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ORIGINAL RESEARCH ARTICLE

Different effectiveness of protein fining agents when tested for interaction and precipitation with tannic acid, a seed polyphenol extract and seven wines

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ABSTRACT

Wine fining comprises any operation resulting in the cleaning, stabilisation and improvement of the organoleptic characteristics of wine. Fining agents, such as certain proteins of varying electrical charges, interact physicochemically with wine polyphenols in ways not yet fully understood with diverse effects on wine attributes. In this study, four widely used protein fining agents (casein, ovalbumin and two gelatins) were compared for their ability to interact with three different types of phenolic-rich solutions: a tannic acid solution, a wine grape seed extract and seven different varietal wines. A phenolic characterisation of both the seed extract and all the wines was carried out. The interaction between the protein fining agents and the phenolic solutions was evaluated by diffusion and precipitation tests on cellulose membranes and protein staining. All four protein fining agents interacted to differing extents by producing soluble and insoluble complexes with the tannic acid, grape seed extract and wines. In the wines, the interactions were found to not only depend highly on the protein composition of the fining agent, but also on the chemical composition of the wine. Notwithstanding, the observed phenolic differences between the wines could not fully account for the differential interactions between wine and the fining agents, thus indicating that the wine matrix as a whole may play a role in those interactions.

KEYWORDS: wine-fining, casein, ovalbumin, gelatin, tannic acid, tannin-protein interaction, polyphenols

INTRODUCTION

Fining in winemaking corresponds to any operation that results in the cleaning, stabilisation and improvement of the organoleptic characteristics of wine. This critical oenological process can occur spontaneously or with the addition of fining agents capable of removing the suspended particles in wine, such as proteins, polysaccharides, phenolic compounds, yeast and bacteria (Marchal *et al*., 2002). Depending on their characteristics, fining agents can be grouped as protein, mineral or synthetic agents (Zoecklein *et al*., 2001). Although the mechanisms of action of fining agents are not fully understood, the fining process would involve interaction between particles of different electrical charge, bond formation and absorption and adsorption processes. For example, protein fining agents are hydrophilic particles with a positive electrical charge and a strong affinity for hydrophobic negatively charged wine polyphenols. Interactions between polyphenols and proteins normally result in insoluble negative hydrophobic complexes that precipitate due to their density (Obreque-Slier *et al*., 2010a; Obreque-Slier *et al*., 2010b). On the other hand, phenolic compounds are a highly diverse group of secondary plant metabolites that provide wine with different sensory properties, such as colour, aroma, bitterness and astringency. Two major subgroups of phenolic compounds are recognized: non-flavonoids and flavonoids (Obreque-Slier *et al*., 2021). Non-flavonoids are characterised as having a diversely substituted benzene ring and they comprise the stilbene, benzoic acid and cinnamic acid groups. Flavonoids are made up of two benzene rings linked by a chain of three carbon atoms and they comprise the subgroups of flavonols (responsible for the yellow colour of white wines), anthocyanins (responsible for the bluish-red pigmentation of red grape skins and wines) and flavanols (commonly associated with bitter taste and astringency) (Ma *et al*., 2014; De la Fuente-Blanco *et al.,* 2017; Haslam and Lilley, 1988; Rossetti *et al*., 2009). Furthermore, proteins used as fining agents for wine constitute a highly diverse family of macromolecules. Despite current major efforts to identify more effective, culturally acceptable and innocuous fining agents, gelatins, ovalbumin and caseins, which are from animal sources, are still the most commonly used protein fining agents in winemaking (Río Segade *et al*., 2019; Deckwart *et al*., 2014; Versari *et al*., 2022, Kemp *et al*., 2022). Gelatins are partial hydrolysates of collagen from animal skin, tendon and bone by-products (Molina, 1994). The main general characteristics of these proteins are their aminoacidic composition mainly based on three aminoacids (glycine, proline /hydroxyproline and glutamic acid) and, hence their fibrilar non-globular structure (Fratzl *et al*., 1998). Gelatins have been found to reduce the polyphenolic content of white wines, thus providing higher stability in terms of oxidation and precipitation in the wine (Delanoe *et al*., 1988; Ribéreau-Gayón *et al*., 2006). In red wines, gelatins are used to improve the organoleptic characteristics of wine due to their clarifying effect, and to ensure the microbiological stability and the stability of the colouring matter (Flanzy, 2000). In a comparative study, a gelatin product comprising smaller polypeptides (< 43 kDa) was found to be more efficacious than other protein fining agents (including casein and a > 43 kDa gelatin product) in removing polymeric proanthocyanidins from white wine and oligomeric and polymeric proanthocyanidins from red wine, thus positively impacting certain sensorial attributes of these products (Braga *et al*., 2007). According to Castellari *et al*. (1997), fining treatment of commercial wines with gelatin reduces the concentrations of *trans*- and *cis*-resveratrol by 2-5 %. Ovalbumin, the main protein component of egg white, is widely used as a fining agent for high-end red wines (Llaudy *et al*., 2004; Hassam *et al*., 2011). This small phosphoglycoprotein (45 kDa) is used in wine fining operations due to its ability to interact with phenolic compounds. Martínez-Lapuente *et al*. (2017) reported that ovalbumin reduces the content of proanthocyanidins in Tempranillo, Garnacha and Graciano wines by around 14 and 23 %. Casein is a proline-rich phosphoheteroprotein accounting for about 80 % of the milk protein (Eskin and Goff, 2012). Bovine casein for oenological use consists of four protein monomers (αS1, 38 %; αS2, 10 %; β, 10 % and κ, 34 %) (Marchal and Waters, 2010; Atamer *et al*., 2017), and has low solubility at pH 4.6 and a micelle organisation (Cosenza *et al*., 2004; De Kruif *et al*., 2012). Casein is positively charged and it flocculates in acid media, such as wine, during which it adsorbs and removes suspended matter (Marchal and Waters, 2010). On the whole, the structural diversity of these widely-used protein fining agents can translate into functional diversity due to the potential for differing mechanisms of interaction between them and the also diverse family of wine phenolic compounds. However, the numerous and diverse factors that impact protein-polyphenol interactions (Chira *et al*., 2009; Obreque-Slier *et al*., 2010a; Obreque-Slier *et al*., 2010b; Fanzone *et al*., 2012; Obreque-Slier *et al*., 2021), together with a paucity of comparative experimental data from studies on either single proteins or simple polyphenols (Bennick, 2002; Llaudy *et al*., 2004, Bajec and Pickering, 2008), are partly responsable for the lack of clarity on the issue. This study aimed to compare four protein fining agents (pure ovoalbumin, pure casein and two commercial gelatins) in terms of their physicochemical interactions with nine polyphenol-rich products (a commercial gallotannin-rich product, a grape seed polyphenol extract, six red wines and one white wine).

