranger (1998b). For the FI fraction, the absorbance at 500 nm was read after a reaction with vanillin at 30ºC for 15 min using a Unicam UV-vis UV4 spectrophotometer (Unicam, Cambridge, U.K.). For the FII and FIII fractions the reaction was at room temperature and left until the maximum absorbance value at 500 nm was achieved (approximately between 20 and 35 min). Quantification was carried out by means of standards curves prepared from monomers (FI), oligomers (FII), and polymers of flavan-3-ol (FIII) isolated from grape seeds, as described earlier (Sun et al., 1998a, 1998b; Sun, Spranger, Roque-do-Vale, Leandro, & Belchior, 2001).

Characterisation of wine proanthocyanidins (fractionated by C_{18} Sep-Pak cartridges) by acid-catalysed depolymerisation in the presence of toluene– α-thiol followed by reversed-phase HPLC analysis

The proanthocyanidins were depolymerised in the presence of a nucleophilic agent (toluene–α-thiol) in an acid medium. Depolymerisation allows the distinction between terminal units, which are released as flavan-3-ols, and extension units released as their benzylthioethers (Maury, Sarni-Manchado, Lefebvre, Cheynier, & Moutounet, 2001; Souquet, Cheynier, & Moutounet, 2000). Reversed-phase HPLC analysis of the products formed allows determination of the structural composition of proanthocyanidins, which are characterised by the nature of their constitutive extension units (released as their benzylthioethers) and terminal units (released as flavan-3-ols). It also allows calculation of their structural characteristics such as the mean degree of polymerisation (mDP), the average molecular mass (mM), the cis:trans ratio, the fraction of prodelphinidins (% prodelph) and the fraction of galloylation (% gal) (Kennedy, Matthews, & Waterhouse, 2000; Prieur, Rigaud, Cheynier, & Moutounet, 1994; Ricardo-da-Silva, Rigaud, Cheynier, Cheminat, & Moutounet, 1991b; Rigaud, Perez-Ilzarbe, Ricardo-da-Silva, & Cheynier, 1991).

To carry out the acid-catalysed degradation, 100 μL of sample were placed in a glass tube with a hermetic seal together with 100 μL of a solution of toluene-α-thiol in methanol containing HCl (0.2M). After closing, the mixture was mixed gently and incubated at 55ºC for 7 min by which time the depolymerisation yield was around 70% (Monagas, Gómez-Cordovés, Bartolomé, Laureano, & Ricardo-da-Silva, 2003). The thiolysed sample was cooled and then analysed by reversed-phase HPLC. The HPLC system used included a Waters 2487 dual λ absorbance detector set at 280 nm, and a Merck Hitachi Intelligent pump model L-6200A (Tokyo, Japan), coupled to a Konikrom data chromatography treatment system version 6.2 (Konik Instruments, Konik-Tech, Barcelona, Spain). The column was a reversed-phase C_{18}Lichrosphere 100 (250 mm x 4.6 mm, 5 μm) (Merck, Darmstadt, Germany), and the separation was performed at room temperature. The elution condition were as follows: 1.0 mL/min, flow rate, solvent A; (water/formic acid, 98/2, v/v), solvent B; (acetonitrile/formic acid/water 80/2/18, v/v/v) 5-30% B linear from 0 to 40 min 30-50% B linear from 40 to 60 min, 50–80% B linear from 60 to 70 min, followed by washing (acetonitrile/formic acid/water 80/2/18, v/v/v) and reconditioning of the column from 75 to 97 min. The amounts of monomers (terminal units) and toluene-α-

thiol adducts (extension units) released from the depolymerisation reaction in the presence of toluene-α-thiol, were calculated from the areas of the chromatographic peaks at 280 nm by comparison with calibration curves (Kennedy et al., 2000; Prieur et al., 1994; Rigaud et al., 1991).

Separation of monomeric and small oligomeric flavan-3-ols (dimers and trimers) by polyamide column chromatography and quantification by HPLC analysis

Procyanidins separation was performed according to Ricardo-da-Silva, Rosec, Bourzeix, and Heredia (1990). The HPLC system used was the same as that employed for the HPLC analysis of the products released by acid-catalysed depolymerisation in the presence of toluene-α-thiol. The elution conditions for monomeric flavan-3-ols were as follows: 0.9 mL/min flow rate, solvent A; (distilled water/acetic acid, 97.5/2.5, v/v), solvent B; (acetonitrile/solvent A 80/20, v/v), 7-25% B linear from 0 to 31 min followed by washing (methanol/distilled water, 50/50, v/v) from 32 to 50 min and reconditioning of the column from 51 to 65 min under initial gradient conditions. The elution conditions for oligomeric procyanidins (dimeric and trimeric) were as follows: 1.0 mL/min, flow rate, solvent A, (distilled water), solvent B, (distilled water /acetic acid 90/10, v/v), 10-70% B linear from 0 to 45 min, 70–90% B linear from 45 to 70 min, 90% B isocratic from 70 to 82 min, 90-100% B linear from 82 to 85 min, 100% B isocratic from 85 to 90 min, followed by washing (methanol/ distilled water 50/50, v/v) from 91 to 100 min and reconditioning of the column from 101 to 120 min under initial gradient conditions. Identification (Ricardo-da-Silva et al., 1991b; Rigaud et al., 1991) and quantification (Dallas, Ricardo-da-Silva, & Laureano, 1995; Dallas, Ricardo-da-Silva, & Laureano, 1996a; Dallas, Ricardo-da-Silva, & Laureano, 1996b; Ricardo-da-Silva et al., 1990;) of monomeric flavan-3-ols and oligomeric procyanidins (dimeric and trimeric) was carried out.

Quantification of flavonoid phenols and non-flavonoid phenols

Determination of the phenol content of the wines carried out using the absorbance at 280 nm before and after precipitation of the flavonoids through reaction with formaldehyde, according to Kramling and Singleton (1969), leading to a quantification of flavonoid, non-flavonoid and total phenols in the wines.

Turbidity

Turbidity was evaluated by measuring the optical density at 650 nm before and after centrifugation as described by Feuillat and Bergeret (1966).

Test for browning potential

Test tubes were filled to 75% with the wine to be tested. Controls were sparged thoroughly with nitrogen and test samples sparged with oxygen. All tubes were sealed hermetically and maintained at 55ºC for 5 days. This test was conducted on treated and untreated wine and allows calculation of the difference of browning values measuring the increase in A_{420nm} as recommended by Singleton and Kramling (1976).

Chromatic characterisation

The absorption spectra of the wine samples were recorded with a Unicam UV-vis UV4 spectrophotometer (Unicam, Cambridge, U.K.), scanned over the range 380-770 nm using quartz cells of 1-cm path length. Data were collected at 10 nm intervals, and referenced to 1-cm path length, to calculate L* (lightness), a* (measure of redness), b* (measure of yellowness), coordinates using the CIELab method (OIV, 2006). The spectrophotometer incorporates the software required to calculate the CIElab parameters directly (Chroma version 2.0 Unicam, Cambridge, United Kingdom). The Chroma [$C^* = [(a^*)^2 + (b^*)^2]^{1/2}$] and the hue-angle [$h^o = tang^{-1} (a^*/b^*)$] were also calculated. To differentiate the colour more precisely, the colour difference was obtained using the following expression: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$, in CIELab units. It quantifies the overall colour difference of a sample when compared to a reference sample (untreated sample). Two colours can be distinguished by the human eye when the difference between ΔE^* values is greater than 2 units (Spagna et al., 1996).

Colour

Colour was determined by measuring absorbance at 420 nm (10-mm cell) using a Unicam UV-vis UV4 spectrophotometer (Unicam, Cambridge, U. K.) in line with OIV (2006).

Analysis of conventional oenological parameters

Alcohol content (% v/v), pH, density, titratable and volatile acidities, free and total sulphur dioxide were measured according to Organisation International de la Vigne et du Vin methods (OIV, 2006).

Statistical analysis

The data are presented as means±SD. One-way analysis of variance and comparison of treatment means (LSD, 5% level) were performed using ANOVA Statistica 6.1 software (StatSoft, Tulsa, OK, USA) in respect of the effect of protein fining agents.

5.3. RESULTS AND DISCUSSION

The physico-chemical characteristics of the fining agents used in this study are summarised in Table 5.2, and the structural characteristics of the unfined wine proanthocyanidins are presented on the first lines of Tables 5.3-5.5.

The mDP of the fraction FI, the "monomeric fraction", was close to 1.5. The mDP of the monomeric fraction should be 1, but the FI fraction also includes two unknown compounds as shown by Sun et al. (1998a). It is probable that very few oligomeric proanthocyanidins pass through the C_{18} Sep-Pak during separation.

Table 5.2 - Physic-chemical characteristics of the protein fining agents employed on the fining trial (Cosme et al., 2007).

Fining agents	Molecular weight distribution (kDa)	Surface charge density [a] meq/g product at pH 3.4	Protein content [a] as % Nxk (% w/w, dry weight)	Isoelectric point [a]
IL_1	Polydispersion below 20.1	0.04±0.00	112±4	4.55±0.02
IS_4	Bands above 94.0 between 94.0-43.0 and at 20.1	0.41±0.01	73±3	6.48±0.03
CS_4	Band close to 30.0	0.09±0.01	71±1	4.64±0.06
CKS_1	Band close to 30.0	0.04±0.00	85±2	4.51±0.04
AS_1	Band close to 43.0	0.73±0.01	78±1	5.00±0.02
GL_1	Polydispersion below 43.0	0.11±0.00	92±2	4.20±0.01
GS_2	Polydispersion above 43.0	0.74±0.02	98±1	4.74±0.00
GS_4	No bands between 94.4 and 14.4	0.26±0.00	91±4	4.50±0.00

Isinglass (**IL_1**), isinglass (**IS_4**), casein (**CS_4**), potassium caseinate (**CKS_1**), egg albumin (**AS_1**), gelatine (**GL_1**), gelatine (**GS_2**), gelatine (**GS_4**). k – Multiplication factor, which was 6.68 for egg albumin; 6.25 for isinglass; 6.38 for casein and potassium caseinate; 5.55 for gelatine. a – Mean values of three determinations ± standard deviation (SD).

5.3.1. Effect of the fining agents on the flavan-3-ol fractions

The fining agents that removed the monomeric flavanols (fraction FI) most strongly were casein (46%), gelatine with low molecular weight distribution (GS_4 – 31%) and swim bladder isinglasses (IS_4 – 28%). Casein and potassium caseinate showed an electrophoretic profile with similar MW distribution (MW ≈ 30.0 kDa) (Cosme et al., 2007). However, their affinity for monomeric flavanols was different. Only casein lowered these compounds significantly, whereas this effect was not observed for potassium caseinate. The two isinglasses (IL_1, IS_4) also showed different behaviours in relation to the monomeric flavanols. Of these two proteins, only the isinglass obtained from fish swim bladder decreased these compounds significantly (Table 5.3).

In the case of oligomeric flavanols (fraction FII, mDP= 2.9) the greatest decrease was observed with isinglass IL_1 (55%), gelatine with low molecular weight distribution (GS_4 - 40%) and casein (CS_4 – 40%). Isinglass (IL_1) and gelatine (GS_4) were characterised by a polydispersion of the low molecular weights (< 20.1 kDa). For the oligomeric flavanols, casein and potassium caseinate, despite the similarity of their electrophoretic profiles (MW ≈ 30.0 kDa) (Cosme et al., 2007), their affinities for these compounds were quite different. Again, casein decreased these compounds significantly. Isinglass with MW

distributions below 20.1 kDa (IL_1) decreased these compounds significantly but no statistical differences were observed with swim bladder isinglass (IS_4) (Table 5.3).

Table 5.3 - Monomeric flavanols (FI), oligomeric proanthocyanidins (FII) and polymeric proanthocyanidins (FIII) for both unfined white wine and white wine after different fining treatments (mean±SD).

Fining treatment	F1 (mg/L)	FII (mg/L)	FIII (mg/L)
T	5.3±0.1a	35.1±0.4a	82.8±0.5a
IL_1	4.2±0.1abc	15.8±0.8d	81.8±0.9a
IS_4	3.8±0.2bc	27.9±2.5abc	81.8±0.9a
CS_4	2.9±0.2c	21.1±2.5cd	42.9±0.5c
CKS_1	4.2±0.3abc	32.6±3.1ab	62.8±4.8b
AS_1	4.7±0.2ab	27.8±3.4bc	39.2±2.9cd
GL_1	4.7±0.3ab	24.9±1.7c	35.1±3.8d
GS_2	4.5±0.2ab	25.4±3.9c	35.1±6.3d
GS_4	3.6±0.7bc	21.2±3.5cd	34.5±2.9d

Unfined (**T**), isinglass (**IL_1**), isinglass (**IS_4**), casein (**CS_4**), potassium caseinate (**CKS_1**), egg albumin (**AS_1**), gelatine (**GL_1**), gelatine (**GS_2**), gelatine (**GS_4**). Means (n=2) within a column followed by the same letter are not significantly different (LSD, 5%).

The polymeric flavanols (fraction FIII, mDP = 3.8) were decreased significantly by the three gelatines (58%). Neither of the isinglasses decreased the concentration of these compounds significantly (1%). Bonerz et al. (2004) observed that a proteinaceous fining agent extracted from fish skin selectively removed proanthocyanidins with lower mDP. Casein (48%) decreased these compounds more than the twice as effectively as potassium caseinate (24%) (Table 5.3).

5.3.2. Effect of the fining agents on the structural characteristics of proanthocyanidin fractions

The data regarding the structural characteristics of wine proanthocyanidins obtained by reversed phase HPLC of the depolymerisation products released by thiolysis are presented in Table 5.4.

Fining with protein fining agents lowered the mDP of oligomeric and polymeric proanthocyanidins remaining in fined white wine compared to the unfined wine. These results are in accordance with previous reports, which suggest that the largest proanthocyanidin molecules are precipitated first in fining experiments (Ricardo-da-Silva et al., 1991a). This effect could be due to the higher number of phenolic rings present in the more polymerised proanthocyanidins with an increase in hydrophobicity, rendering their complexes more effectively removed (Baxter, Lilley, Haslam, & Williamson, 1997). Nevertheless, wine fined with potassium caseinate did not show statistical differences in mDP for the polymeric proanthocyanidins remaining in the fined wine. In contrast, only isinglass characterised by a polydispersion below 20.1 kDa brought about a significant decrease in the mDP of oligomeric proanthocyanidins (Table 5.4). However, only this isinglass did not significantly reduce the percentage of prodelphinidin (epigallocatechin units) within the oligomeric proanthocyanidin fraction.

5.3.3. Effect of the fining agents on some monomeric, dimeric and trimeric flavan-3-ols molecules

A detailed HPLC analysis of the most important oligomeric proanthocyanidins such as procyanidin dimers (B1, B2, B3 and B4), trimers (trimer 2 and C1) and dimer gallates (B2-3-*O*-gallate, B2-3'-*O*-gallate and B1-3-*O*-gallate) (Table 5.5) was also performed.

It was observed that the egg albumin, the swim bladder isinglass and the three gelatines, decreased all of the individual dimeric procyanidins (B1, B2, B3 and B4), significantly. In contrast, none of the individual dimeric procyanidins (B1, B2, B3 and B4), were significantly decreased by the addition of potassium caseinate. Regarding the individual trimeric procyanidins (trimer 2 and C1), only swim bladder isinglass and potassium caseinate did not bring about a significant decrease in either of the trimers. The isinglass with a low molecular weight polydispersion (MW < 20.1 kDa), brought about a significant decrease of the dimeric procyanidins esterified by gallic acid B1-3-*O*-gallate. The three gelatines tested significantly decreased the dimeric procyanidins esterified by gallic acid B2-3'-*O*-gallate, however only the gelatine characterised by a polydispersion below 43.0 kDa did not significantly reduce the dimeric procyanidins esterified by gallic acid B2-3-*O*-gallate (Table 5.5).

Treatment with gelatine (GS_4) and with isinglass (IL_1) significantly depressed the amount of total dimeric procyanidins (44% and 37%, respectively), the total trimeric procyanidins (56% and 63%, respectively) and the total content of dimer gallates (46% and 50%, respectively) – all compared with untreated wine (Table 5). These fining agents were characterised by low MW polydispersions (< 20.1 kDa). Potassium caseinate had no statistically different ($P < 0.05$) effect on these compounds which contrasted with casein, which induced significant decreases in all oligomeric procyanidins (total dimers 7 and 23%, total trimers 8 and 52% and total dimer gallates 9 and 49%, respectively). As expected, these observations are in accordance with the results obtained for the oligomeric flavanols (FII). Machado-Nunes et al. (1998) also observed that casein decreased procyanidins in white wines. However, Jouve et al. (1989) did not find significant decreases of oligomeric procyanidins (dimeric and trimeric) with casein.