MATERIALS AND METHODS

1. Materials

All seven wine varieties of *Vitis vinifera* L. [Sauvignon blanc (SB), Carménère (CR), Tempranillo (TP), Malbec (MB), Sangiovese (SG), Cabernet franc (CF) and Petit Verdot (PV)] were donated by Villaseñor Vineyards. The 12-year-old plants were planted in a 2 × 1 espalier pattern in the Cachapoal Valley of the O'Higgins Region in Chile (34°02'05.1"S; 70°42'18.2"W). The growing site, climatological conditions, physical properties of the soils and viticultural practices are described elsewhere (Obreque-Slier *et al*., 2021). The grape yield was 13000 kg/ha (± 1000 kg). In addition, grape seeds

were obtained from 20 plants of Carménère and 5 clusters of grapes per plant, which were randomly selected during the 2021 vintage. Two grapes per cluster were hand-collected to obtain 200-grape samples. Sampling was carried out 60 days after *veraison* (30 April). Protein fining agents were donated by different companies: Casein (Vinicas, batch 159687), Ovalbumin (Partner, batch 236574), Gelatin 1 (Rousellot, batch 40195011) and Gelatin 2 (Rousselot, batch P53U). Whatman(R) grade 1 cellulose sheets were obtained from Whatman Ltd (Maidstone, England). Pro-analysis grade reagents, solvents and HPLC standards were purchased from Merck (Darmstadt, Germany). Coomassie blue R-250 and tannic acid were acquired from Sigma-Aldrich (Saint Louis, MO, USA).

2. Equipment

For the analysis of low molecular weight phenols, an Agilent Technologies 1200 series High Performance Liquid Chromatograph was used (Agilent Technologies Santa Clara, CA., USA). The equipment comprised a model G1311A Quat pump, a model L-7455 aligned photodiode array detector and a model L-7200 automatic injector. For the spectrophotometric and titrimetric assays, a Shimadzu UV-1700 PharmaSpec UV-Vis spectrophotometer (Shimadzu Corporation, Kyoto, Japan) and a Hanna model pH 211 pH meter (Hanna Instruments, Santiago, Chile) were used respectively.

3. Fining agents

Casein, ovalbumin and gelatins (2 g each) were dissolved in distilled water (1000 mL) at 35 °C with constant mechanical stirring for 30 min (pH = 7.0 adjusted with 0.1N NaOH). Following centrifugation (800 g x 5 min), the supernatant was passed through a membrane disc filter with a pore size of 0.45 µm and a diameter of 25 mm. The absorbance of the filtrate at 280 nm was adjusted to 0.7 AU (1 mg/mL for bovine serum albumin as standard) with distilled water.

4. Tannic acid solution and grape seed extract

A stock solution of tannic acid (5 mg/mL) was prepared in distilled water at 20 °C with constant mechanical stirring for 30 minutes and pH was adjusted to 3.7 with 0.1N NaOH. The grapes that had been kept at -80 °C were thawed and the seeds were manually dissected with the aid of sterile steel forceps and then weighed. The phenolic compounds were extracted as described in detail by Izquierdo-Hernández *et al*. (2016). Briefly, 5 g of grape seeds were ground in a marble mortar and macerated with mechanical stirring for 1h at 20 °C in 100 mL of 1:1 v/v methanol/water at pH 3.0. Seeds were recovered by vacuum filtration through a cellulose membrane grade 1 and re-macerated under the same conditions in the presence of 100 mL of 4:1 v/v acetone/water at pH 3.0. Both macerates were mixed, centrifuged at 800 g for 10 min (4 °C) and the supernatant was filtered through a 0.45 µm pore size membrane. The extract was concentrated to up to 70 mL under reduced pressure at 30 °C. Distilled water was then added to produce 100 mL of the corresponding stock solution.

5. Wines

A common winemaking protocol was followed for all seven wine grape varieties in the study. Briefly, wine grapes of each variety were harvested manually (22.5 °Brix ± 0.5 °Brix), destemmed, crushed and macerated for 3 days at 8 °C in 10000 L stainless steel tanks (three tanks per variety). All tanks were inoculated with *Saccharomyces cerevisiae* (20 g/hL) and kept at 25-28 °C for 10 days (red wines) or 14-16 °C for 21 days (white wine). Then, spontaneous malolactic fermentation (only in red wines) was carried out for about 60 days (malic acid < 0.1 g/L). The free SO_2 level was adjusted to 30 mg/L and the wines were racked, cold stabilised and bottled. The wines were analysed after one month of storage at 4 °C.

6. General physicochemical parameters of wines

All seven wines were analysed using the following conventional methods, as described elsewhere (OIV, 2021): a) pH by potentiometry, b) titratable acidity using NaOH as titrant, c) alcoholic strength measured by volume, and d) reducing sugars using the Luff-Schoor method.

7. Analysis of polyphenols in wines and seed extract

All the wines and the seed extract were analysed using the following spectrophotometric and HPLC-DAD methods: a) total phenols by spectrophotometry at 280 nm (García-Barceló, 1990), b) total tannins using the methylcellulose precipitation method (Mercurio *et al*., 2007), c) total anthocyanins using the bisulfite discoloration method (García-Barceló, 1990), d) colour intensity and hue as described by Glories (1984), e) proanthocyanidin fractionation using C18 Sep Pak cartridges (Sun *et al*., 1998), f) low molecular weight phenols by HPLC-DAD chromatography using external standards (Obreque-Slier *et al*., 2021), and g) anthocyanins by HPLC-DAD chromatography using external standards (Obreque-Slier *et al*., 2021).