HPLC analyses of the isomers (+) catechin, and (-) epicatechin, showed that the various fining agents had different efficiencies in removing these two compounds (Table 5). These are actually isomers differing only on the spatial position of one OH group which is either ‘up’, or ‘down’ with respect to the ring. In the event, (-) epicatechin was only significantly removed by swim bladder isinglass, whereas (+) catechin was significantly removed by all of the protein fining agents tested and especially by the gelatines and casein.

Table 5.4 - Structural characterisation of proanthocyanidins (oligomeric and polymeric), mean degree of polymerisation (mDP), fractions of galloylation (% gal), fraction of prodelphinidins (% prodelph), average molecular mass (mM) and the cis/trans (cis:trans) ratio for both unfined white wine and white wine after different fining treatments (mean±SD).

Fining	**Oligomeric proanthocyanidins (FII)**					**Polymeric proanthocyanidins (FIII)**				
treatment	**mDP**	**%gal**	**% prodelph**	**mM**	**cis:trans**	**mDP**	**%gal**	**% prodelph**	**mM**	**cis:trans**
T	2.9±0.2a	12.0±0.8ab	26.9±4.5a	889±63a	2.5±0.2a	3.8±0.2a	13.1±0.0ab	18.6±3.4a	1200±51a	3.1±0.3a
IL_1	2.2±0.0b	13.3±0.2b	25.8±0.9ab	694±25b	2.0±0.1b	3.0±0.2b	6.4±0.3c	15.4±3.0ab	926±60b	2.4±0.0b
IS_4	2.6±0.3ab	12.0±0.1ab	11.8±2.3c	815±86ab	2.4±0.2ab	3.1±0.2b	9.5±1.7ac	17.5±0.4ab	946±63b	2.3±0.1b
CS_4	2.7±0.4a	11.0±1.3ac	16.8±5.9bc	831±123ab	2.4±0.3ab	3.1±0.4b	8.5±1.4c	15.9±4.6ab	940±110b	2.7±0.5ab
CKS_1	2.7±0.1a	9.2±2.3c	14.2±1.8c	812±35ab	2.5±0.0a	3.4±0.3ab	9.9±1.2ac	20.0±1.1a	1021±80ab	2.5±0.1ab
AS_1	2.6±0.0ab	13.0±1.3ab	15.7±6.0bc	821±5ab	2.5±0.2a	2.8±0.4b	14.5±1.8b	11.1±2.4b	861±103b	2.5±0.3ab
GL_1	2.8±0.0a	12.9±0.6ab	16.7±0.5bc	884±12a	2.7±0.2ac	3.0±0.3b	14.4±2.1b	15.8±4.4ab	952±103b	2.9±0.7ab
GS_2	2.7±0.0a	12.5±0.3ab	13.2±2.2c	827±11ab	2.1±0.2b	3.1±0.1b	9.4±1.7c	18.4±2.4a	955±35b	2.8±0.6ab
GS_4	2.8±0.2a	11.9±0.5ab	16.3±7.4bc	876±73a	3.0±0.1c	2.9±0.3b	15.4±2.5b	14.4±0.7ab	917±92b	2.2±0.1b

Unfined (T), isinglass (IL_1), isinglass (IS_4), casein (CS_4), potassium caseinate (CKS_1), egg albumin (AS_1), gelatine (GL_1), gelatine (GS_2) and gelatine (GS_4). Means (n=2) within a column followed by the same letter are not significantly different (LSD, 5%).

Table 5.5 - Monomeric flavan-3-ols, dimeric, trimeric and dimeric procyanidins esterified by gallic acid, analysed by HPLC for both unfined white wine and for white wine after different fining treatments (mean±SD).

Fining	Monomers		Dimers					Trimers			Dimer gallates			
treatment	(+)-Catechin (mg/L)	(-)-Epicatechin (mg/L)	B3 (mg/L)	B1 (mg/L)	B4 (mg/L)	B2 (mg/L)	∑dimeric (mg/L)	T2 (mg/L)	C1 (mg/L)	∑trimeric (mg/L)	B2-3-O gallate (mg/L)	B2-3'-O-gallate (mg/L)	B1-3-O-gallate (mg/L)	∑galates (mg/L)
T	5.6±0.1a	1.7±0.1ab	2.2±0.2a	9.4±0.1a	1.5±0.1a	2.7±0.1a	15.76±0.14a	3.1±0.0a	1.1±0.0a	4.11±0.04a	0.5±0.1a	0.2±0.1a	0.3±0.1a	1.04±0.24a
IL_1	4.4±0.1b	1.3±0.0ac	1.4±0.0d	5.4±0.1d	1.4±0.0a	1.5±0.0cd	9.73±0.02c	1.1±0.0f	0.4±0.0d	1.52±0.04e	0.2±0.0d	0.2±0.0ab	0.2±0.0b	0.53±0.03c
IS_4	4.2±0.1c	1.1±0.0c	1.7±0.3cd	7.0±0.1bc	1.1±0.2bc	2.1±0.1b	11.87±0.54b	3.1±0.1a	0.9±0.2ab	3.98±0.22a	0.4±0.0ab	0.2±0.0a	0.3±0.1a	0.94±0.12ab
CS_4	3.4±0.0f	1.4±0.2abc	1.9±0.1abc	7.5±0.5b	0.9±0.1bc	1.8±0.3bc	12.11±1.05b	1.4±0.0de	0.6±0.0cd	1.96±0.05cd	0.3±0.0cd	0.1±0.1d	0.2±0.0ab	0.53±0.11c
CKS_1	4.4±0.0b	1.1±0.1ac	2.2±0.0ab	8.9±0.3a	1.2±0.1ab	2.4±0.1a	14.62±0.51a	3.0±0.1a	0.8±0.1abc	3.77±0.21a	0.5±0.1a	0.2±0.0abc	0.3±0.0a	0.95±0.11ab
AS_1	4.5±0.1b	1.7±0.1abc	1.8±0.2cd	7.0±0.5bc	0.9±0.1bc	2.0±0.2b	11.75±0.99b	1.5±0.1cd	0.8±0.2abc	2.32±0.34bc	0.4±0.1ab	0.1±0.0d	0.2±0.1ab	0.70±0.22bc
GL_1	3.6±0.1d	1.7±0.1ab	1.8±0.2bc	7.0±0.5bc	0.9±0.2bc	2.0±0.2b	11.80±1.07b	1.8±0.2b	0.8±0.0abc	2.58±0.22b	0.4±0.0abc	0.1±0.0cd	0.2±0.0ab	0.71±0.02bc
GS_2	3.1±0.0g	1.2±0.0ac	1.6±0.1cd	6.8±0.1c	1.0±0.0bc	2.0±0.0b	11.35±0.12b	1.7±0.0bc	0.7±0.2bc	2.42±0.18b	0.4±0.1bc	0.1±0.0bcd	0.2±0.1ab	0.65±0.13bc
GS_4	1.7±0.0h	1.4±0.0abc	1.5±0.0d	5.1±0.2d	0.8±0.1c	1.5±0.1d	8.87±0.11c	1.2±0.1ef	0.7±0.2bcd	1.82±0.20de	0.3±0.0bcd	0.1±0.0d	0.2±0.1ab	0.57±0.10c

Unfined (**T**), isinglass (**IL_1**), isinglass (**IS_4**), casein (**CS_4**), potassium caseinat (**CKS_1**), egg albumin (**AS_1**), gelatine (**GL_1**), gelatine (**GS_2**) and gelatine (**GS_4**). Means (n=2) within a column followed by the same letter are not significantly different (LSD, 5%).

5.3.4. Effect of the fining agents on flavanoid and non-flavanoid compounds, colour, chromatic characteristic, limpidity and browning potential

The function of protein fining is mainly to clarify and to remove by adsorptive precipitation those compounds that lead to turbidity or to changes in colour. The results showed that protein fining decreased the amount of flavonoid (0.1-7.1%) and non-flavonoid (0.3-3.0%) compounds. As was shown by Lee and Jaworski (1988) the phenolic compounds are not all subjected to oxidation equally. In general the monomeric catechins and the dimeric procyanidins brown more intensely than other phenolics. The flavonoid compounds most important in white wine oxidation are also most easily removed by fining. However, significant decreases were observed only with casein (7.1%) and with potassium caseinate (2.8%) (Table 5.6). The results for flavonoids agree with those of other authors (Amati et al., 1979; Machado-Nunes et al., 1998; Puig-Deu, López-Tamames, Buxaderas, & Torre-Boronat, 1996), indicating that the protein fining agents have a greater effect on flavonoids than on other polyphenols. For non-flavonoid compounds, the other fining agents studied did not show significant effects with the exception of swim bladder isinglass and of potassium caseinate (Table 5.6).

White wine colour (expressed as the absorbance at 420 nm) and browning potential both showed a significant decrease with casein and with potassium caseinate as well as with swim bladder isinglass (Table 5.6). Similar observations have been reported by Schneider (1988), Castino (1992) and Sims at al. (1995) for casein and by Amati et al. (1979) for potassium caseinate. The wines fined with casein, potassium caseinate and swim bladder isinglass were more stable to oxidation. The increase of absorbance (A_{420nm}) produced by the browning test was less in these wines. This effect is probably related to the fact that swim bladder isinglass and potassium caseinate reduced the non-flavonoid compounds significantly, while casein reduced the level of flavonoid compounds significantly (Table 5.6). In contrast, the loss in white wine colour (A_{420nm}) was not significant for the gelatines. Sims at al. (1995) reported similar results. The reduction of polyphenols was very low with gelatine, which agrees with Sims et al. (1995) and Fischerleitner et al. (2002, 2003).

Table 5.6 - Non-flavonoids, flavonoids, total phenols, turbidity, browning potential, chromatic characteristics and colour A_{420}, of both fined and unfined white wine (mean±SD).

Fining treatment	Non-flavonoid phenols (mg/L gallic acid)	Flavonoid phenols (mg/L gallic acid)	Total phenols (mg/L gallic acid)	Turbidity	Browning Potential[b]	Chromatic Characteristics						Colour[a]
						L*(%)	a*	b*	C*	hº	Δ E*	A420nm
T	167±1a	332±3a	499±3a	7.1±0.4a	0.023	90.5±0.0a	-0.63±0.09a	15.21±0.06ab	15.22±0.06ab	92.32±0.35ab	-	0.329±0.006a
IL_1	167±1a	332±3a	498±2a	3.8±0.1c	0.013	93.9±0.5de	-0.67±0.04a	15.37±0.45bd	15.38±0.44bd	92.50±0.23ab	3.49±0.37a	0.311±0.006ab
IS_4	162±2c	332±1a	495±5a	3.8±0.4c	0.005	94.7±0.6e	-0.76±0.07ab	14.36±0.55a	14.39±0.55a	93.03±0.40a	4.35±0.40b	0.288±0.005b
CS_4	166±2a	309±3c	475±1c	1.8±0.1d	0.005	95.5±0.3e	-0.94±0.04b	13.08±0.38c	13.11±0.38c	94.11±0.07c	5.52±0.35c	0.224±0.002c
CKS_1	162±2bc	323±2b	485±9b	2.0±0.3d	0.005	95.4±0.0e	-0.90±0.03b	13.24±0.28c	13.27±0.29c	93.89±0.04c	5.35±0.12c	0.225±0.003c
AS_1	165±2abc	331±3a	499±3a	3.9±0.2c	0.020	92.5±0.9cd	-0.57±0.15ac	16.22±0.44de	16.23±0.43de	92.00±0.58bd	2.34±0.49d	0.318±0.002a
GL_1	165±0abc	332±2a	499±1a	6.8±0.6a	0.018	90.1±1.2a	-0.07±0.16d	18.21±0.64g	18.21±0.64g	90.36±0.33e	3.18±0.62ae	0.328±0.004a
GS_2	165±1ab	332±1a	498±2a	5.5±0.3b	0.019	90.8±1.3ab	-0.19±0.07d	17.85±0.23fg	17.85±0.23fg	90.61±0.21e	2.84±0.31de	0.329±0.005a
GS_4	166±1a	332±1a	496±2a	5.3±0.2b	0.016	92.3±0.3bc	-0.41±0.04c	17.00±0.06ef	17.00±0.06ef	91.37±0.11d	2.56±0.21d	0.305±0.006ab

Unfined (**T**), isinglass (**IL_1**), isinglass (**IS_4**), casein (**CS_4**), potassium caseinate (**CKS_1**), egg albumin (**AS_1**), gelatine (**GL_1**), gelatine (**GS_2**) and gelatine (**GS_4**) a - absorbence unit; b- difference of the increase of absorbance A_{420} between the wine with and without nitrogen; L* - lightness, a* - redness, b*- yellowness, C* - chroma, hº - hue angle, ΔE – total colour difference. The values corresponding to ΔE* were obtained taking as a reference the unfined wine (**T**). Means (n=2) within a column followed by the same letter are not significantly different (LSD, 5%).

The results obtained with the CIELab method for the chromatic characteristics of the unfined and fined wine with different proteins, showed that they changed after fining (Table 5.6). In the wines fined with casein, potassium caseinate, isinglasses, egg albumin and gelatine with a polydispersion on the low molecular weight, lightness (L*) increased significantly, suggesting a clarifying action. These results fit in with the turbidity data. The values of chroma (C*) decreased significantly after the addition of casein and potassium caseinate. Also, hue-angle (hº) values increased after addition of these two fining agents. Higher values of hº are due to lower absorbance at 420 nm (yellow pigments – 90º). This observation on hº values could indicate that some yellow pigments were removed after addition of casein and potassium caseinate. The values obtained for colour difference (ΔE), between each fined and unfined wine (Table 6), all show values higher than 2 CIElab units, indicating that these colour differences can be discriminated visually (Spagna et al., 1996). The largest values for colour variation ΔE^* were found for potassium caseinate and for casein, followed by both isinglasses and all detectable by eye. The results also show that the values for b* decreased with casein or potassium caseinate. These fining agents all reduced the yellow intensity.

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6. GELATINE, CASEIN AND POTASSIUM CASEINATE AS WINE FINING AGENTS: DIFFERENT EFFECTS ON COLOUR, PHENOLIC COMPOUNDS AND SENSORY CHARACTERISTICS

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Gelatine, Casein and Potassium Caseinate as Wine Fining Agents: Different Effects on Colour, Phenolic Compounds and Sensory Characteristics

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ABSTRACT

Aims: Describe and compare some characteristics, such as molecular weight (MW) distribution and surface charge density of commercial protein fining agents and to enhance the understanding of their effect on wine chemical and sensory characteristics.

Methods and Results: Protein (casein, potassium caseinate and gelatine) MW distribution was characterised by electrophoresis. These proteins were added to a red and a white wine, in order to evaluate its effect on colour, phenolic compounds and sensory attributes.

Conclusion: A band at 30.0 kDa characterised casein and potassium caseinate. Gelatines showed polydispersion on the MW distribution, gelatine GSQ on the higher MW (> 43.0 kDa) and gelatine GL on the lower MW (< 43.0 kDa). Despite the fact that casein and potassium caseinate had similar MW distribution, casein decreased essentially the monomeric ((+) - catechin and (-) - epicatechin) while the potassium caseinate showed a lower influence on these compounds. Also, among the two gelatines used, a different behaviour was observed. The gelatine characterised by a polydispersion below 43.0 kDa depleted more the polymeric tannin fractions than the gelatine characterised by a polydispersion above 43.0 kDa. That gelatine has also decreased colour intensity and coloured anthocyanins of red wine but the hue remains unchanged. Addition of fining agents did not affect greatly the concentration of monomeric anthocyanins. Sensory analysis showed that wines fined with the different proteins presented distinct characteristics.

Significance and Impact of study: The knowledge of the physico-chemical characteristics, such as MW distribution and surface charge density, is important for wine fining optimisation and consequently for the wine quality.

Key words: fining agent, anthocyanins, condensed tannins, surface charge density, wine..

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6.1. INTRODUCTION

Fining allows wine clarification, stabilisation and the improvement of sensory characteristics. However, it is necessary to know and to understand the fining mechanisms, to reach the intended objectives.

The main protein fining agents used in wine are gelatine, casein, potassium caseinate, egg albumin and isinglass. However, in the recent years, plant proteins (wheat, gluten and other origins) have also been studied for wine fining (MARCHAL et al. 2000a; b, PANERO et al. 2001, MAURY et al. 2003, FISCHERLEITNER et al. 2002, 2003).

Proteins used as wine fining agents present diverse physico-chemical characteristics mainly molecular weight distribution and surface charge density. The knowledge of these characteristics is important for wine fining optimisation and consequently for the wine quality.

The great diversity of gelatines available in the market is a result of the collagen origin and the nature of the production process. Collagen could be found in the skin, bones or cartilages. Hydrolysis of the collagen could be chemical (alkaline or acid) or enzymatic. For the chemical process, the hydrolysis degree is function of the temperature and the time (LAGUNE and GLORIES 1996a). The gelatines obtained by enzymatic hydrolysis present protein fractions with MW lower than 13.7 kDa (PAETZOLD and GLORIES 1990); the presence of gelatines with a polydispersion on the higher MW as well as on the lower MW was also verified by several authors (PAETZOLD and GLORIES 1990; MARCHAL et al. 1993; 2000a; b; 2002; VERSARI et al. 1998; 1999; COSME et al. 2007). The MW distribution of gelatine affects both the quantity and the type of phenolic compounds removed from red wines (HRAZDINA et al. 1969; YOKOTSUKA and SINGLETON 1987; RICARDO-DA-SILVA et al. 1991a; LAGUNE et al. 1996; SCOTTI and POINSAUT 1997; VERSARI et al. 1998; LEFEBVRE et al. 1999; SARNI-MANCHADO et al. 1999; MAURY et al. 2001). According to the type of gelatine and the pH of the medium, surface charge densities ranged from 0.02 to 1.2 mEq/g (PAETZOLD and GLORIES 1990; LAGUNE and GLORIES 1996b; LAMADON et al. 1997).

Furthermore, a major band at 30 kDa and other minor bands with lower MW, as well as higher MW characterised casein and potassium caseinate fining agents (MARCHAL et al. 2000 a; b; COSME et al. 2007).

Wine phenolic compounds interact with protein fining agents. For example, the two main types of interaction between proteins and tannins are: hydrogen bonds and hydrophobic interactions (MURRAY et al. 1994). The complexes formed could be soluble or insoluble. The precipitation occurs in two steps: association between proteins and tannins leads to the formation of soluble complexes that could, in a following step, aggregate each other and precipitate. This last step depends on the capacity of the tannin to establish linkages between protein molecules (CHEYNIER et al. 1998). Environmental conditions such as pH, alcohol and temperature also influence the formation of tannin-protein complexes (CALDERON et al. 1968, RIBÉREAU-GAYON et al. 1977, 1998).

Thereby, protein fining agents could be used to remove specific phenolic compounds and consequently astringency or bitterness of wines. The sensation of astringency is due to the interaction of salivary proteins (rich in proline) with wine phenolic compounds, mainly condensed tannins (KALLITHRAKA et al. 1998; SAINT-CRICQ-DE-GAULEJAC et al. 1999). However, LEA and ARNOLD (1978) have suggested that not all wine phenolic compounds contribute in the same way to wine astringency. These authors have concluded that the sensation of astringency is essentially due to the more polymerised tannins and those esterified with gallic acid. Gelatines like salivary proteins present higher levels of proline than most of the proteins (LAGUNE and GLORIES 1996a). Therefore, addition of gelatine to the wine leads to a reduction in the tannin content, mainly concerning the more polymerised tannins and those esterified with gallic acid (SARNI-MANCHADO et al. 1999; MAURY et al. 2001). This indicates that gelatine addition could decrease wine astringency. In a study with flavanol monomers and several flavanol dimers and trimers, esterified or not with gallic acid, RICARDO-DA-SILVA et al. (1991a) have observed that gelatine and casein interact more intensely with the more polymerised proanthocyanidins and also those esterified with gallic acid.

Therefore, the main objective of this work was to describe and compare some characteristics, such as molecular weight distribution, surface charge density and protein content of several distinct commercial protein fining agents (gelatines, casein and potassium caseinate) and to enhance the understanding of their effect on wine (white and red) colour, chromatic characteristics, monomeric anthocyanins, phenolic compounds and sensory characteristics.

6.2. MATERIALS AND METHODS

6.2.1. Fining agents characterisation

Protein fining agents

In this work, four protein fining agents have been characterised: two gelatines (GSQ, GL), one potassium caseinate (CK) and one casein (CS) (Table 6.1).

Total nitrogen

The total nitrogen content was determined by the Kjeldahl method based on mineralisation, distillation and titration with 0.1 N HCl (MANFREDINI 1989; OIV 2006b).

Protein quantification

The protein content was determined by the Bradford method as modified by READ and NORTHCOTE (1981). Analyses were carried out by adding different proteins [protein fining agents and standard proteins (bovine serum albumin)] to a dye reagent [Coomassie brilliant blue G-250 (Acros Organics, USA), ethanol, phosphoric acid and deionised water], which resulted in an increased absorbance at 595 nm, due to the formation of a protein-dye complex.

Table 6.1 - Fining agents characterised and used for white and red wine fining.

Fining agents	**Code**	**Concentration**	
		White wine	**Red wine**
Gelatine	GSQ	8 g/hL	10 g/hL
Gelatine	GL	5 mL/hL	6 mL/hL
Casein	CS	15 g/hL	15 g/hL
Potassium caseinate	CK	20 g/hL	20 g/hL

Protein MW distribution characterised by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The MW distribution of oenological protein fining agents was studied using a SDS-PAGE method as described by LAEMMLI (1970) and adapted for protein fining agents by MARCHAL et al. (2000a; b; 2002). Standard proteins covering a 14.4-94.0 kDa range were

used to estimate the MW [Low Molecular Weight (LMW) Amersham Biotech, London, United Kingdom]. Samples and standard proteins were treated with buffer [(0.125 M Tris-Cl, 4 % SDS, 20 % glycerol, 2% 2-mercaptoetanol, pH 6.8)] (v/v) and denatured at 100 ºC for 5 minutes. The gel with 0.75 mm thickness was run in a mini-vertical gel electrophoresis unit (Mighty-Small II SE 250, Hoefer, San Francisco, USA) at a constant voltage (75 V) at 20 ºC until the bromophenol blue raised the bottom of the gel. After migration, proteins were stained in a solution made up of one part Coomassie blue R-350 (Amersham Bioscience, Uppsala, Sweden) and nine parts of a solution with methanol: acetic acid: water (3:1:6) and destained in a mixture of acetic acid: methanol: water (1:2:7) (MARCHAL et al. 2000a; b; 2002).

pH

For solid gelatine, pH was measured on a 1 % solution of the initial product (w/v). As concerns solid potassium caseinate, pH was measured on a 5 % solution of the initial product (w/v), and solid casein on a 10 % solution of the initial product (w/v). As regards liquid gelatine, pH was measured directly in the colloidal solution. pH determination was based on the International Codex of Oenology (OIV 2006b).

Weight loss on drying

Weight loss on drying was determined according to the International Codex of Oenology (OIV 2006 b) at 100-105 ºC on a 2 g sample of the following proteins: casein, potassium caseinate and gelatine. In the case of a colloidal solution of gelatine, a 10 g sample was used, which was dried over water at 100 ºC for four hours, and then dried in an oven at 100-105 ºC for three hours.

Surface charge density

The surface charge density was determined with a particle charge detector – produced by MÜTEK (Herrsching, Germany) model PCD 03 pH – by titration with a charge compensating polyelectrolyte 0.001 N electropositive-polydiallyldimethylammonium [polyDADMAC (Herrsching, Germany)] or 0.001 N electronegative-sodium polyethylensulfate [PES-Na (Herrsching, Germany)] (PAETZOLD and GLORIES 1990; DIETRICH and SCHÄFER 1991) until the streaming potential is 0 mV, which corresponds to the point where all charges are neutralised. The volume of

polyelectrolyte required for the neutralisation allowed to estimate the surface charge density of the product, expressed in milliequivalent of polyelectrolyte per gram of fining agent (mEq/g).

Gelatine fining agents were dispersed in a model wine solution without ethanol (VERNHET et al. 1996).

Casein and potassium caseinate were first dissolved in 0.1 N KOH and subsequently dispersed in the model solution. The surface charge density of these fining agents was measured at pH 3.4 (adjusted with 50 % HCl and centrifuged at 4.000 rpm during 15 minutes).

6.2.2. White and red wine fining trials

Chemicals

Vanillin was purchased from Merck (Darmstadt, Germany). Solvents and acids used were of HPLC grade.

Wines

White and red wines of the 2003 vintage used in this study were elaborated with different grapes from Vitis vinifera varieties from the Óbidos Region (Adega Cooperativa do Bombarral) and from Lisbon (Tapada da Ajuda – Instituto Superior de Agronomia) respectively. Table 6.2 shows the analytical composition of both wines before the fining treatment.

Fining experiments

Experiments involved the addition of standard quantities of the protein fining agents (gelatines, casein and potassium caseinate) prepared as recommended by the producers (Table 6.1). Trials were conducted at the laboratory scale in 1000 mL volumes of wine. Untreated wine was used as control. The fining agents were thoroughly mixed and allowed to remain in contact with the wine for 7 days; the samples were then centrifuged at 4000 rpm for 15 min. before analysis

Physic-chemical analysis of wine

Alcohol content % (v/v), pH, density, titratable and volatile acidities, free sulphur dioxide, malic acid and residual sugars were analysed according to the Organisation Internationale de la Vigne et du Vin methods (OIV 2006a).

Table 6.2 - Physic-chemical characteristics of the white and the red wines used before fining treatment.

Parameters	Red wine	White wine
PH	3.53	3.48
Free sulphur dioxide (mg/L)	16	26
Volatile acidity (g/L acetic acid)	0.81	0.44
Titratable acidity (g/L tartaric acid)	6.0	5.5
Reducing sugars (g/L)	2.8	1.2
Alcohol content (% v/v)	13.9	10.4
Density (g/cm^3)	0.9960	0.9937
Malolactic fermentation	Occurred	Occurred

Fractionation of proanthocyanidins according to the degree of polymerisation by C18 Sep-Pak cartridges and determination of the flavan-3-ol content by the vanillin assay

The separation of flavanols was performed on a C18 Sep-Pak cartridge (Waters, Milford, Ireland) according to the degree of polymerisation in three fractions, monomers, oligomers and polymers of flavan-3-ol in agreement with the method described by SUN et al. (1998a). Quantification of the total flavan-3-ol content in each fraction was performed using the vanillin assay according to the method described by SUN et al. (1998a, b). For the monomeric fraction, the absorbance at 500 nm was read after reaction at 30 ºC for 15 min. using a Unicam UV-vis UV4 spectrophotometer (Unicam, Cambrigde, U.K.). For the oligomeric and polymeric fractions, the reaction was performed at room temperature and left until the maximum absorbance value at 500 nm was reached. Quantification was carried out by means of standard curves prepared from monomers, oligomers, and polymers of flavan-3-ol isolated from grape seeds, as described earlier (SUN et al. 1998a, 2001).

Separation of monomeric and small oligomeric flavan-3-ols (dimers and trimers) by polyamide column chromatography and quantification by HPLC analysis

Procyanidin separation was performed according to RICARDO-DA-SILVA et al. (1990). High Performance Liquid Chromatography (HPLC) analyses were carried out using a HPLC system including a Konik Instruments (Konik Instruments, Konik-Tech, Barcelona, Spain) UV-vis detector (Uvis 200) set at 280 nm, and a Merck Hitachi Intelligent pump model L-6200A (Tokyo, Japan), coupled to a Konikrom data chromatography treatment system version 6.2 (Konik Instrument, Konik-Tech, Barcelona, Spain). The column was a reverse-phase C18Lichrosphere 100 (250 mm x 4.6 mm, 5 μm) (Merck, Darmstadt, Germany). Separation was performed at room temperature. The elution conditions for monomeric flavan-3-ols were as follows: 0.9 mL/min., flow rate, solvent A; (water/acetic acid, 97.5/2.5, v/v), solvent B; (acetonitrile/solvent A 80/20, v/v), 7-25 % B linear gradient from 0 to 31 min. followed by washing (methanol/water, 50/50, v/v) from 32 to 50 min and reconditioning of the column from 51 to 65 under initial gradient conditions. The elution conditions for oligomeric procyanidins (dimeric and trimeric) were as follows: 1.0 mL/min., flow rate, solvent A, (distilled water), solvent B, (water/acetic acid, 90/10, v/v), 10-70% B linear gradient from 0 to 45 min., 70 – 90 % B linear gradient from 45 to 70 min., 90 % B isocratic from 70 to 82 min., 90-100% B linear gradient from 82 to 85 min., 100 % B isocratic from 85 to 90 min., followed by washing (methanol/ water, 50/50, v/v) from 91 to 100 min. and reconditioning of the column from 101 to 120 min. under initial gradient conditions. Identification (RICARDO-DA-SILVA et al. 1991b; RIGAUD et al. 1991) and quantification (RICARDO-DA-SILVA et al. 1990; DALLAS et al. 1995, DALLAS et al. 1996a, b) of monomeric flavan-3-ols and oligomeric procyanidins (dimeric and trimeric) were performed.

Monomeric anthocyanins

Monomeric anthocyanin analysis was carried out by HPLC according to DALLAS and LAUREANO (1994). The equipment used was a Perkin-Elmer (Norwalk, USA) system, equipped with a model L-7100 Lachrom Merck Hitachi-High-Technologies pump (Tokyo, Japan), a model LC-95 UV-Vis detector set at 520 nm coupled to a version 6.2 Konikrom data chromatography treatment system (Konik Instruments, Konik-Tech, Barcelona, Spain). The column was a reverse-phase C18 Lichrosphere 100 (5 □m packing, 250mm x 4.6 mm i.d.) (Merck, Darmstadt, Germany) protected with a guard column of the

same material. The separation was carried out at room temperature. The elution conditions for monomeric anthocyanins were as followed: 0.7 mL/min., flow rate, solvent A was 40 % formic acid, solvent B was CH3CN and solvent C was bidistilled water. The initial conditions were 25 % of A, 6 % of B and 69 % of C for 15 min. followed by a linear gradient to 25 % of A, 25.5 % of B 49.5 % of C during 70 min., and 20 min. of 25 % A, 25.5 % of B and 49.5 % of C.

Quantification of monomeric anthocyanins in wine was performed by means of standard curves prepared by using different concentrations of malvidin 3-glucoside chloride in methanol 0.1 % HCl. The peak area was converted to mg/L of malvidin 3-glucoside equivalent. Twenty μL of each sample were injected in triplicate.

Quantification of non-flavonoid phenols

Determination of the phenolic content of wines was carried out by absorbance measurement at 280 nm before and after precipitation of the flavonoids through reaction with formaldehyde according to KRAMLING and SINGLETON (1969), leading to a quantification of non-flavonoid compounds in the wine.

Chromatic characterisation, colour and pigments

Absorption spectra of the wine samples were recorded with a Unicam UV-vis UV4 spectrophotometer (Unicam, Cambridge, U.K.), scanned over the range 380 to 770 nm using quartz cells. Data were collected at 10 nm intervals, and referred to a 1-cm path length to calculate L* (lightness), a* (measurement of redness), b* (measurement of yellowness), coordinates using the CIELab method (OIV 2006a). The spectrophotometer incorporates the software required to calculate the CIELab parameters, directly (Chroma version 2.0 Unicam, Cambridge, United Kingdom). Colour intensity was calculated by summation of the absorbances at three wavelengths 620, 520 and 420 nm (1-mm cell) using a Unicam UV-vis UV4 spectrophotometer (Unicam, Cambridge, U. K.). Hue was expressed as the ratio of absorbance at 420 nm and 520 nm. The content of total and coloured anthocyanins and total and polymeric pigments were determined according to the method proposed by SOMERS and EVANS (1977).

Sensory evaluation

The wines were subjected to sensory analysis to assess the differences between the unfined and the fined wines. A panel composed by nine trained members evaluated the wines. The wines were presented in two sessions; one for white wines and another for red ones (unfined and fined wines). Wines were presented to the panel at random. A code with three arbitrary numbers was attributed to each wine. White wines were assessed for limpidity, colour, aromatic intensity and quality, taste intensity and quality, fullness and global appreciation. Red wines were assessed for colour intensity, hue, aromatic intensity and quality, taste intensity and quality, fullness, astringency and global appreciation. There was a structured scale with numbers from 0 to 4 for colour evaluation and from 1 to 7 for the other characteristics.

A principal component analysis (PCA) was carried out on the results of the averages of the sensory analysis data for each attribute. For statistical analysis, the Statistica 6.0 program was used.

6.3. RESULTS AND DISCUSSION

6.3.1. Fining agents characterisation

Loss during drying, pH, total nitrogen and protein content

The liquid gelatine (GL) had a loss during drying of 86 % (w/w). As expected, the value was higher than those obtained for fining agents in a solid state [8 – 11 % (w/w)]. Losses during drying are in accordance with the recommendations of the International Codex of Oenology (OIV 2006b) (Table 6.3).