8. Interaction of fining agents with tannic acid, seed extract and wines

Diffusion and precipitation assays of binary mixes of each one of the fining agents in the study (casein, ovalbumin and gelatins) with polyphenolic solutions agents (tannic acid, seed extract and wines) were conducted (Obreque-Slier *et al*., 2010a). Briefly, a constant volume (500 µL) of each one of the fining agent-containing solutions was mixed thoroughly with a variable volume (range from 20 to 500 µL) of a stock solution of tannic acid or seed extract (prepared as previously described). Once distilled water had been added to the whole series of mixtures to obtain a volume of 1000 µL, they were left to stand for 5 min at 20 °C. Likewise, 500-µL aliquots from each fining agent-containing solution were mixed with progressively increasing volumes (25, 50, 75, 100, 125 y 150 µL) of the various wines and completed with distilled water to obtain a final volume of 1000 µL. After vortexing again, a horizontally positioned cellulose membrane was spotted with 15-µL aliquots from each particular mixture

of the different series in the study, which were allowed to diffuse. Several cellulose membranes were used per assay. Each membrane was dried under a light-lamp, fixed for 5 min in 5 % trichloroacetic acid, rinsed for 5 min in 80 % ethanol, stained for protein detection for 20 min in 0.5 % Coomassie blue R-250 dissolved in 45 % isopropanol/10 % acetic acid, and washed in 7 % acetic acid until a clear background had been obtained. After rinsing in distilled water the membrane was dried again under a light-lamp. In such diffusion assay on cellulose membranes, a blue-stained circular spot usually represents the diffusion area of the protein present in the binary mix. In a variant complementary assay, the binary mixtures were centrifuged at 800 g for 3 minutes and 15-µL aliquots were taken from each supernatant to be spotted onto a cellulose membrane (precipitation assay). The membrane was processed for protein detection as described above. Digital images of the blue spots on the membrane were obtained using an Epson 4855 scanner. The blue–stained diffusion area of protein distribution was measured using Image J 1.45 software. Each of the assays was performed at least three times.

9. Statistical Analysis

The different experimental treatments were subjected to an ANOVA analysis. When statistically significant differences were observed, a Fisher LSD multiple comparison test with a confidence level of 5 % was applied. Statistical analyses were processed using Info Stat software (Info Stat, 2018).

RESULTS

1. Interaction between protein fining agents and tannic acid: diffusion and precipitation assays

Figure 1 shows the interaction of a fixed volume (500 µL) of the fining agents in the study with increasing volumes (20-500 µL range) of the stock solution of tannic acid. The diffusion assay (Figure 1, spots 1A-2V) shows that in the absence of tannic acid (control condition), ovalbumin, casein and gelatins 1 and 2 diffused radially and homogeneously on the cellulose membrane (monophasic diffusion) (Figure 1, spots 1A, 1M, 1Y and 2K). Gelatins were less diffusive than the smaller proteins ovalbumin and casein. After the addition of increasing amounts of tannic acid to the fining agents, a progressive reduction in the total area of distribution/diffusion of the protein was observed. The diffusion area of ovalbumin decreased significantly (with respect to the corresponding control) after the addition of 100 µL of tannic acid (Supplementary data, Table S1). The monophasic mode of diffusion of ovalbumin in the control condition became biphasic (i.e., both a non-diffusible fraction or NDF surrounded by a strip of diffusible fraction or DF can be distinguished) with the addition of 240 µL of tannic acid (spot 1I). In the case of casein, the addition of 60 µL of tannic acid resulted in a significant reduction in the area of diffusion (Figure 1, spot 1P), and 180-240 µL resulted in the transition from monophasic to biphasic mode of diffusion (Figure 1, spots 1T and 1U). Volumes of tannic acid higher

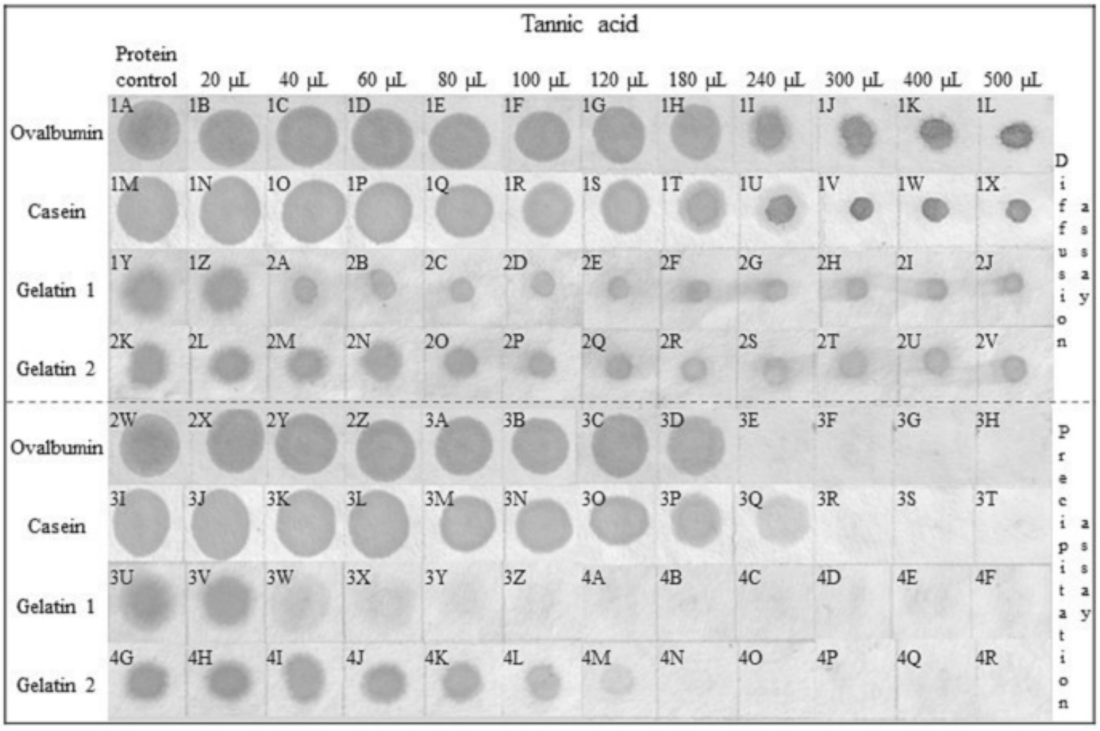


FIGURE 1. Effect of growing volumes of tannic acid on the diffusion (upper panel) and precipitation (lower panel) of protein fining agents on cellulose membranes. Numbers on top of the panel represent volumes of tannic acid mixed with a constant volume of stock solutions of ovalbumin, casein or gelatins.

than those found to be necessary to produce the monophasic/biphasic transition in the mode of diffusion of both casein and ovalbumin led finally to a fully non-diffusible tannic acid/fining agent binary complex (absence of DF). In the case of the less diffusible gelatins 1 and 2, the lowest dose of tannic acid in the assay (20 µL) produced a significant anti-diffusive effect. As with ovalbumin and casein, increasing the concentrations of tannic acid led to a complete suppression of gelatin diffusion. This effect was observed with much lower doses of tannic acid (Figure 1, spots 2C and 2P onwards for gelatins 1 and 2, respectively).