All of the fining agents analysed had acidic or almost neutral pH (Table 6.3).

Total nitrogen values of solid and liquid gelatines were respectively, 14.0 and 18.9 % (w/w, dry weight) and, for potassium caseinate and casein, the values were 14.5 and 10.7 % (w/w, dry weight), respectively (Table 6.3).

Table 6.3 - Weight loss on drying, pH, total nitrogen, total protein and surface charge density (mean±SD).

Fining agent	Weight loss (%w/w)	pH	Total Nitrogen (% w/w, dry weight)	Protein content by Bradford method (μg BSA/g fining agent)	Surface charge density (mEq/g product, at pH 3.4)
GSQ	11±0.2	4.2±0.02	14.0±1.1	42.0±1.6	0.96±0.00
GL	86±0.1	2.5±0.01	18.9±1.6	54.7±2.0	0.52±0.02
CS	8±0.0	7.2±0.02	10.7±1.2	101.1±1.4	0.20±0.00
CK	8±0.2	6.7±0.02	14.5±1.0	93.3±2.1	0.24±0.00

GSQ – gelatine; GL – gelatine; CS – casein; CK – potassium caseinate.

Protein molecular weight distribution

The MW distribution of casein and potassium caseinate observed in the SDS-PAGE electrophoretic pattern (Fig. 6.1) showed that both fining agents are characterised by a major band at 30.0 kDa. This was also observed by other authors for casein (MARCHAL et al. 2000a; b; COSME et al. 2007) and potassium caseinate (COSME et al. 2007). The gelatines GSQ and GL showed polydispersion according to MW distribution, which was also observed by other authors (MARCHAL et al. 1993; 2000a; b; 2002, COSME et al. 2007). However, gelatine GSQ showed a polydispersion on the higher MW (MW > 43.0 kDa) whereas gelatine GL showed a polydispersion on the low MW (MW < 43.0 kDa) (Fig. 6.1). Knowledge of the MW distribution of the protein fining agents is important for tannin-protein interactions (SARNI-MANCHADO et al. 1999; MAURY et al. 2001)

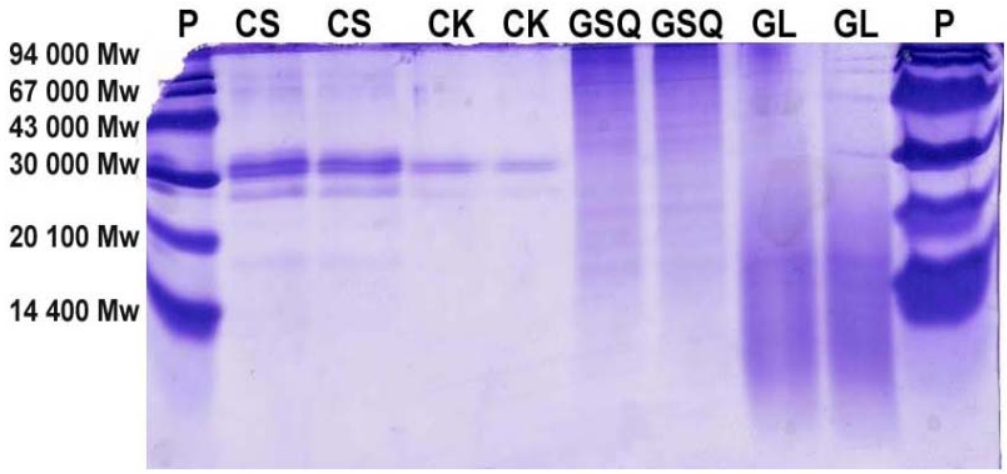

Fig. 6.1 - Electrophoretic patterns of casein – CS, potassium caseinate – CK and gelatine – GSQ, GL. MW standard – P, are given on the left and right side.

Surface charge density

The highest surface charge density was measured in solid gelatine (GSQ) (Table 3), which could be related with a lower degree of hydrolysis of these proteins (SCOTTI and POINSAUT 1997; LAMADON et al. 1997).

As described earlier, casein and potassium caseinate were initially dissolved in KOH and afterwards dispersed in a model solution lacking ethanol at a pH adjusted to 3.4. The surface charge densities of these fining agents were measured at the pH of dissolution (CS - pH 9.7, CK - pH 10.6) and subsequently at pH 3.4. It was observed that the surface charge density of these fining agents changed after pH adjustment (from -1.09 to 0.20 and -1.3 to 0.24 mEq/g of product, respectively).

6.3.2. White wine fining trials

Phenolic compounds and chromatic characteristics

The objective of this study was to know which tannin fraction (monomeric, oligomeric and polymeric flavan-3-ol) is quantitatively more depleted after addition of the diverse protein fining agents.

The fining agent that more depleted the monomeric flavanols was casein (58%), followed by potassium caseinate (29%) (Table 6.4). This result agrees with those of AMATI et al. (1979) for potassium caseinate (20-34% decrease in catechin). Gelatine did not considerably remove the monomeric flavanols, which is in accordance with the work done by SARNI-MANCHADO et al. (1999).

Table 6.4 - Monomeric flavanols, oligomeric and polymeric proanthocyanidins, non-flavonoids compounds and chromatic characteristics of both fined and unfined white wine (means ± SD).

Fining agent	Monomeric flavanols (mg/L)	Oligomeric proanthocyanidins (mg/L)	Polymeric proanthocyanidins (mg/L)	Non-flavonoids phenols (mg/L gallic acid)	L*(%)	a*	b*
T	1.5±0.3	3.5±0.2	12.7±0.4	581±4	94.6±0.0	0.37±0.01	11.30±0.01
GSQ	1.4±0.1	1.9±0.2	7.3±0.2	443±1	94.8±0.1	0.29±0.04	10.68±0.04
GL	1.4±0.2	2.3±0.3	3.7±0.3	428±2	95.2±0.1	0.29±0.04	10.26±0.02
CS	0.6±0.2	1.8±0.3	7.1±0.3	583±3	94.5±0.0	0.12±0.06	10.86±0.02
CK	1.0±0.0	2.5±0.1	8.8±0.2	461±3	93.3±0.1	0.21±0.04	12.00±0.03

Unfined (T), gelatine (GSQ), gelatine (GL), casein (CS), potassium caseinate (CK). L* - lightness, a* - redness, b*- yellowness.

All fining agents used in this study decreased the oligomeric flavanol content; however, gelatine with a polydispersion above 43.0 kDa and casein were found to be the fining agents that decreased to a greater extend these compounds (50%). Gelatine with a polydispersion below 43.0 kDa and potassium caseinate removed oligomeric flavanols, but to a lesser extend (20-25%). It was observed that casein and potassium caseinate showed different affinities for these compounds, despite the similarity of their electrophoretic profiles (MW ≈ 30 kDa) and surface charge densities (≈ 0.20 mEq/g). Casein decreased to a greater extend the oligomeric flavanol content in white wines (Table 6.4) than did potassium caseinate.

The gelatine with a polydispersion below 43.0 kDa removed the polymeric flavanols at a higher quantity (71%), which agrees with the results found by MAURY et al. (2001). These authors showed that proteins of lower MW (16 kDa), presented a greater affinity to polymeric tannins than proteins with higher MW (190 kDa). The other fining agents used in this study removed similar quantities of these compounds (30-45%).

Proanthocyanidins (oligomeric and polymeric) were more influenced by proteic fining agents than monomeric flavanols, which is probably related to their higher degree of polymerisation. This observation has already been done by ROSSI and SINGLETON (1966), CHEYNIER et al. (1997) and by SARNI-MANCHADO et al. (1999). According to these results, gelatine with a polydispersion below 43.0 kDa and casein were the fining agents that promote the highest decrease of proanthocyanidins and monomeric flavanols, respectively.

As could be observed in Table 6.4, addition of fining agents diminishes the content of non-flavonoid (20.6-26.3%) compounds. The results obtained with the CIELab method for determining the chromatic characteristics of the unfined and fined wine with diverse proteins are presented in Table 6.4. In the wine fined with gelatine with a polydispersion below 43.0 kDa (GL), lightness slightly increased (L*); this suggests a clarifying action on this wine. Yellowness (b*) decreased after gelatine and casein addition, as well

Effect of protein fining on sensory evaluation

The wine fined with gelatine with a polydispersion above 43.0 kDa (GSQ), showed a high aroma intensity. Attributes that also were few pointed are colour and taste quality. In contrast, the wine fined with gelatine with a polydispersion below 43.0 kDa, showed the best visual characteristics (colour and limpidity). The wines fined with casein and

potassium caseinate showed similar sensory characteristics. These two wines presented the highest values for body and global appreciation (Fig. 6.2).

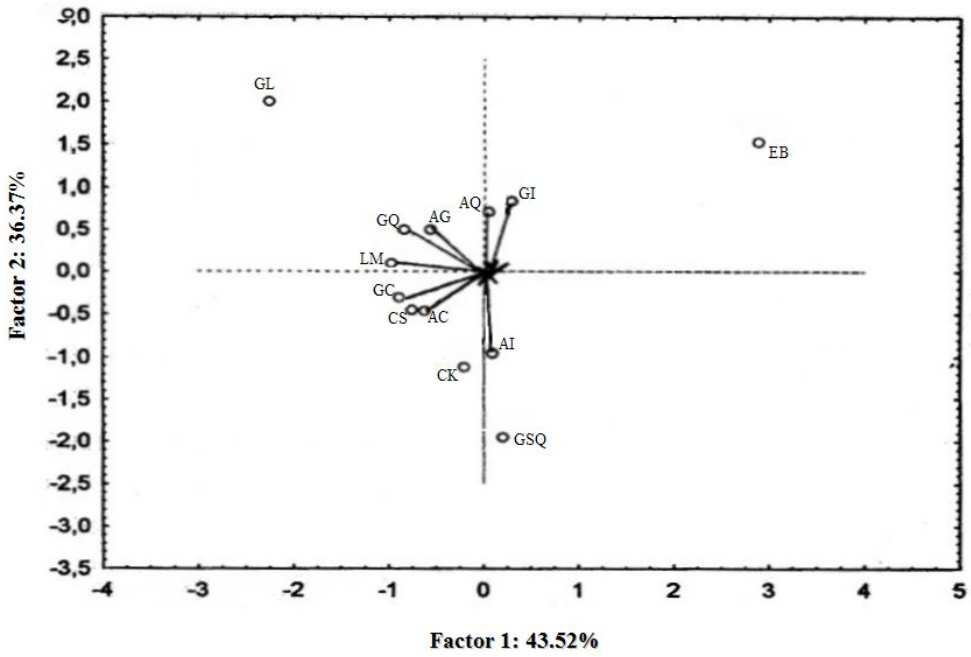

Fig. 6.2 - Principal components analyse of white wine. GL – gelatine, GSQ - gelatine, CS – casein, CK – potassium caseinate, EB – unfined wine. LM – limpidity, AC – colour, AI – aroma intensity, AQ – aroma quality, GQ – taste quality, GI – taste intensity, GC – taste body, AG – global appreciation.

6.3.3. Red wine fining trials

Phenolic compounds and colour

Gelatine with a polydispersion below 43.0 kDa was the fining agent that promotes the highest decrease of the oligomeric and polymeric flavanol content. As previously observed by YOKOTSUKA and SINGLETON (1995), the amount of proanthocyanidins removed, diminishes with the increase of the MW of the proteic fining agent. Gelatine GL was characterised by protein fractions with lower MW distribution (MW<43kDa) whereas gelatine GSQ was characterised by protein fractions with higher MW distribution (MW>43kDa) (Fig. 6.1); thereby the results observed in Table 6.5 are in accordance with those of YOKOTSUKA and SINGLETON (1995)

The monomeric flavanols [(+) - catechin and (-) – epicatechin] were separated by HPLC (Table 6.6). Analyses evidenced that the diverse proteic fining agents have different efficiencies in depleting these two compounds. Consequently, addition of gelatines did not considerably decrease these compounds, which was in accordance with the data of

RICARDO-DA-SILVA et al. (1991a). In contrast, casein and potassium caseinate decreased the concentration of (+) - catechin and (-) - epicatechin.

Table 6.5 - Oligomeric and polymeric proanthocyanidin contents of both fined and unfined red wine (means ± SD).

Fining agent	Oligomeric proanthocyanidins (mg/L)	Polymeric proanthocyanidins (mg/L)
T	280.8±4.7	790.5±22.2
GSQ	267.2±8.9	569.8±26.2
GL	163.9±1.2	545.0±38.2
CS	248.2±7.6	599.1±19.1
CK	211.1±1.2	743.2±26.4

Unfined (T), gelatine (GSQ), gelatine (GL), casein (CS), potassium caseinate (CK).

The dimeric procyanidin B3 was little influenced by protein fining with the exception of casein which removes 40 % of this compound. One can point out that, casein and potassium caseinate despite their similarities concerning surface charge densities and MW distribution (MW ≈ 30.0 kDa), showed different affinity to this compound (procyanidin B3). Addition of gelatines did not influence procyanidins B1. Procyanidin B4 was mainly removed by gelatine with a polydispersion above 43.0 kDa (GSQ) and potassium caseinate; however procyanidin B2 was mainly removed by casein.

Trimeric 2, trimeric procyanidin C1 as well as dimeric procyanidins esterified with gallic acid were decreased by all the fining agents.

These results are not in accordance with those of RICARDO-DA-SILVA et al. (1991a), which verified that none of these procyanidins are influenced by the addition of proteic fining agents in a young red wine. This could probably be explained by the high level of anthocyanins and tannins present in the wine elaborated from the Mourvèdre grapevine variety, as observed by RICARDO-DA-SILVA et al. (1991a). This high phenolic content can protect the smaller wine tannins from the action of fining agents. In contrast, MACHADO-NUNES et al. (1995) also verified that proteic fining agents decreases wine procyanidins.

Table 6.6 - (+) - Catechin, (-) - epicatechin, dimeric, trimeric and dimeric procyanidins esterified by gallic acid (mg/L) as analysed by HPLC for both fined and unfined red wines (means ± SD).

	Monomers		Dimers				Trimers		Dimer gallates		
Fining agent	**(+) -Catechin**	**(-) - Epicatechin**	**B3**	**B1**	**B4**	**B2**	**Trimer 2**	**Trimer C1**	**B2-3-*O*-gallate**	**B2-3'-*O*-gallate**	**B1-3-*O*-gallate**
T	31.4±0.3	18.1±0.5	7.0±0.0	32.0±0.7	12.7±0.1	19.0±0.9	7.3±0.1	4.9±0.3	2.5±0.8	1.9±0.0	1.6±0.2
GSQ	30.2±0.8	17.7±0.6	5.9±0.2	33.3±0.8	8.6±0.5	19.9±1.4	3.2±0.9	3.4±0.9	1.9±0.8	0.6±0.2	1.1±0.2
GL	29.3±0.1	17.5±0.9	6.3±0.6	33.2±0.8	12.7±0.1	20.1±0.8	4.2±0.3	3.8±1.2	1.0±0.3	0.5±0.2	1.0±0.4
CS	22.0±0.0	15.8±0.6	4.1±0.1	20.7±0.9	12.6±0.2	13.0±1.5	4.4±0.8	2.8±0.2	1.2±0.2	0.4±0.1	0.9±0.4
CK	26.4±0.5	16.0±0.9	6.0±0.5	26.8±0.3	10.9±0.3	17.0±1.6	3.8±0.8	2.2±0.2	1.0±0.1	0.9±0.2	1.0±0.2

Unfined (T), gelatine (GSQ), gelatine (GL), casein (CS), potassium caseinate (CK).

Monomeric anthocyanins, colour, pigments and chromatic characteristics

The following monomeric anthocyanins were separated by HPLC: Delphinidin-3-glucoside, Petunidin-3-glucoside, Peonidin-3-glucoside, Malvidin-3-glucoside, Malvidin-3-acetylglucoside and Malvidin-3-p-coumarylglucoside (Table 6.7). Addition of casein promotes the highest decrease of monomeric anthocyanins, but this decrease was very low. These findings agree with those of LOVINO et al. (1999) and COSME et al. (2007) who observed that fining red wine with casein decreases the level of monomeric anthocyanins.

Table 6.7 - Monomeric anthocyanin contents (mg/L malvidin-3-glucoside) for both fined and unfined red wine (means ± SD)

	T	GSQ	GL	CS	CK
Delphinidin-3-glucoside	6.1±0.7	5.7±0.1	5.5±0.1	5.8±0.5	5.9±0.1
Cyanidin-3-glucoside	n.q.	n.q.	n.q.	n.q.	n.q.
Petunidin-3-glucoside	8.0±0.2	8.1±0.3	7.8±0.2	7.5±0.9	8.5±0.1
Peonidin-3-glucoside	6.6±0.4	7.2±0.1	6.8±0.3	5.9±0.8	5.3±0.4
Malvidin-3-glucoside	82.7±0.3	82.6±0.1	82.2±0.8	79.8±0.5	82.4±0.3
Malvidin-3-acetylglucoside	18.3±0.6	17.7±0.2	17.2±0.2	17.4±1.1	18.5±0.1
Malvidin-3-p-coumarylgucoside	7.9±0.2	8.0±0.1	7.6±0.1	8.3±0.2	8.3±0.1
Σ monomeric anthocyanins	129.6±0.4	129.3±0.2	127.1±0.3	124.7±0.7	129.1±0.2

Colour intensity only decreased after addition of gelatine with a polydispersion below 43.0 kDa (GL) and the wine hue was not affected by protein fining (Table 6.8). These results are in accordance with the works of RICARDO-DE-SILVA et al. (1991a), MACHADO-NUNES et al. (1995), VERSARI et al. (1998), LOVINO et al. (1999) and PANERO et al. (2001.

Casein promotes a slight decrease in the total anthocyanin content of wine (Table 8). Fining agents also have a little effect on total pigments. Colour intensity and coloured anthocyanins are related, the decrease of coloured anthocyanins leading to a reduction of the colour intensity, as it was observed following the addition of gelatin with a polydispersion below 43.0 kDa (GL) (Table 6.8).

Determination of polymeric pigments gives an indication of the amount of anthocyanins combined with tannins. The lowest contents of polymeric pigments were found in wines fined with gelatine with a polydispersion below 43.0 kDa (GL).

Table 6.8 - Total anthocyanins, coloured anthocyanins, total pigments, polymeric pigments, colour intensity, hue and chromatic characteristics of both fined and unfined red wine (means ± SD).

Fining agents	Total anthocyanins (mg/L)	Coloured anthocyanins (mg/L)	Total pigments[a]	Polymeric pigments[a]	Colour intensity[a]	Colour hue[a]	L*(%)	a*	b*
T	228±16	59.3±1.3	16.5±0.6	3.0±0.2	11.1±0.03	0.62±0.01	68.6±0.4	32.34±0.28	0.03±0.25
GSQ	215±4	58.4±0.8	15.5±0.4	2.9±0.1	11.0±0.03	0.66±0.03	70.5±0.5	31.35±0.67	-0.28±0.18
GL	221±19	50.7±1.0	15.0±0.9	2.4±0.1	9.0±0.04	0.61±0.01	73.0±0.4	29.21±0.38	-0.61±0.01
CS	213±18	57.0±0.3	15.6±0.9	3.0±0.0	11.7±0.01	0.61±0.00	68.8±0.5	32.29±0.35	-0.09±0.06
CK	218±12	58.1±1.0	16.1±0.6	3.1±0.0	11.3±0.01	0.64±0.00	69.3±1.1	32.07±0.99	-0.08±0.12

Unfined (T), gelatine (GSQ), gelatine (GL), casein (CS), potassium caseinate (CK). L* - lightness, a* - redness, b*- yellowness, a - absornance units

The results obtained with the CIELab method for the chromatic characteristics of the unfined and fined red wine with diverse proteins showed that changes occurred after fining (Table 6.8). In wines fined with gelatine, lightness (L*) increased, which appears to be related with the low redness (a*) values due to the removal of pigments as earlier observed by GIL-MUÑOZ et al. (1997).

Effect of protein fining on sensory evaluation

Fig. 6.3 showed that the global appreciation was strongly correlated with astringency and aroma quality as well as aroma intensity and colour intensity. The wine fined with gelatine with a polydispersion above 43.0 kDa (GSQ) showed a colour hue, a taste intensity and quality that differentiate this wine from the other. However, the wine fined with gelatine with a polydispersion below 43.0 kDa was characterised as being the more astringent, with a higher aroma quality and a better global appreciation. The wine fined with casein showed more colour and more aroma intensity. The appreciation of the wine fined with potassium caseinate was very similar to the wine fined with gelatine with a polydispersion below 43.0 kDa (GL).

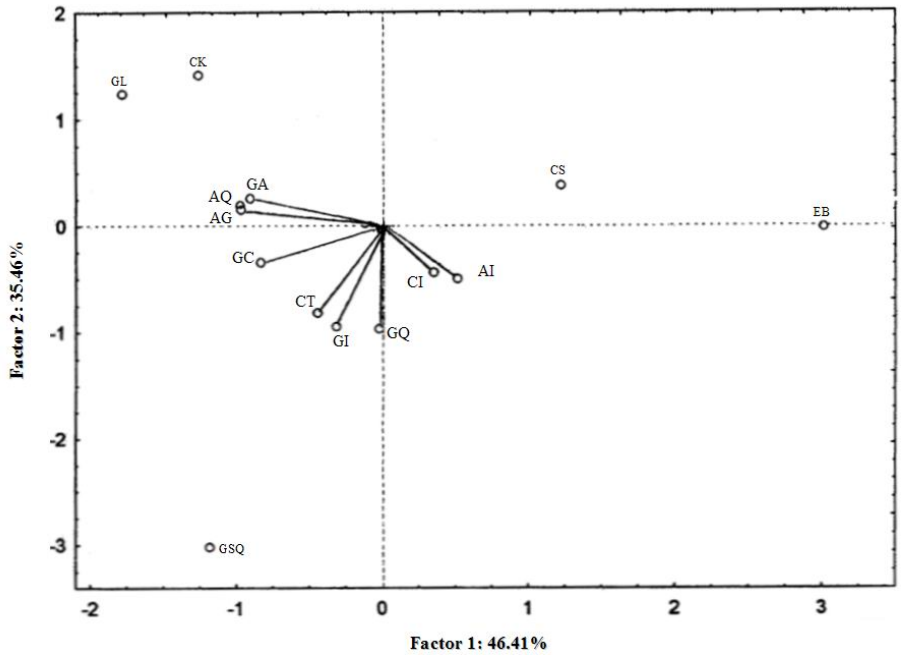

Fig. 6.3 - Principal components analyse of red wine. GL – gelatine, GSQ - gelatine, CS – casein, CK – potassium caseinate, ET – unfined wine. CI – colour intensity, CT – colour hue, AI – aroma intensity, AQ – aroma quality, GQ – taste quality, GI – taste intensity, GC – taste body, GA – taste astringency, AG – global appreciation.

6.4. CONCLUSIONS

The fining agents studied here presented a similar total nitrogen content (11-19% w/w). However, casein and potassium caseinate showed higher quantities of total protein, when compared to gelatines. They also present different molecular weight distribution. The electrophoretic pattern of gelatines was characterised by a polydispersion. However, gelatine GSQ is characterised by a polydispersion on the high molecular weight (MW > 43.0 kDa), contrary to gelatine GL that was characterised by a polydispersion on the low molecular weights (MW < 43.0 kDa). Casein and potassium caseinate were both characterised by a band at 30.0 kDa.

In white wine, the monomeric and oligomeric flavanol contents decreased after casein addition. However, polymeric proanthocyanidins in white wine and oligomeric and polymeric proanthocyanidins in red wine were more depleted by the gelatine characterised by a polydispersion below 43.0 kDa than by the gelatine characterised by a polydispersion above 43.0 kDa. These results show that, the same type of protein, as was gelatine, could influence in a different way the diverse flavan-3-ols.

Colour intensity and molecules linked with wine colour are less influenced by protein fining, but they could also be selectively decreased by a specific fining protein. For example, gelatine with a polydispersion below 43.0 kDa diminishes more intensively the colour intensity and the coloured anthocyanins.

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7. Reaction Between Protein Fining Agents and Proanthocyanidins with Different Degrees of Polymerization in Wine-like Model Solutions: Effect of Proanthocyanidin Structure, pH, Temperature and Concentration

Under internal discussion.

Reaction between protein fining agents and proanthocyanidins with different degrees of polymerization in wine-like model solutions: Effect of proanthocyanidin structure, pH, temperature and concentration

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ABSTRACT

In order to analyse the effect of proanthocyanidin chemical structure, pH, temperature and concentration on protein-proanthocyanidin interaction, fining trials were conducted in wine-like model solutions (12 % ethanol, pH 3.2, 20ºC). In each solution a proanthocyanidin fraction (obtained from *Vitis Vinifera* L. cv. Touriga Nacional wine extract) was mixed with commercial fining proteins (gelatin, casein, isinglass and egg albumin). Eight solutions were prepared, differing on the mean degree of polymerisation (mDP) of the proanthocyanidins. Furthermore, a fining trial with isinglass was carried out in wine-like model solutions (12 % ethanol) of pH 3.2 and 3.8 on three proanthocyanidin fractions (3.1, 5.1 or 9.5 mDP). After seven days at 10ºC or 20ºC the quantity of proanthocyanidins remained after fining was determined. In the case of isinglasses and gelatines it was observed a statistically significant correlation ($r=0.52$ and $r=-0.49$, respectively; $P < 0.05$) between the decrease of the proanthocyanidins (%) and the mDP of the proanthocyanidin fraction existed in the wine-like model solution. No correlation was observed for casein and egg albumin between the decrease of the proanthocyanidins (%) and the mDP of the proanthocyanidin fractions. Isinglasses and gelatines seem to deplete more the percentage of galloylation and the decrease was higher on the fractions richer in gallates. Gelatines were also the protein that more decreased the percentage of prodelphinidins. The results also revealed that the proanthocyanidin decrease after proteins (isinglass) fining is higher at 10ºC than at 20ºC. The proanthocyanidin concentration as well as the decrease of the pH in 0.6 pH units effect the quantity of proanthocyanidins remained after protein fining.

Keywords: fining, protein, fining agents, proanthocyanidins, thiolysis, pH, temperature.

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7.1. INTRODUCTION

Currently, a great diversity of protein fining agents is used in wine fining, such as: gelatine, casein, potassium caseinate, egg albumin or isinglass and, more recently some plant proteins (Marchal et al. 2000a; b; 2002; Panero et al. 2001; Maury et al. 2003). The various protein fining agents seems to behave differently.

Proanthocyanidins bind and precipitate most strongly proteins with a high content of amino acid proline in their sequence as it was shown by Hagerman and Butler (1981) using a competitive binding assay to compare the affinity of various proteins such as calfskin gelatine, polyproline, bovine serum albumin, hen ovalbumin and synthetic polymers for the tannin obtained from *Sorghum bicolor* (Linn.) Moench. Also, in studies using salivary proline-rich protein and plant polyphenol (galls of *Rhus semialata*) was suggested that proline residues form the key element of the binding site (Murray et al. 1994).

The interaction is mainly a hydrophobic association between proline residues and the aromatic rings of the tannins, but secondary hydrogen bonding effects help to stabilise the complex, as it was shown in woks using salivary proline-rich protein and plant polyphenol (galls of *Rhus semialata*) (Murray et al. 1994) or gelatine and tannic acid (Siebert et al. 1996).

Yokotsuka and Singleton (1987) in studies with gelatine and grape seeds tannin fractions (catechin or dimeric and oligomeric condensed tannin) observed a statistical significant correlation between the proline quantity and the tannin precipitation, while a high amount of amino acid proline contributes to a hydrophobic character of the protein. Nevertheless, the tannin binding site on proteins, is probably not only on amino acid proline. It was demonstrated that the protein-tannin interaction also takes place with proteins including a high quantity of histidine residues, such as human salivary histatins (Naurato et al. 1999) as well as with arginine and phenylalanine hydrophobic side chains (Murray et al. 1994, Charlton et al. 2002). The tannin-protein interaction also depends on protein structure. On linear proteins the aromatic groups of polyphenols are believed to be involved with the amino acid residues, whereas in globular proteins (compact secondary and tertiary structure) the interaction with polyphenols involved only surface exposed residues, therefore the affinity for proanthocyanidins to those last proteins is lower (Hagerman and Butler 1981, Haslam 1996, Baxter et al. 1997).

It was also suggested that the protein-proanthocyanidin interaction is stronger close to the isoelectric point of the protein, where protein-protein repulsion is diminished (Hagerman and Butler 1978). It was shown that proteins are precipitated by tannic acid or proanthocyanidins most efficiently at pH values close to the isoelectric point of the protein (Calderon et al. 1968, Van Buren and Robinson 1969, Hagerman and Butler 1980, 1981, Hagerman and Klucher 1986, Hagerman et al. 1998). Complexation is essentially a surface phenomenon increased at or close to the isoelectric point of the protein (Haslam 1996). Protein precipitation appears also to be favourable at low temperature (Kawamoto and Nakatsubo 1997). Luck et al. (1994) and Kawamoto and Nakatsubo (1997), considered that the reversible complexation of proanthocyanidins may be due to a process composed by two-stage and they believed that the protein amount influenced principally the first step whereas temperature, pH and ionic strength influenced mainly the last step.

One of the first studies performed on fining used three different phenolic fractions separated from grape seeds (catechins, proanthocyanidins and condensed tannins) in wine-like model solution with protein fining agents were carried out by Rossi and Singleton (1966). These authors found out that fining wine-like model solution with gelatine, isinglass or casein, the condensed tannins were most effectively removed than the monomeric catechins. These could probably be explained by the fact that the capacity of a protein type fining agent for phenol should depend on the number of potential hydrogen-bonding sites per unit weight and on the accessibility of the sites (Singleton 1967).

It was established that protein-proanthocyanidin interaction increased with an increase in the mDP of the proanthocyanidin fractions, as it was shown with must proteins (Yokotsuka et al. 1983), salivary proteins (Lea and Arnold 1978, Arnold et al. 1980, De Freitas and Mateus 2001), bovine serum albumin (Artz et al. 1987), gelatine (Ricardo-da-Silva et al. 1991; Yokotsuka and Singleton 1995, Sarni-Manchado et al. 1999, Maury et al. 2001), and with the portions of galloylated units of proanthocyanidins (Ricardo-da-Silva et al. 1991; Cheynier et al. 1997, Sarni-Manchado et al. 1999; Maury et al. 2001).

Charlton et al. (1996) and Baxter et al. (1997) demonstrated that human salivary proline-rich proteins interacted not only with proanthocyanidins with high mDP but also with monomeric flavanols. However, they also showed that the proanthocyanidins with high mDP interacted more strongly with human salivary proline-rich proteins than the proanthocyanidins with low mDP. Recently, Poncet-Legrand et al. (2006) found out that the structural composition of proanthocyanidins presented an important influence on protein-

proanthocyanidin interaction. Those authors showed that epicatechin and epigallocatechin did not form aggregates with poly (L-proline) however epicatechin gallate, epigallocatechin gallate and catechin form aggregates. Consequently, the protein-proanthocyanidin interaction depends on both proanthocyanidin and protein chemical structure (Zhu et al. 1997, Hagerman et al. 1998).

There have been a small number of works that studied the interaction of well characterised protein fining agents with proanthocyanidin fractions with diverse mDP. As far as we could know there is a lack of information on the quantity and structural characteristics (mDP, % gal and % prodelph) of proanthocyanidins remaining in wine-like model solutions after fining with different type of proteins as well as the influence of environmental factors (pH, temperature and concentration). An enhanced understanding of all the molecules implicated on fining and there behaviour at different environmental conditions could lead to an improved control and thus to an optimisation of this enological practice.

7.2. MATERIALS AND METHODS

Reagents

Toluene-α-thiol was purchased from Fluka (Buchs, Switzerland). Solvents and acids used were of HPLC grade.

Protein fining agents

The fining agents previously characterised by Cosme et al. (2007) and already used in wine studies (Cosme et al. 2007, 2008) were used in this work: one egg albumin (AS_1), two isinglasses (IL_1, IS_4), one potassium caseinate (CKS_1), one casein (CS_4) and three gelatines (GL_1, GS_2 and GS_4) (Table 7.1).

Preparation of proanthocyanidin wine extracts using LiChroprep RP-18

Wine extract was prepared from a 2004 *Vitis vinifera* cv. Touriga Nacional red wine, from the Tapada da Ajuda (Instituto Superior de Agronomia, Lisbon). The wine was concentrated by evaporation at <30 ºC under vacuum, and than pre-fractionated by chromatography on an open column (LiChroprep RP-18) using a method adapted from Sun

et al. (1999). The concentrated wine was loaded onto an open column packed with LiChroprep RP-18 (40-63 μm particle size, Merck, Darmstadt, Germany) previously conditioned with methanol, distilled water and phosphate buffer at pH 7.0. Phenolic acids were first eliminated by elution with 100 mL of phosphate buffer at pH 7.0, followed by 200 mL of methanol to elute the proanthocyanidins. The wine extract previously obtained was then purified using a C18 Sep-Pak cartridge (Sun et al. 1998).