In the precipitation assay (Figure 1, spots 2W-4R), all the binary mixtures were centrifuged prior to spotting the membrane with aliquots from the supernatants; therefore, the absence of a spot can be interpreted as full precipitation having occurred. In the assay, the addition of 240, 300, 80 µL and 180 µL of tannic acid to a single amount of the fining agent resulted in the full precipitation of ovalbumin, casein, gelatin 1 and gelatin 2, respectively (Figure 1, spots 3E, 3R, 3Y and 4N). In this assay, much lower volumes of tannic acid produced a significant reduction in the area of diffusion displayed by the soluble or non-precipitated fining agent, compared to the volumes shown to be necessary to produce full precipitation of the corresponding protein (Figure 1, spots 2Y, 3L, 3V, 4J for ovalbumin, casein, gelatin 1 and gelatin 2, respectively) (Supplementary data, Table S2). This phenomenon can be accounted for by soluble fining agent-tannic acid complexes resulting from the interactions.

2. Interaction between fining agents and a grape seed extract

A seed extract from the grapes of the Carménère variety (*Vitis vinifera* L) was prepared and characterised as described in Materials and Methods. The HPLC-DAD analysis of the extract revealed the presence of monomeric flavan-3-ols ((+)-catechin and (-)-epicatechin) and oligomeric procyanidins, such as the B1, B2, B3 and B4 dimers and the C1 trimer. Only one non-flavonoid compound (gallic acid) was identified. Size exclusion chromatography showed that the polymeric fraction of proanthocyanidins was by far the most abundant in the extract (Table 1). In the diffusion assay, 500-µL aliquots of each of the stock solutions of the fining agents were mixed with increasing volumes (20-500 µL range) of the grape seed extract and completed to 1000 µL with distilled water (Figure 2, spots 1A-2V). Fifteen-µL aliquots from each mixture were applied on a horizontally positioned cellulose membrane. All four oenological products diffused radially on the membrane (Figure 2, spots 1A, 1M, 1Y and 2K). The addition of increasing volumes of the seed extract usually resulted in a progressive decrease in diffusion area of each of the fining agents used in the study - to such an extent that even the lowest volume of seed extract (20 µL) provoked a significant effect (Figure 2, spots 1B, 1N, 1Z y 2L) (Supplementary data, Table S3). The anti-diffusive effect of the seed extract on gelatin 2 was clearly higher than that observed for the other three protein fining agents. In all the cases, increasing amounts of seed extract produced a

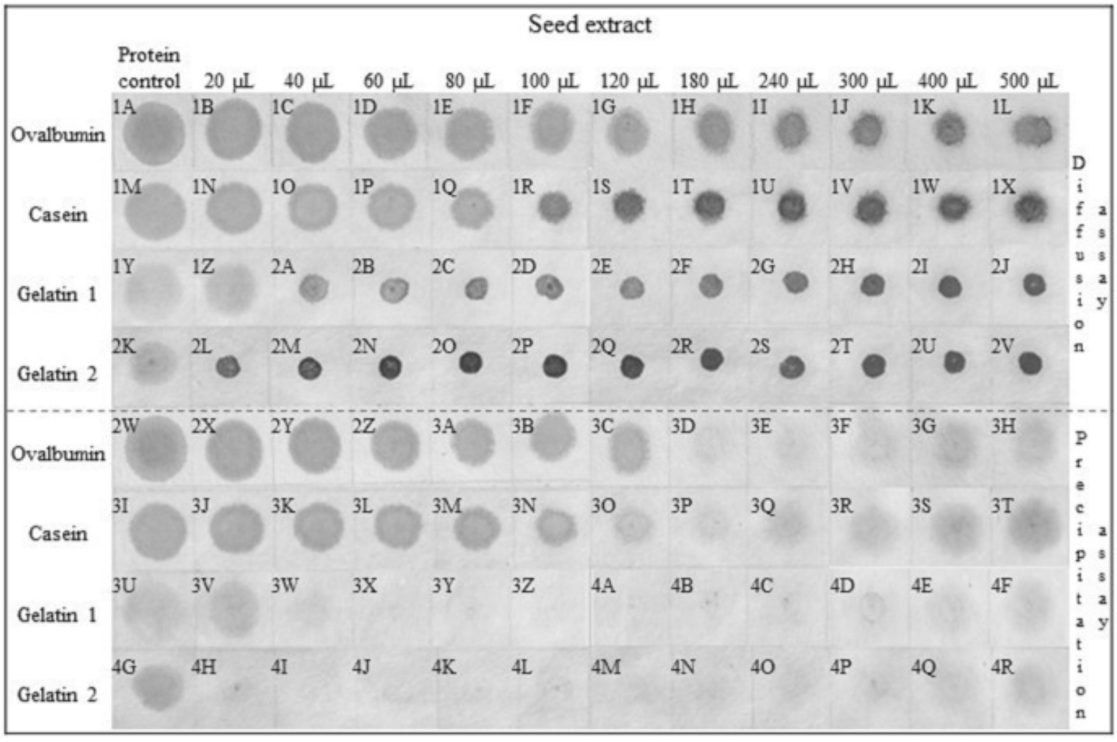


FIGURE 2. Effect of growing volumes of a seed extract on the diffusion (upper panel) and precipitation (lower panel) of protein fining agents on cellulose membranes. Numbers on top of the panel represent volumes of seed extract mixed with a constant volume of stock solutions of ovalbumin, casein or gelatins.

progressively darker and denser non-diffusible fraction (NDF) at the spotting site. In the cases of ovalbumin and casein, this behaviour was observed with the addition of around 100 µL of the extract (Figure 2, spots 1F and 1R), while in the cases of gelatin 1 and gelatin 2, 40 µL and 20 µL of seed extract, respectively, were needed (Figure 2, spots 2A and 2L). After centrifuging all the mixtures and spotting the membrane with an aliquot from each supernatant (Figure 2, precipitation assay, spots 2W-4R), only the spots corresponding to the control conditions (Figure 2, no mixing with the seed extract; i.e., spots 2W, 3I, 3U, 4G) showed no changes compared to the corresponding ones in the diffusion assay (Figure 2, spots 1A, 1M, 1Y, 2K). This indicates that no precipitation of the pure protein solutions occurred. By contrast, in this precipitation assay, a number of binary mixtures of a protein fining agent with the seed extract resulted in the complete disappearance of any spot revealed by the protein dye. Thus, precipitation was observed in the supernatants corresponding to ovalbumin (Figure 2, spot 3D), casein (Figure 2, spot 3P), gelatin 1 (Figure 2, spot 3W) and gelatin 2 (Figure 2, spot 4H) at different protein/seed extract (v/v) ratios. In addition, the series of spots in the precipitation assay corresponding to ovalbumin and casein (gelatins 1 and 2 were nonexistent due to full precipitation by addition of low volumes of the extract) also showed a progressive decrease in the area of diffusion, in connection with the volume of seed extract before full precipitation (Figure 2, from 2W to 3C for ovalbumin and from 3I to 3O for casein). This decrease can be accounted for by the presence of soluble complexes between the protein fining agent and phenolic components of the seed extract.

3. Interaction between fining agents and seven varietal wines

Wines from six red wine grape varieties (Sangiovese, Petit Verdot, Cabernet Franc, Tempranillo, Carménère and Malbec) and one from a white variety (Sauvignon blanc) were produced following a common protocol, as described in Materials and Methods. The phenolic characterisation of the wines is shown in Table 2. Some significant differences in the phenolic profiles of these wines were observed. Briefly, the SG and SB wines showed the lowest and PV and CF wines the highest pH values. Likewise, the SB wine showed the highest total acidity content. The alcohol content of most of the wines in the study was in the range of 12.5 % (v/v) (TM) to 14.0 % (v/v) (CR). The residual sugar content varied between 2.3 and 3.6 g glucose/L in red wines and 4.4 g glucose/L in the white wine. With regards to the phenolic content, the PV wine stood out in terms of its high concentrations of total phenols, total anthocyanins and mono-, oligo- and polymer fractions of proanthocyanidins. In the HPLC-DAD analysis, PV showed the highest concentrations of hydroxybenzoic acids, hydroxycinnamic acids, total non-flavonoids, flavanols, flavonoids and acetylated anthocyanins. Meanwhile, the CR wine had the highest concentrations of total tannins (together with CF), total flavonols and glycosylated anthocyanins. In contrast, the SG wine showed the lowest concentrations of phenols, tannins and total anthocyanins out of the red wines.

TABLE 1. Physicochemical characteristics of the grape seed extract.

	Concentration
Total phenols [1]	30.5 ± 6.6
Total tannins [2]	31.8 ± 9.0
Color intensity [3]	0.3 ± 0.1
Hue	2.4 ± 0.3
(-)-epicatechin [2]	1600.7 ± 991.6
(+)-catechin [2]	1838.2 ± 1646.5
Gallic acid [1]	70.6 ± 25.4
B1-3′-*O*-Gallate [2]	192.0 ± 115.6
B4-3′-*O*-Gallate [2]	75.9 ± 30.2
Epicatechin-3-*O*-gallate [2]	418.8 ± 418.8
Proanthocyanidin B1 [2]	94.2 ± 47.1
Proanthocyanidin B2 [2]	289.8 ± 90.7
Proanthocyanidin B3 [2]	131.2 ± 84.7
Proanthocyanidin B4 [2]	179.5 ± 80.1
Procyanidin gallates [2]	354.0 ± 234.0
Trimer C1 [2]	60.4 ± 27.4
Monomers of proanthocyanidins [2]	0.5 ± 0.3
Oligomers of proanthocyanidins [2]	4.4 ± 1.4
Polymers of proanthocyanidins [2]	46.0 ± 9.2

Figures represent mean ± standard deviation (triplicates).[1] mg gallic acid equivalent/Kg;[2] mg epicatechin equivalent/Kg;[3] Absorbance units.

TABLE 2. Physicochemical characteristics of seven wines in the study.