Table 7.1 - Physic-chemical characteristics of the protein fining agents employed on the fining trial (Cosme et al. 2007).

Fining agents	Molecular weight distribution (kDa)	Quantity added	Surface charge density [a] meq/g product at pH 3.4	Protein content [a] as % Nxk (% w/w, dry weight)	Isoelectric point [a]
IL^{b}_{1}	Polydispersion below 20.1	50 mL/hL	0.04±0.00	112±4	4.55±0.02
IS^{c}_{4}	Bands above 94.0 between 94.0-43.0 and at 20.1	2.25 g/hL	0.41±0.01	73±3	6.48±0.03
CS_4	Band close to 30.0	40 g/hL	0.09±0.01	71±1	4.64±0.06
CKS_1	Band close to 30.0	40 g/hl	0.04±0.00	85±2	4.51±0.04
AS_1	Band close to 43.0	12.5 g/hL	0.73±0.01	78±1	5.00±0.02
GL_1	Polydispersion below 43.0	50 mL/hL	0.11±0.00	92±2	4.20±0.01
GS_2	Polydispersion above 43.0	8 g/hL	0.74±0.02	98±1	4.74±0.00
GS^{d}_{4}	No bands between 94.4 and 14.4	8 g/hL	0.26±0.00	91±4	4.50±0.00

Isinglass (**IL_1**), isinglass (**IS_4**), casein (**CS_4**), potassium caseinate (**CKS_1**), egg albumin (**AS_1**), gelatine (**GL_1**), gelatine (**GS_2**), gelatine (**GS_4**). k – Multiplication factor, which was 6.68 for egg albumin; 6.25 for isinglass; 6.38 for casein and potassium caseinate; 5.55 for gelatine. a - mean values of three determinations ± Standard Deviation (SD), b – obtained from collagen hydrolysis from fish skin, c – obtained from fish swim bladder, d – with high degree of hydrolysis.

Fractionation of wine extracted proanthocyanidins according to their degree of polymerisation using a sequential dissolving procedure on an inert glass powder column

The wine extracted proanthocyanidins were separated into eight fractions according to their degree of polymerisation following the method described by Labarbe et al. (1999). The elution (methanol/chloroform) gradient applied was the followed FI-25:75 (v/v); FII-30:70 (v/v); FIII-35:65 (v/v); FIV-40:60 (v/v); FV-45:55 (v/v); FVI-50:50 (v/v); FVII-55:45 (v/v); FVIII-100:0 (v/v).

Those tannin fractions were analysed by HPLC after thiolysis, to estimate their structural characteristics (mDP, % gal and % prodelph) and to determine their concentration (Table 7.2).

Table 7.2 - Quantity and structural characteristics (mean degree of polimerisation – mDP, percentage of galloylation -%gal, percentage od prodelphinidins - % prodelph) of proanthocyanidin fractions extracted from *Vitis vinifera* cv. Touriga Nacional wine (mean±SD).

Proanthocyanidin fractions	Concentration (mg/L)	mDP	%gal	% prodelph
FI	220.0±22.7	3.0±0.1	3.8±0.5	23.1±0.5
FII	44.6±4.1	3.7±0.2	8.9±0.1	6.7±0.3
FIII	34.7±3.2	4.4±0.2	7.8±0.5	16.9±0.3
FIV	77.3±3.8	5.0±0.1	5.3±0.3	14.1±0.7
FV	130.1±11.2	5.7±0.3	3.1±0.6	9.6±0.2
FVI	9.8±3.8	6.3±0.1	2.9±0.1	8.7±0.2
FVII	116.8±9.3	8.6±0.1	2.3±0.4	23.2±0.7
FVIII	102.9±7.5	10.2±0.2	8.7±0.1	11.4±0.3

The eight proanthocyanidin fractions were obtained according to the extraction and fractionation method previously described.

Preparation of the wine-like model solutions containing wine extracted proanthocyanidins with different mDP

The effect of protein fining agents with distinct physic-chemical characteristics on the interaction with different mDP proanthocyanidin fractions (separated from the wine extract) was studied in a wine-like model solution (12 % ethanol, pH 3.2, 20 ºC). Each wine-like model solution was prepared with absolute ethanol, potassium hydrogen tartarate, istilled water, dissolution of each proanthocyanidin fraction with distinct mDP (Table 2) and pH adjusted to 3.2 with HCl. Eight wine-like model solutions were obtained with 12 % ethanol, pH 3.2 and mDP ranging from 3.0 to 10.2. The wine-like model solutions presented the concentration and mDP similar to those existed in a tannic profile of a Touriga Nacional wine (Table 7.2).

Furthermore, other experiments with different environmental conditions were performed. So, proanthocyanidin fractions (mDP 3.1, 5.1 and 9.5) were, respectively dissolved in wine-like model solutions prepared as explained above at two concentrations for each fraction (200 mg/L and 100 mg/L; 70 mg/L and 35 mg/L; 100 mg/L and 50 mg/L, respectively) and at two pH (3.2 and 3.8) commonly encountered in wine, and they were kept at two controlled temperatures (10 ºC and 20 ºC).

Fining experiments on wine-like model solution

Experiments involved the addition of standard quantities of the protein fining agents prepared as suggested by the manufacturers (Table 7.1). The trials were conducted at laboratory scale in 20 mL volumes of wine-like model solutions (12 % ethanol, pH 3.2, 20 ºC). Untreated wine-like model solution was used as control. The fining agents (casein, potassium caseinate, egg albumin, isinglasses and gelatines) were thoroughly mixed and allowed to remain in contact with the wine-like model solution for 7 days at 20 ºC, the samples were then centrifuged at 4,000 rpm for 15 min before analysis. All experiments were performed in duplicate.

In addition, fining experiments in wine-like model solutions with different environmental conditions were carried out as mentioned previously. The trials involved the addition of standard quantities of isinglass (IL_1 and IS_4). Isinglass was chosen for these experiments because it is a fewer studied protein in fining experiments.

Characterisation of wine-like model solution proanthocyanidins by acid-catalysed depolymerisation in the presence of toluene– α-thiol followed by reversed-phase HPLC analysis

The acid-catalysed degradation was carried out according to Monagas et al. (2003) and the thiolysed sample were then analysed by reversed-phase HPLC. The equipment and elution conditions employed for analytical HPLC were the same used by Cosme et al (2008). The amounts of monomers (terminal units) and toluene-α-thiol adducts (extension units) released from the depolymerisation reaction in the presence of toluene-α-thiol, were calculated from the areas of the chromatographic peaks at 280 nm by comparison with calibration curves (Rigaud et al. 1991; Prieur et al. 1994, Kennedy et al. 2000).

Statistical analysis

The data are presented as means±SD. Correlations were performed using Statistica 6.1 software (StatSoft, Tulsa, OK, USA).

7.3. RESULTS AND DISCUSSION

7.3.1. Effect of proanthocyanidin structural characteristics (mDP, % gal and % prodelph) on the interaction with protein fining agents in wine-like model solution

The wines showed a great structural diversity of proanthocyanidins depending on the grape variety, winemaking technology and on the wine aging process (Dallas et al. 1995, Sun et al. 2001, Monagas et al. 2003, Cheynier et al. 2006). Therefore, proanthocyanidin fractions (ranging from 3.0 to 10.2) extracted from Touriga Nacional wine were added separately to diverse wine fining proteins. The physic-chemical characteristics of the proteins used in this assay are summarised in Table 7.1 and the structural characteristics of each one of the eight proanthocyanidin fractions are presented in Table 7.2.

Overall, gelatines were the proteins that more lowered the quantity of each proanthocyanidin fraction compared to the other proteins (Table 7.3). This observation was in accordance to Hagerman and Butler (1981) who found that proteins rich in proline presented the highest affinity for proanthocyanidins. In fact, from the four proteins studied gelatine is recognized to present the highest amount of amino acid proline (12.8 to 18.8 %) (Singleton 1967, Schreiber 1976, Ricardo-da-Silva et al. 1991).

It is to point out that only isinglasses and gelatines presented a statistically significant correlation ($r=0.52$ and $r=-0.49$, respectively; $P < 0.05$) between the decrease of the percentage of proanthocyanidins and the mDP of the proanthocyanidin fraction presented in the wine-like model solution. However, when the correlations are analysed separately for each isinglass, it was observed that only swim bladder isinglass (IS_4) presented a statistical significant correlation ($r=0.71$; $P < 0.05$). Isinglasses are characterised by an amino acid profile containing high lever of glycine and proline and almost unique on containing both hydroxyproline and hydroxylysine, nevertheless no differences on the amino acid composition were observed by several authors between isinglass obtained from swim

bladder and isinglass obtained from fish skin (Eastoe 1957, Gómez-Guillén et al. 2002). Likewise, if the gelatines correlations were analysed in separate, gelatine characterised by a polydispersion below 43.0 kDa (GL_1) do not evidenced a statistically significant correlation ($r=-0.27$; $P < 0.05$). In the case of gelatines, the statistically significant correlation observed was negative. Poncet-Legrand et al. (2003) verified that the mDP of grape seed tannins (3, 5, 8 and 15 mDP) had a complex effect, thereby it was observed that aggregation augmented with mDP, for molecular weight fractions with maximum at mDP 5, and the aggregation decreased for fractions with higher mDP (8 and 15 mDP). As suggested by Poncet-Legrand et al. (2003) this advised that the higher molecular weight fractions can assume a conformation that improved their solubility. The data for gelatines shown in Fig. 7.1 seems to be in accordance to the observations of those authors, as above an mDP of 5, the decrease of proanthocyanidins seemed to be less important. In addition, Ricardo-da-Silva et al. (1991) also observed that the trimeric procyanidins presented a loss close to 30 %.

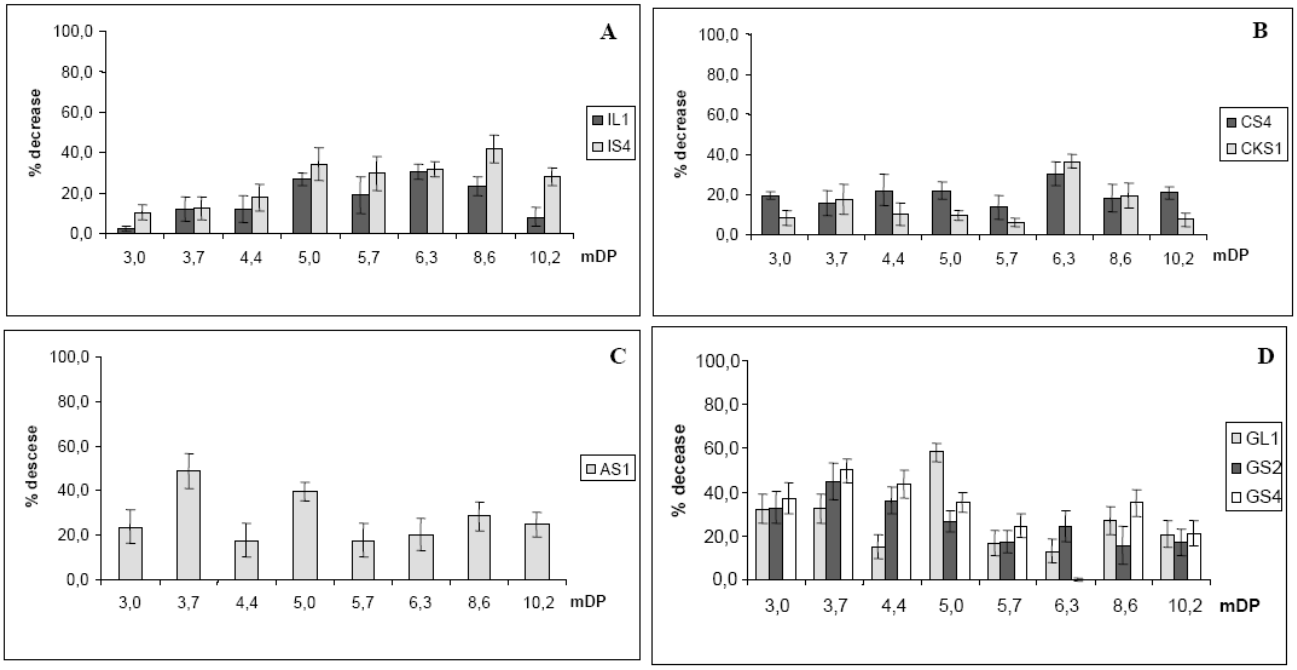

Fig. 7.1 - Quantity decrease (%) of the proanthocyanidin fractions with different mDP presented on each wine-like model solution fined with (A) isinglass, (B) casein, potassium caseinate, (C) egg albumin and (D) gelatine. Isinglass (IL_1), isinglass (IS_4), casein (CS_4), potassium caseinate (CKS_1), egg albumin (AS_1), gelatine (GL_1), gelatine (GS_2), gelatine (GS_4). The error bars indicated in the fig. represented the standard deviations.

The mDP of the distinct proanthocyanidin fractions remained in the fined wine-like model solutions decreased in all trials. These differences were related with the protein used (Table 7.3). Furthermore, it was observed, that frequently the proanthocyanidin fractions with higher mDP (8.6 and 10.2) showed a greater decrease on their mDP after fining.

Isinglasses, egg albumin and gelatines seem to deplete more the percentage of galloylation and the decrease was higher on the factions richer in gallates. These results are similarly to the observed by other authors (Ricardo-da-Silva et al. 1991, Bacon and Rhodes 1998) who showed that tannin with a high level of galloylation presented higher affinity for proteins such as poly-L-proline and salivary parotid proteins. The galloyl rings give additional aromatic cycles and favour hydrophobic complexation whereas the additional hydroxyl groups are sites for hydrogen bond formation (Maury et al. 2003). It is also to point out that for isinglass obtained from fish swim bladder (IS_4) and for gelatines characterized by a polydispertion on the low molecular weight (GL_1 and GS_4), a statistically positive significant ($P < 0.05$) correlation ($r=0.757$, $r=0.722$, $r=0.747$, respectively) was established between decrease of the percentage of galloylation and the fraction of gallates presented on the distinct proanthocyanidin solutions. Casein (CS_4) and potassium caseinate (CKS_1) were the fining agents that less decreased the percentage of galloylation. Nevertheless, on the less galloylated fractions (2.3; 2.9; 3.1 and 3.8 % gal) casein (CS_4) removes more gallates than potassium caseinate (CKS_1), in opposition potassium caseinate (CKS_1) was more effective on the fractions with higher percentage of galloylation (7.8 and 8.7 % gal) as shown on Figure 7.2.

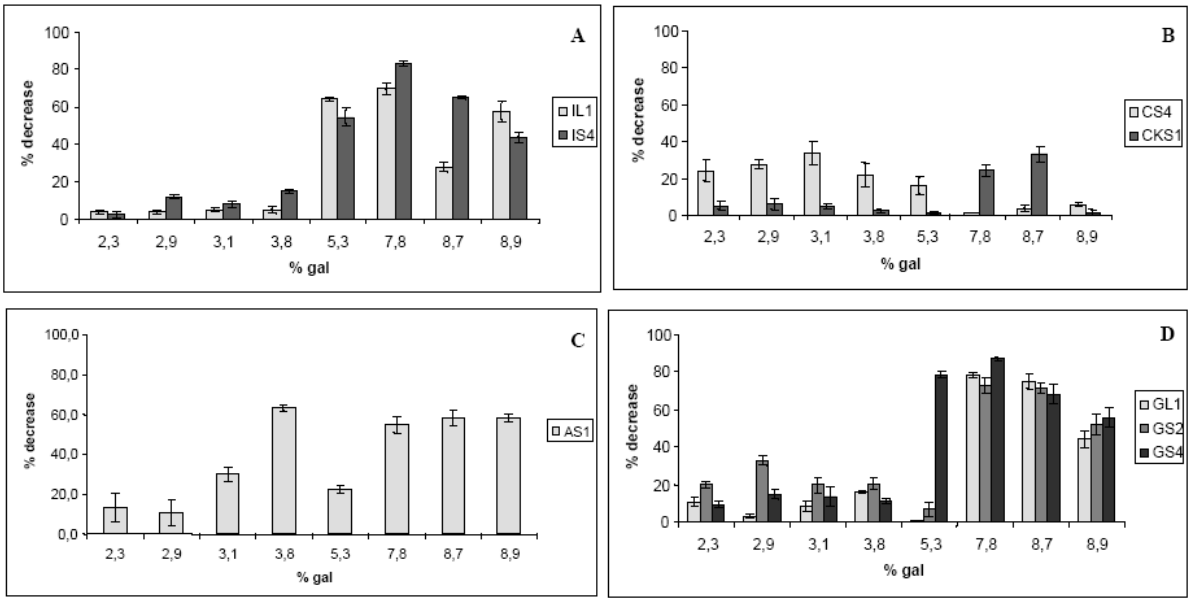

Fig. 7.2 - Galloylation decrease (%) of the proanthocyanidin fractions with different mDP on wine-like model solution fined with (A) isinglass, (B) casein, potassium caseinate, (C) egg albumin and (D) gelatine. Isinglass (IL_1), isinglass (IS_4), casein (CS_4), potassium caseinate (CKS_1), egg albumin (AS_1), gelatine (GL_1), gelatine (GS_2), gelatine (GS_4). The error bars indicated in the fig. represented the standard deviations.

The percentage of prodelphinidins (containing epigallocatechin units) on the diverse proanthocyanidin fractions remained after fining in wine-like model solution was lower for all the treatments; however the decreases observed on the percentage of prodelphinidins were lower than those observed for the other structural characteristics studied (Figure 7.3). From all the four types of fining proteins assayed gelatines were the fining agents that more decreased the percentage of prodelphinidins, and the reduction was higher on the fractions richer in those types of tannins (Figure 7.3). Regarding the two isinglasses tested, it was observed that both presented an identical behaviour in relation to the decrease of the percentage of prodelphinidins, a similar behaviour was also observed among casein and potassium caseinate.

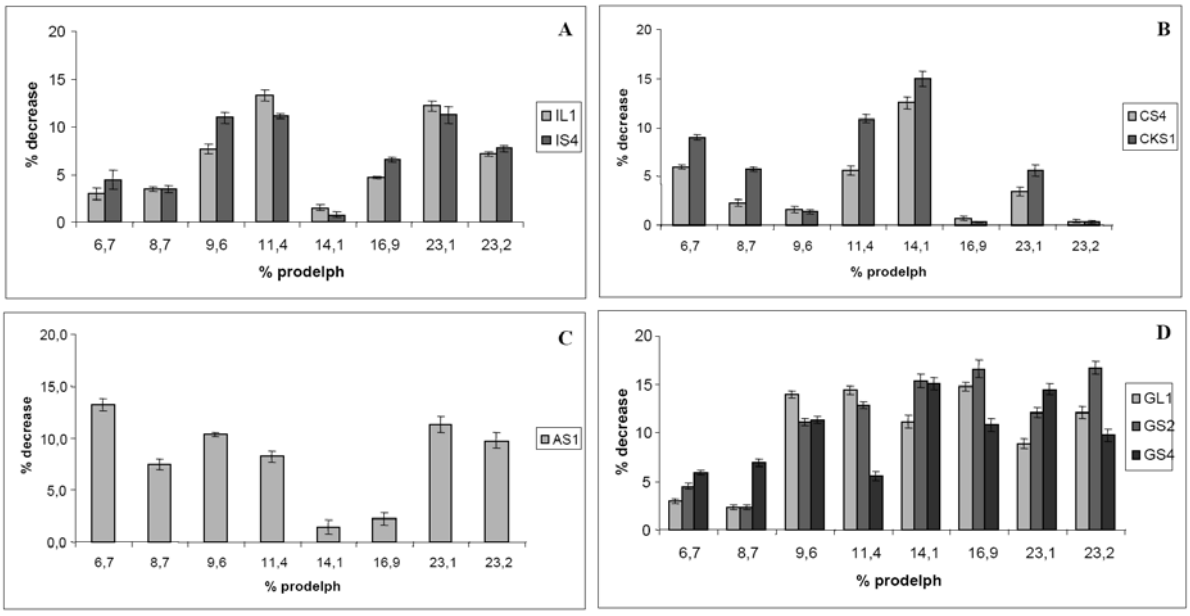

Fig. 7.3 -Prodelphinidin decrease (%) of the proanthocyanidin fractions with different mDP on wine-like model solution fined with (A) isinglass, (B) casein, potassium caseinate, (C) egg albumin and (D) gelatine. Isinglass (IL_1), isinglass (IS_4), casein (CS_4), potassium caseinate (CKS_1), egg albumin (AS_1), gelatine (GL_1), gelatine (GS_2), gelatine (GS_4). The error bars indicated in the fig. represented the standard deviations.

Table 7.3 - Concentration and mean degree of polymerisation (mDP) of the proanthocyanidin fractions of both fined and unfined wine-like model solutions (mean±SD).

	T		**IL_1**		**IS_4**		**CS_4**		**CKS_1**		**AS_1**		**GL_1**		**GS_2**		**GS_4**	
Fractions	**mDP**	**mg/L**	**mDP**	**mg/L**	**mDP**	**mg/L**	**mDP**	**mg/L**	**mDP**	**mg/L**	**mDP**	**mg/L**	**mDP**	**mg/L**	**mDP**	**mg/L**	**mDP**	**mg/L**
FI	3.0±0.1	220.0±22.7	2.8±0.2	214.2±19.9	2.5±0.2	196.7±18.0	2.4±0.6	177.5±15.9	3.0±0.1	202.1±19.6	3.0±0.4	167.9±14.3	2.0±0.3	148.9±20.1	2.6±0.2	147.6±13.0	2.3±0.4	138.0±11.6
FII	3.7±0.2	44.6±4.1	3.3±0.4	39.3±5.2	2.7±0.2	38.9±2.6	3.4±0.2	37.7±5.7	3.6±0.8	36.7±2.2	3.3±0.1	22.9±4.3	2.6±0.6	30.1±3.1	2.9±0.1	24.6±4.0	3.3±0.2	22.4±2.0
FIII	4.4±0.2	34.7±3.2	4.1±0.3	30.5±2.3	3.8±0.4	28.4±3.3	3.9±0.1	27.0±3.9	4.7±0.8	31.1±3.9	4.3±0.6	28.6±2.4	2.7±0.1	29.4±2.9	3.3±0.2	32.2±1.0	4.2±0.3	19.6±2.1
FIV	5.0±0.1	77.3±3.8	4.3±0.8	56.6±3.2	4.1±0.5	50.6±6.2	4.9±0.2	60.4±3.0	5.2±0.3	70.0±7.1	5.1±0.3	46.8±3.1	3.0±0.1	32.3±3.6	4.8±0.2	66.8±2.8	4.5±0.3	50.2±3.6
FV	5.7±0.3	130.1±11.2	5.2±0.6	105.2±9.2	4.4±0.2	91.3±7.6	5.7±0.2	112.5±8.1	5.6±0.3	122.5±7.6	5.7±0.6	107.2±18.0	4.8±0.3	108.2±10.1	5.3±0.4	107.8±9.0	4.7±0.2	98.0±8.0
FVI	6.3±0.1	9.8±3.8	5.9±0.2	6.8±1.0	4.8±0.3	6.7±1.1	6.1±0.6	6.8±1.0	5.8±0.6	6.2±0.9	6.3±0.9	7.8±1.5	5.9±0.4	8.5±2.0	6.4±0.1	7.4±2.8	5.7±0.6	10.6±1.3
FVII	8.6±0.1	116.8±9.3	7.3±0.5	89.4±4.4	6.4±0.9	67.7±5.9	7.5±0.1	95.6±6.7	6.7±0.1	94.2±4.9	6.5±0.7	83.6±6.2	7.7±0.1	85.1±6.3	7.8±0.2	98.5±9.1	6.5±0.3	75.9±6.5
FVIII	10.2±0.2	102.9±7.5	10.1±0.6	94.4±2.4	9.6±0.1	73.9±5.7	8.3±0.2	81.6±5.4	9.9±0.1	95.4±3.5	8.0±0.9	77.7±4.1	8.3±0.2	81.5±5.3	8.7±0.4	85.3±4.6	9.1±0.3	80.9±4.3

Unfined wine (**T**), Isinglass (**IL_1**), isinglass (**IS_4**), casein (**CS_4**), potassium caseinate (**CKS_1**), egg albumin (**AS_1**), gelatine (**GL_1**), gelatine (**GS_2**), gelatine (**GS_4**).

7.3.2. Effect of temperature, pH and proanthocyanidin concentration on the quantity of proanthocyanidins remained after fining with isinglass

Additionally, trials with diverse environmental conditions were carried out. Three proanthocyanidin fractions (mDP 3.1, 5.1 and 9.5) at two concentrations normally encountered in wine (200 mg/L and 100 mg/L; 70 mg/L and 35 mg/L; 100 mg/L and 50 mg/L, respectively) and at two pH (3.2 and 3.8) were kept at two controlled temperatures (10 ºC and 20 ºC). Two isinglass with different molecular weight distribution, isoelectic point, surface charge density and protein content were used for these assays (Table 7.1), since it is a less studied protein in fining experiments.

It was verified after fining with both isinglasses, a considerably higher decrease of the proanthocyanidins of each fraction at 10ºC than at 20ºC (Figure 7.4). The temperature effect on the gelatin-proanthocyanidin interaction was previously shown by Versari et al. (1999), who observed a higher removal at a temperature of 0 ºC than at 15 ºC. These observations could probably be explained by the fact that the affinities between proteins and proanthocyanidins become weaker as the temperature increased, as it was verified by Charlton et al. (2002). Protein precipitation appears also to be favourable at low temperature (Kawamoto and Nakatsubo 1997).

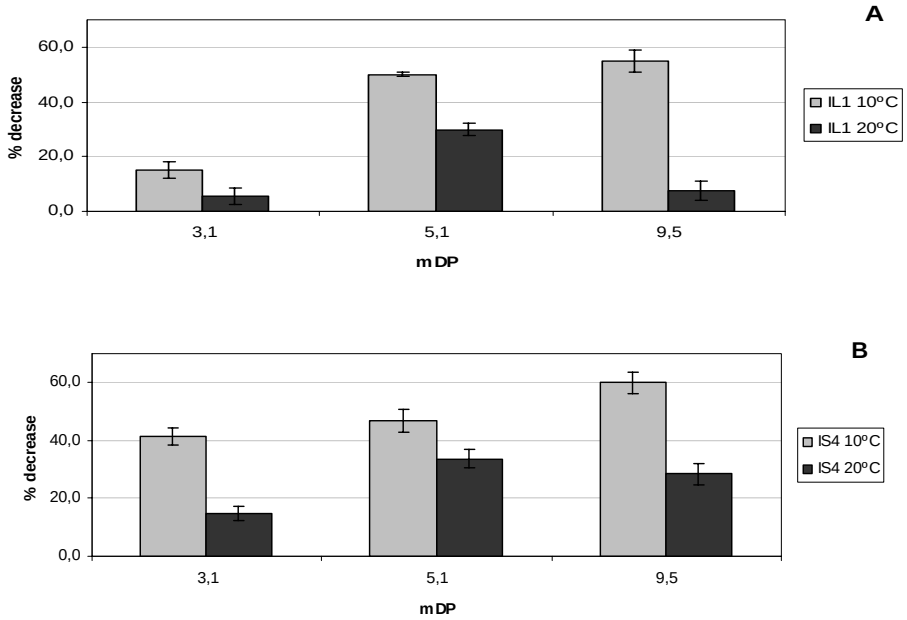

Fig. 7.4 - Decrease (%) of the proanthocyanidin fractions with different mDP on wine-like model solution fined with isinglass (A – IL_1, B – IS_4) at 10 ºC and 20ºC. The error bars indicated in the fig. represented the standard deviations.

Proanthocyanidin concentration of the fraction with mDP 3.1 do not influenced notably the proanthocyanidin decrease after addition of both isinglasses (IL_1 and IS_4). However, for the other proanthocyanidin fractions (mDP 5.1 and 9.5) assayed the percentage of the decrease observed was greater at the higher proanthocyanidin concentration (Figure 7.5). These results agree with Calderon et al. (1968), in that they showed that the amount of tannin-gelatine precipitation in influenced by the concentration.

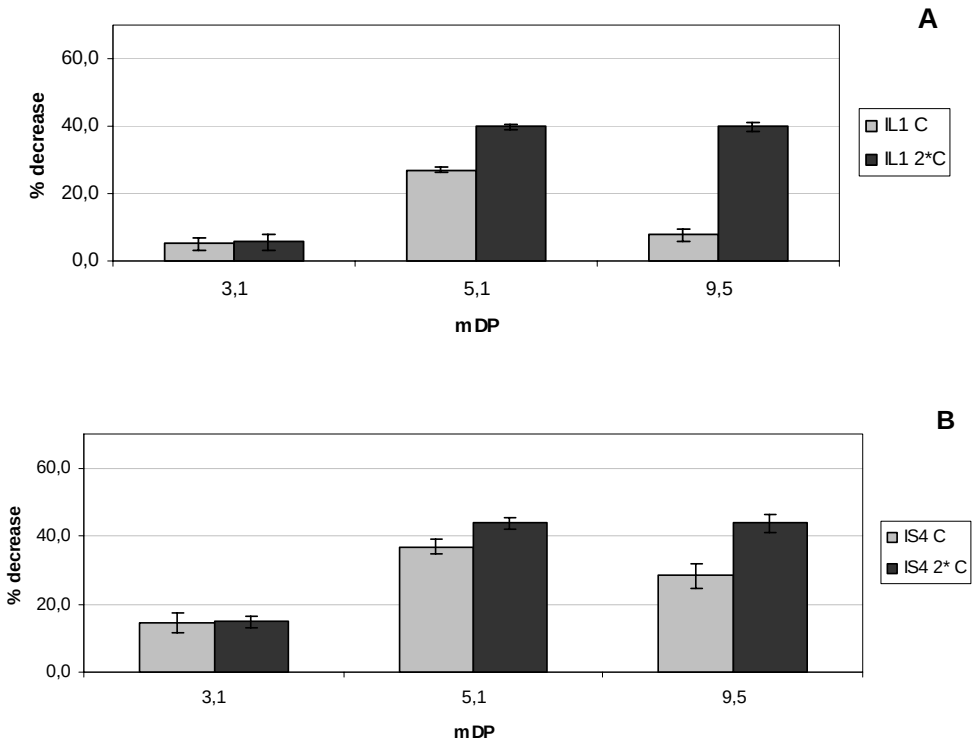

Fig. 7.5 - Decrease (%) of the proanthocyanidin fractions with different mDP at two concentrations on wine-like model solution fined with isinglass (A – IL_1, B – IS_4) at pH 3.2 and pH 3.8. The error bars indicated in the fig. represented the standard deviations

The pH effect on fining with two isinglasses (IL_1 and IS_4) is shown in Figure 7.6. Only for the proanthocyanidin fraction with mDP 9.5 in assays with isinglass obtained from fish skin and mDP 3.1 in trials with isinglass obtained from fish swim bladder, the decrease of proanthocyanidin shown was not considerably influenced by the pH value. Nevertheless, for the other proanthocyanidin fractions (mDP 3.1 and 5.1 – IL_1 mDP 5.1 and 9.5 - IS_4) the decrease observed was always higher at pH 3.2. These observation could probability be related to the isoelectic point of the protein and therefore at a lower pH, the protein

measured a higher surface charge density. This suggested that the protein isoelectric point could have an influence on the quantity of proanthocyanidins removed.

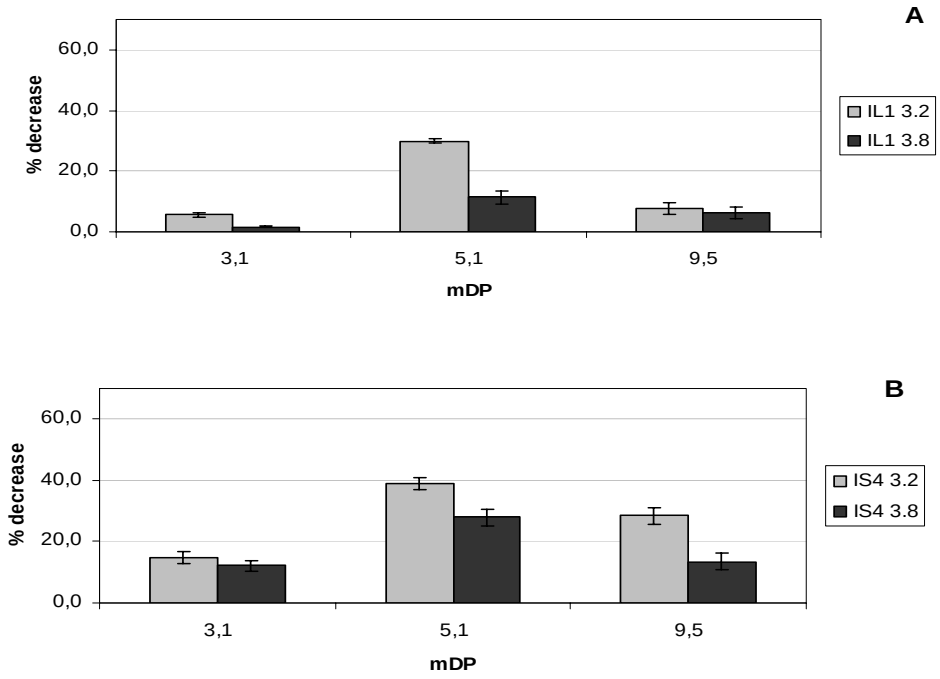

Fig. 7.6 - Decrease (%) of the proanthocyanidin fractions with different mDP at pH 3.2 and pH 3.8. on wine-like model solutions fined with isinglass (A – IL_1, B – IS_4). The error bars indicated in the fig. represented the standard deviations.