	Petit Verdot		Carménère		Malbec		Cabernet Franc		Tempranillo		Sangiovese		Sauvignon blanc	
pH	3.9 ± 0.0	a	3.7 ± 0.0	c	3.7 ± 0.0	c	3.8 ± 0.1	b	3.6 ± 0.0	d	3.5 ± 0.1	e	3.0 ± 0.0	f
Titratable acidity [1]	5.1 ± 0.1	cd	5.4 ± 0.2	b	5.3 ± 0.2	bc	4.6 ± 0.2	e	5.0 ± 0.1	d	5.0 ± 0.0	d	7.0 ± 0.1	a
Alcohol degree [2]	13.9 ± 0.5	a	14.0 ± 0.2	a	13.5 ± 0.2	b	13.9 ± 0.3	a	12.5 ± 0.1	d	13.4 ± 0.2	b	13.0 ± 0.0	c
Residual sugar [3]	3.5 ± 0.3	b	3.6 ± 0.6	b	2.6 ± 0.2	c	2.7 ± 0.1	c	2.3 ± 0.21	c	2.4 ± 0.2	c	4.4 ± 0.2	a
Total phenols [4]	2676.1 ± 10.2	a	1783.9 ± 0.8	b	1630.7 ± 3.6	d	1664.6 ± 14.5	c	1336.6 ± 10.1	e	1141.6 ± 18.8	f	190.2 ± 0.8	g
Total tannins [5]	1751.0 ± 8.6	c	2399.8 ± 47.1	a	1829.8 ± 52.8	c	2420.6 ± 26.8	a	1910.2 ± 64.7	b	1490.7 ± 21.5	e	214.1 ± 1.5	f
Total anthocyanins [6]	1153.9 ± 9.5	a	1097.5 ± 5.4	b	842.1 ± 23.8	c	753.0 ± 40.2	d	567.8 ± 39.4	e	413.9 ± 4.8	f	N.D.	
Color intensity [7]	19.7 ± 0.0	a	16.2 ± 0.5	b	10.2 ± 0.4	c	9.9 ± 0.15	c	6.9 ± 0.1	d	5.5 ± 0.0	e	0.8 ± 0.1	f
Hue	0.6 ± 0.0	cd	0.5 ± 0.0	d	0.6 ± 0.0	cd	0.7 ± 0.0	bc	0.7 ± 0.0	bc	0.8 ± 0.0	b	4.7 ± 0.2	a
Monomers of proanthocyanidins [5]	11.6 ± 0.4	a	3.0 ± 0.2	c	2.4 ± 0.5	c	5.4 ± 0.9	b	1.7 ± 0.0	d	2.6 ± 0.0	c	0.1 ± 0.0	e
Oligomers of proanthocyanidins [5]	50.1 ± 0.6	a	19.4 ± 1.2	c	21.2 ± 2.3	c	33.6 ± 6.7	b	18.1 ± 0.7	c	17.5 ± 0.8	c	0.2 ± 0.0	d
Polymers of proanthocyanidins [5]	711.2 ± 18.0	a	282.0 ± 16.5	e	356.7 ± 0.9	d	430.9 ± 5.6	b	387.6 ± 3.4	c	246.0 ± 6.3	f	3.7 ± 0.6	g
Total hydroxybenzoic acids [8]	59.8 ± 2.7	a	26.1 ± 1.6	c	25.6 ± 0.4	c	47.8 ± 1.9	b	28.6 ± 2.7	c	23.1 ± 6.8	c	3.0 ± 0.0	d
Total hydroxycinnamic acids [9]	41.4 ± 2.1	a	37.0 ± 3.8	abc	31.5 ± 2.2	c	33.7 ± 4.2	bc	35.8 ± 2.9	abc	40.7 ± 2.6	ab	8.8 ± 0.3	d
Total non-flavonoid phenolics [10]	139.7 ± 1.2	a	112.0 ± 9.8	b	82.3 ± 1.2	c	117.3 ± 4.3	b	82.7 ± 7.1	c	80.5 ± 11.2	c	5.9 ± 0.0	d
Total flavanols [5]	188.5 ± 4.7	a	92.8 ± 10.0	c	126.7 ± 3.3	b	119.4 ± 11.8	b	78.8 ± 1.8	c	50.1 ± 5.6	d	N.D.	
Total flavonols [11]	82.0 ± 5.2	ab	109.1 ± 22.6	a	69.8 ± 1.1	bc	72.7 ± 14.6	bc	51.3 ± 4.5	c	55.5 ± 4.4	bc	N.D.	
Total flavonoid phenolics [10]	270.5 ± 10.0	a	202.0 ± 32.6	b	196.5 ± 4.2	b	192.1 ± 26.4	b	130.1 ± 6.4	c	105.6 ± 10.0	c	17.7 ± 0.8	d
Glucoside anthocyanins [6]	260.1 ± 2.8	c	361.2 ± 1.8	a	269.9 ± 2.7	b	217.9 ± 0.6	d	188.8 ± 2.0	e	158.0 ± 1.5	f	N.D.	
Acetyl glucoside anthocyanins [6]	104.5 ± 1.4	a	56.1 ± 0.5	c	46.6 ± 0.8	d	69.9 ± 0.3	b	18.07 ± 0.38	e	7.2 ± 0.0	f	N.D.	
Coumaroyl glucoside anthocyanins [6]	24.4 ± 0.2	b	28.2 ± 0.5	a	29.2 ± 0.8	a	15.3 ± 0.4	d	17.6 ± 0.1	c	5.1 ± 0.2	e	N.D.	

Figures represent mean ± standard deviation (triplicates).[1] g tartaric acid/L;[2] % v/v;[3] g glucose/L;[4] mg gallic acid equivalent/L;5 mg epicatechin equivalent/L;[6] mg malvidin-3-O-glucoside equivalent/L;[7] Absorbance units;[8] mg gallic acid equivalent/L;[9] mg caffeic acid equivalent/L;[10] mg/L;[11] mg quercetin equivalent/L. Different small letters in single rows stand for statistically significant differences between wine varieties (LSD Fisher test, $p < 0.05$). N.D.: Not detected.