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8. GENERAL CONCLUSIONS

From the results obtained in this work the following conclusions can be achieved:

1. It was studied the tannic profile of five grapes (*Vitis vinifera* L. cv. Touriga Nacional, Trincadeira, Castelão, Syrah and Cabernet Sauvignon) and from their monovarietal wines.

It was observed that the concentration and the structural characteristics of the proanthocyanidins from grape seeds and skins differed among the *Vitis* V*inifera* L. cv grape varieties studied and that the quantity was always higher in seeds. In seeds and skins depending on the variety, the polymeric fraction represented, respectively, 77-85% and 91-99 % of the total proanthocyanidins.

The mean degree of polymerisation of the seed proanthocyanidins ranged from 2.8 to 12.8, however different distributions were observed among the varieties analysed. The percentage of galloylation ranged from 9.4 to 32.2 %, and it was observed that the degree of galloylation of the proanthocyanidins increased with an increase of the mean degree of polymerisation.

Skin proanthocyanidins differed from seed proanthocyanidins by their lower percentage of galloylation (2.3 to 7.3 %), higher mean degree of polymerisation (3.8 to 81.0) and presence of epigalhocatechin units (12.9 to 42.1 %). Also in the skins, was observed a different distribution among the varieties analysed of the different proanthocyanidin fractions.

In the monovarietal wines, the mean degree of polymerisation of the proanthocyanidins ranged from 2.1 to 9.6. The polymeric fraction represented 77-91% and 82-95% of the total proanthocyanidins, respectively, in vintage 2004 and 2005, but the concentration in 2005 was higher. The wine proanthocyanidins of Trincadeira and Cabernet Sauvignon, in the two vintages, showed a similar tannic profile. It is also to point out, that for the two vintages; wines from Castelão do not show proanthocyanidin fractions with mean degree of polymerisation lesser than 3 and the wines from Cabernet Sauvignon with mean degree of polymerisation above 7.

After 6 month, total proanthocyanidin concentration diminished clearly (39-59 %), and the distribution of the diverse proanthocyanidin fractions changed. The fractions with lower mean degree of polymerisation seems too polymerised and the fraction with higher mean degree of polymerisation seems to be lossen. On the structural characteristic was

shown that the percentage of prodelphinidin decrease slightly but the percentage of galloylation and the mean degree of polymerisation remained unchanged.

2. The second objective of this study was to characterise commercial fining agent products, and the following conclusions were obtained.

The diverse protein fining agents showed different physic-chemical characteristics such as molecular weight distribution and surface charge densities. These differences are not only verified between different proteins as it would be expected, but also in fining agents obtained from the same type of protein

Caseins and potassium caseinate products are characterised by a band at 30.0 kDa and egg albumins by a band close to 43.0 kDa. The electrophoretic patterns of several isinglasses were not similar. Swim bladder isinglass has bands at 20.1, between 94.0 – 43.0 and above 94.0 kDa. The other isinglasses analysed showed a polydispersion. Two of the gelatines studied do not have any band in the molecular weight range studied. The other gelatines studies showed a polydispersion on the lower molecular weight as well as on the higher molecular weight.

Egg albumin, issinglass and gelatine had shown higher surface charge densities when they are presented on their solid state. The highest surface charge densities were found in solid egg albumin and in solid gelatin characterised by a polydispersion above 43.0 kDa, which could be related with a lower degree of hydrolysis of these proteins. The isoelectric point of the proteins ranged from 4.20 to 6.48. All of the fining agents studied had acidic or almost neutral pH.

3. The characterised commercial protein fining agents were added to white and red wines in order to enhance the understanding of their action on wine chemical and sensory characteristics. The conclusions obtained were the follow:

With respect to limpidity, it was shown that proteins with higher surface charge density increased wine limpidity. A linear correlation was found between total surface charge density and decrease of turbidity.

Fining agents induced a reduction from 1.1 to 7.8 % on the total phenolic compounds of red wines. Among the gelatines studied differences were observed.

Therefore, gelatine characterised by a polydispersion on the low molecular weight removed considerably more total phenolic compounds from red wine than the gelatine characterised by a polydispersion on the high molecular weight. Regarding the non-flavonoid and flavonoid compounds, the gelatine characterised by a polydispersion below 43.0 kDa mainly reduced the non-flavonoid compounds in red wine while gelatine characterised by a polydispersion below 14.4 kDa mainly reduced the flavonoids, which seems to be related with the decrease in anthocyanins. Also differences were observed between the two isinglasses studied. Swim bladder isinglass exerted a greater effect on the total phenolic compounds than isinglass obtained from fish skin, by decreasing a major amount of non-flavonoid compounds. While casein and potassium caseinate have similar protein molecular weight distributions and isoelectric points, the results regarding the depletion of flavonoids and non-flavonoids in red wine differed. Casein mainly removed non-flavonoid compounds, while the potassium caseinate only induced a lesser decrease of these compounds.

In white wines the protein fining agents decreased the quantity of flavonoid (0.1-7.1%) and non-flavonoid (0.3-3.0%) compounds, however, significant decreases on flavonoids were only observed with casein and potassium caseinate and on non-flavonoid compounds with swim bladder isinglass and potassium caseinate.

The different protein fining agents which has been physic-chemically characterised were assayed on their effects on the flavones fractions in red and white wine - these having fractions with mean degrees of polymerisation of 4.9, 3.4 and. 1.5 in red wine and 3.8, 2.9 and 1.5 in white wines.

Proanthocyanidins with mean degree of polymerisation of 4.9, probably associated with astringency were removed notably by swim bladder isinglass, egg albumin and by the two types of gelatines characterised by a polydispersion on the low molecular weight. The two isinglasses showed distinct behaviours in relation to this fraction. Of these two proteins, only the isinglass obtained from fish swim bladder lowered these compounds considerably.

The three gelatines decreased the proanthocyanidins with mean degree of polymerisation of 3.8 significantly. Any of the isinglasses decreased the concentration of these compounds significantly. Casein diminished these compounds more than the twice as effectively as potassium caseinate.

The proanthocyanidins with mean degree of polymerisation of 3.4 were greatly decreased by egg albumin, casein and by the three gelatines studied. With both casein and potassium caseinate, the proanthocyanidins with mean degree of polymerisation of 3.4 tended to be removed but this effect was considerable only for casein. The isinglasses did not lower the concentration of these compounds noticeably.

In the case of proanthocyanidins with mean degree of polymerisation 2.9 the greatest decrease was observed with isinglass characterised by a polydispertion below 20.1, gelatine with low molecular weight distribution and casein.

Casein, swim bladder isinglass, and the low molecular weight gelatines significantly removed the monomeric flavanols, generally associated with bitterness. Casein and potassium caseinate showed an electrophoretic profile with similar molecular weight distribution (MW ≈ 30.0 kDa). However, their affinity for monomeric flavanols was different. Casein decreased these compounds significantly while this was not observed for potassium caseinate. The two isinglasses also showed different behaviours in relation to the monomeric flavanols. Of these two proteins, only the isinglass obtained from fish swim bladder decreased these compounds significantly. Egg albumin did not lower the monomeric flavanols significantly.

From the addition of the different protein fining agents to red and white wines could be conclude that the decrease observed depends on the fining agent but also on the mean degree of polymerisation of the proanthocyanidins fraction.

It was shown that the isinglasses with a polydispersion below 20.1 kDa, do not decreased the proanthocyanidins with a mean degree of polymerisation of 3.8 in white wine as well as the proanthocyanidin fractions with an mean degree of polymerisation of 3.4 in red wine. These observations suggested that the fining agents acted in function of the mean degree of polymerisation of the proanthocyanidins; independently they come from red or white wine.

However, it should also be noted that the knowledge of the electrophoretical profile of the fining agents is not enough, while fining agents with similar electrophoretical profiles (casein and potassium caseinate) showed different behaviours for some studied compounds, which suggested that other factors influenced the affinities of proteins for proathocyanidins.

The addition of protein fining agent to the red and white wine changed the structural characteristics of the proanthocyanidins remained after fining. Fining with protein fining

agents lowered the mean degree of polymerisation of the proanthocyanidins remaining in the fined red and white wine compared to the unfined wine. However, only the wine fined with egg albumin and isinglass obtained from swim bladder leads to a considerable decrease in the mean degree of polymerisation of proanthocyanidins remaining in the red fined wine. This allows us to admit that these fining agents could selectively remove proanthocyanidins with higher mean degree of polymerisation. The fining treatment with gelatines and egg albumin decreased appreciably the percentage of galloylation in the polymeric proanthocyanidins of red wine. The percentage of prodelphinidins within the polymeric proanthocyanidins fraction was notably lower for all the treatments, except when gelatine characterised by a polydispertion on the low molecular weight was employed in red wine.

A detailed HPLC analysis of the most important oligomeric proanthocyanidins, such as procyanidin dimers (B1, B2, B3 and B4), trimers (trimer 2 and C1) and dimer gallates (B2-3-*O*-gallate, B2-3'-*O*-gallate and B1-3-*O*-gallate) that are included in the proanthocyanidin fraction with mean degree of polymerisation 3.4 in red wine and 2.9 in white wine was performed and the main conclusion are shown.

The three gelatines employed in red wine, lowed all of the individual dimeric procyanidins, however none, were notably diminished by egg albumin. In the white wine was found that the three gelatines, egg albumin and swim bladder isinglass decreased all of the individual dimeric procyanidins, significantly, but not any of the individual dimeric procyanidins, were significantly decreased by the addition of potassium caseinate.

Regarding the individual trimeric procyanidins (trimer 2 and C1) in red wine, only gelatine characterised by a polydispersion above 43.0 kDa induced an important decrease of the trimer C1. A large decrease of the three dimeric procyanidin esterified by gallic acid was only observed by the use of gelatine characterised by a polydispersion above 43.0 kDa.

Overall, it was established that gelatines were the fining agents that more depleted the quantity of total dimeric and trimeric procyanidins in red wine, which agrees with the results found for the proanthocyanidin fraction with mean degree of polymerisation of 3.4.

Isinglass obtained from swim bladder and egg albumin decrease the quantity of total dimeric procyanidins esterified by gallic acid in red wine to a greater extent when compared with the corresponding nongalloylated procyanidins. Gelatine with a polydispersion above 43.0 kDa also showed a better effect on this type of molecule. However, this effect was not observed for all protein fining agents evaluated.

Treatment of white wine with gelatine characterised by a polydispersion on the low molecular weight and with isinglass obtained from fish skin depleted significantly the quantity of total dimeric procyanidins, the total trimeric procyanidins and the total quantity of dimer gallates – all compared with untreated wine. These fining agents were characterised by low molecular weight polydispersions (< 20.1 kDa). Potassium caseinate had no statistically different effect on these compounds which contrasted with casein which induced significant decreases in all oligomeric procyanidins. As expected, these observations are in accordance with the results obtained for the fraction with mean degree of polymerisation of 2.9.

HPLC analyses of the isomers (+) catechin, and (-) epicatechin, showed that the different fining agents had distinct ability in removing these two compounds. It was observed that the protein fining agents induced a higher decrease in (+) - catechin than in (-) - epicatechin. Apart from casein in red wine and swim bladder isinglass in white wine, none of the other fining agents decreased (-) – epicatechin appreciably. The (+) – catechin was considerably lowered by casein and by the three gelatines assayed in both wines.

Colour intensity and molecules related to wine colour were shown to be less influenced by protein fining agents than the proanthocyanidins. On the total monomeric anthocyanin concentration isinglasses and gelatine characterised by a polydispersion on the low molecular weight had the least effect and the highest effect were observed by casein and potassium caseinate. It is to point out that potassium caseinate diminished the quantity of total monomeric anthocyanins 66 mg/L and casein 116 mg/L, however these two fining agents had analogous electrophoretic profiles and isoelectric points. It is also to be noted that the decreases measured for casein were mainly due by a reduction in peonidin-3-glucoside and malvidin-3-glucoside. However, all these decreases were not statistically different.

The CIELab method evidenced that in each fined wines, lightness (L*) increased significantly, which seemed to be correlated with less redness a*, due to the removal of pigments. This data are in accordance with the results obtained for monomeric anthocyanins and for total and polymeric pigments. The colour difference (ΔE), between each wine and the unfined wine with exception of wine fined with fish skin isinglass all the others had values higher than one CIElab unit, indicating that the colour differences can be detected visually

White wine colour (expressed as the absorbance at 420 nm) and browning potential both showed a significant decrease with casein and with potassium caseinate as well as with swim bladder isinglass. The wines fined with casein, potassium caseinate and swim bladder isinglass were more stable to oxidation.

The increase of absorbance (A_{420nm}) produced by the browning test was less in these white wines. This effect is probably related to the facts that swim bladder isinglass and potassium caseinate reduced the non-flavonoid compounds significantly, while casein reduced the level of flavonoid compounds significantly. In contrast, the loss in white wine colour (A_{420nm}) was not significant for the gelatines.

In white wines fined with casein, potassium caseinate, isinglasses, egg albumin and gelatine with a polydispersion on the low molecular weight, the CIELab method showed that lightness (L*) increased significantly, suggesting a clarifying action of these fining agents. The values of chroma (C*) decreased significantly after the addition of casein and potassium caseinate. The colour differences (ΔE) obtained, between each fined and unfined wine, indicates that they could be discriminated visually. The largest values for colour variation ΔE^* were found for potassium caseinate and for casein, followed by both isinglasses.

The sensory evaluation of the white and red wines after addition of gelatine, casein and potassium caseinate was also performed and the following conclusion were obtained.

The white wine fined with gelatine with a polydispersion above 43.0 kDa, showed high aroma intensity. In contrast, the wine fined with gelatine with a polydispersion below 43.0 kDa, showed the best visual characteristics. Wines fined with casein and potassium caseinate showed the highest values for body and global appreciation.

The global appreciation in red wine was strongly correlated with astringency and aroma quality as well as aroma and colour intensity. The wine fined with gelatine with a polydispersion above 43.0 kDa showed a colour hue, a taste intensity and quality that differentiate this wine from the other. However, the wine fined with gelatine with a polydispersion below 43.0 kDa was characterised as being the more astringent, with a higher aroma quality and a better global appreciation. The wine fined with casein showed more colour and more aroma intensity. The appreciation of the wine fined with potassium caseinate was very similar to the wine fined with gelatine with a polydispersion below 43.0 kDa.

4. To study the effect of proanthocyanidin chemical structure, pH, temperature and concentration on protein-proanthocyanidin interaction, protein fining agents were added to proanthocyanidins differing on the mean degree of polymerisation in wine-like model solutions. The following conclusions were obtained.

Isinglasses and gelatines showed a statistically significant correlation (r=0.52 and r=-0.49, respectively; $P < 0.05$) between the decrease of the percentage of proanthocyanidins and the mean degree of polymerization of the proanthocyanidin fractions present in the wine-like model solution. Within the four protein types testes, isinglasses and gelatines decreases more the percentage of galloylation and the reduction was higher on the fractions richer in gallates. Gelatines were also the protein that more decreased the percentage of prodelphinidin.

In assayes carried out with isinglass was also observed that the proanthocyanidin decrease is always higher at 10ºC than at 20ºC. At a higher proanthocyanidin concentration, a greater decrease was shown for the proanthocyanidin fractions with higher mean degree of polymerisation. The pH did not influenced the decrease of proanthocyanidin fractions with higher mean degree of polymerisation after fining with swim bladder isinglass, and the proanthocyanidin fractions with lower mean degree of polymerisation after fining with isinglass obtained from fish skin.

Future research work

1) **Influence of characterised protein fining agents on the wine aromatic profile.** Study the existence of possible changes of aromatic compounds after fining and there perception/influence on sensory analysis.

2) **Influence of adding characterised protein fining agents before or during alcoholic fermentation on the wine characteristics.** Study the existence of possible changes on the wine composition related to the moment of the additon of the fining agent and consequently there perception on wine sensory analysis.

3) **Influence of fining with characterised protein fining agents on the final wine stability such as tartaric stability.** Study the existence of possible changes on the final wine stability related to the addition of the fining agent.

DIVULGAÇÃO DOS RESULTADOS NO ÂMBITO DESTA TESE

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