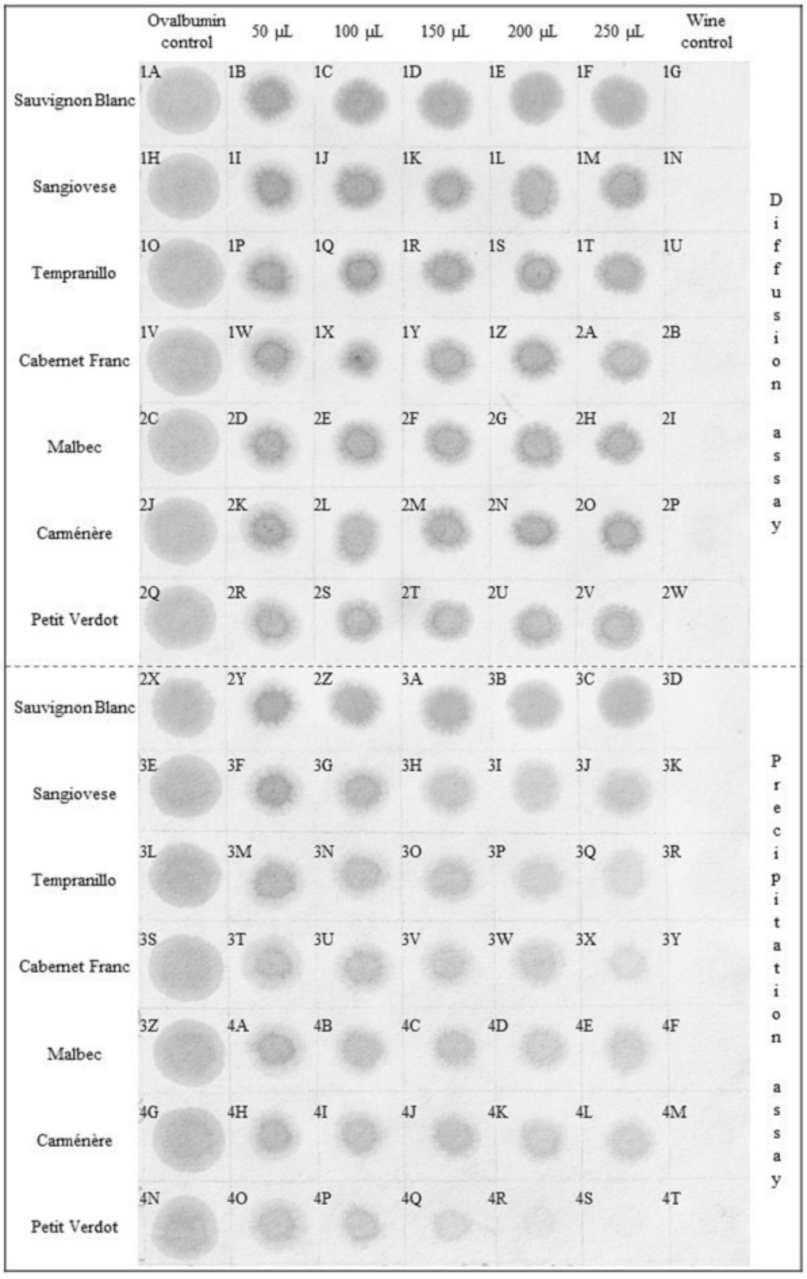


FIGURE 3. Effect of growing volumes of different wines on the diffusion (upper panel) and precipitation (lower panel) of a fix amount of ovalbumin on cellulose membranes. Numbers on top of the panel represent volumes of wine mixed with a constant volume of a stock solution of ovalbumin.

In terms of all the phenolic parameters, the lowest values were obtained for the SB wine, while average values were obtained for the MB and TP wines.

In order to assess the interactions between the wines and the protein fining agents, increasing volumes of wines (50-250 µL range) were added to a fixed amount of each of the four protein fining agents in the study (500 µL of the corresponding stock solution); distilled water was added to obtain a volume of 1000 µL (Figures 3-6). After approximately 20 min, a 15-µL aliquot taken from each mixture was placed on a cellulose membrane and processed as indicated in Materials and Methods (diffusion assay). In the assay of ovalbumin (Figure 3, spots 1A-2W; Supplementary data, Tables S5-S8), the addition of the lowest volume of any of the wines in the assay (50 µL) resulted in a drastic decrease in the diffusion of the fining agent, along with the appearance of a NDF in the centre of the spot (Figure 3, spots 1B, 1I, 1P, 1W, 2D, 2K and 2R). The addition of higher volumes of wine produced neither a further decrease in the area of protein diffusion nor an effect on the presence of a non-diffusible fraction. This assay on ovalbumin showed no major differences between red wines or between red and white wines. Regarding the diffusion assay on casein (Figure 4, 1A-2W; Supplementary data, Tables S9-S10), both similarities and differences with respect to ovalbumin were observed.

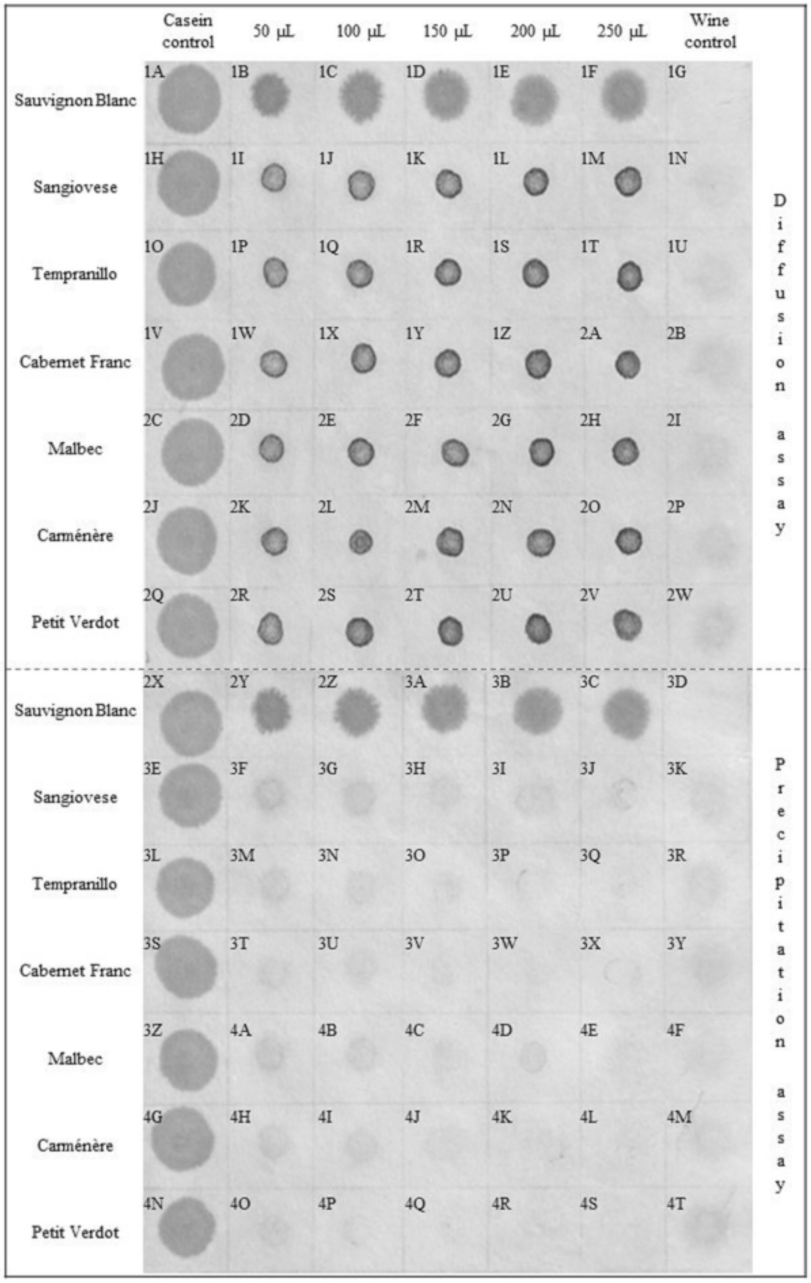


FIGURE 4. Effect of growing volumes of different wines on the diffusion (upper panel) and precipitation (lower panel) of fix amount of casein on cellulose membranes. Numbers on top of the panel represent volumes of wine mixed with a constant volume of a stock solution of casein.

Thus, the lowest volume of each wine (50 µL) was enough to fully suppress the diffusion of the fining agent (Figure 4, spots 1B, 1I, 1P, 1W, 2D, 2K and 2R). This time, the diffusion of casein in contact with SB was characteristically different from those of red wines (Figure 4, spots 1B-1F). Indeed, in the white wine a certain decrease in diffusivity or reduced compactness of the NDF were observed. By contrast, red wines put in contact with any volume of casein showed no diffusion whatsoever of the fining agent and, consequently, an apparently highly compact non-diffusible fraction. In the assay on gelatin 1 (Figure 5, 1A-2W; Supplementary data, Tables S11-S12), a significant restriction in protein diffusion was once again recorded, even in the lowest volume (50 uL) of any of the red wines or in the white wine (Figure 5, spots 1I, 1P, 1W, 2D, 2K, 2R and 1B). The addition of increasing volumes of any of the red wines resulted in a progressive, although diverse, decrease in the diffusion area of the fining agent. Such an effect was not observed for SB (Figure 5, spots 1B-1F). The diffusion assay showed that the mixture of gelatin 2 with the different wines had a differential anti-diffusion effect on the fining agent (Figure 6, 1A-2W; Supplementary data, Tables S13-S14). Indeed, 100-µL volumes of the PV, CR, MB, CF and TP wines were usually necessary to produce a significant restriction in the diffusion of this fining agent. By contrast, higher volumes of SG wine (200-250 µL) were necessary to

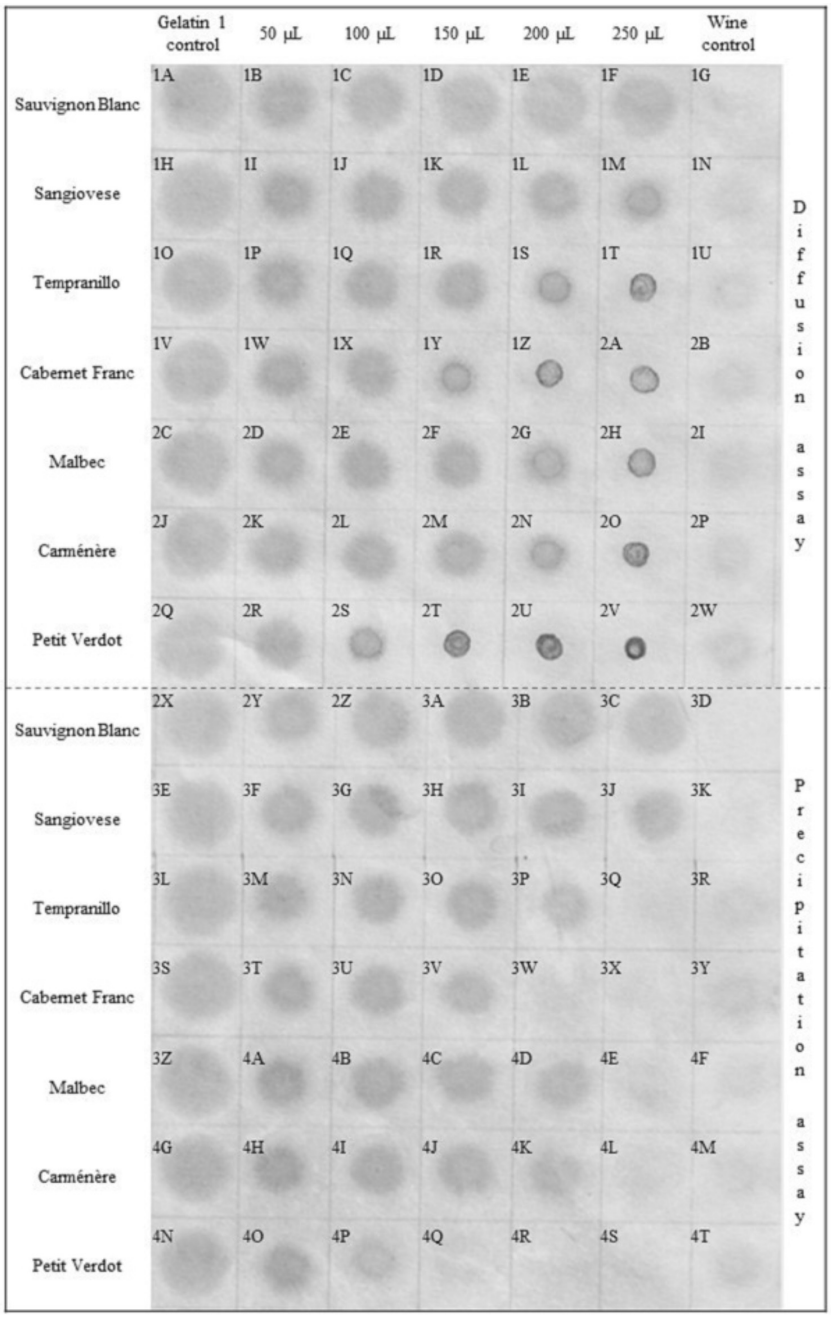


FIGURE 5. Effect of growing volumes of different wines on the diffusion (upper panel) and precipitation (lower panel) of a fix amount of gelatin 1 on cellulose membranes. Numbers on top of the panel represent volumes of wine mixed with a constant volume of a stock solution of gelatin 1.

produce a significant restriction in diffusion. In the case of most of the red wines, increasingly higher volumes resulted in progressive reductions in the diffusion area of the binary mixes, as well as in the progressive compaction of the NDF. The SB wine showed no anti-diffusive effect on gelatin 2 whatsoever. By contrast, spots corresponding to SB/gelatin 2 binary mixes showed progressively higher diffusion areas.

After the diffusion test, interactions between the protein fining agents and wines were assessed by means of a precipitation test. The wine-fining agent binary mixes were centrifuged and a 15-uL aliquot from each of supernatant was spotted on a horizontally positioned cellulose membrane (bottom panels of Figures 3-6; Supplementary data, Tables S5-S14). In the case of ovalbumin (Figure 3, 2X-4T), centrifugation showed no effect on the morphology of the spots corresponding to pure albumin in the diffusion test (Figure 3, spots 2X, 3E, 3L, 3S, 3Z, 4G, 4N), thus confirming the soluble character of the fining agent in the stock solution. By contrast, the spots of most of the binary mixtures became fainter compared to the corresponding spots in the diffusion assay, thus suggesting partial precipitation; for most of the wines, this was progressive and directly related to the content of wine in the binary mixture. The binary mixtures including white wine (SB) produced the darkest spots in the precipitation

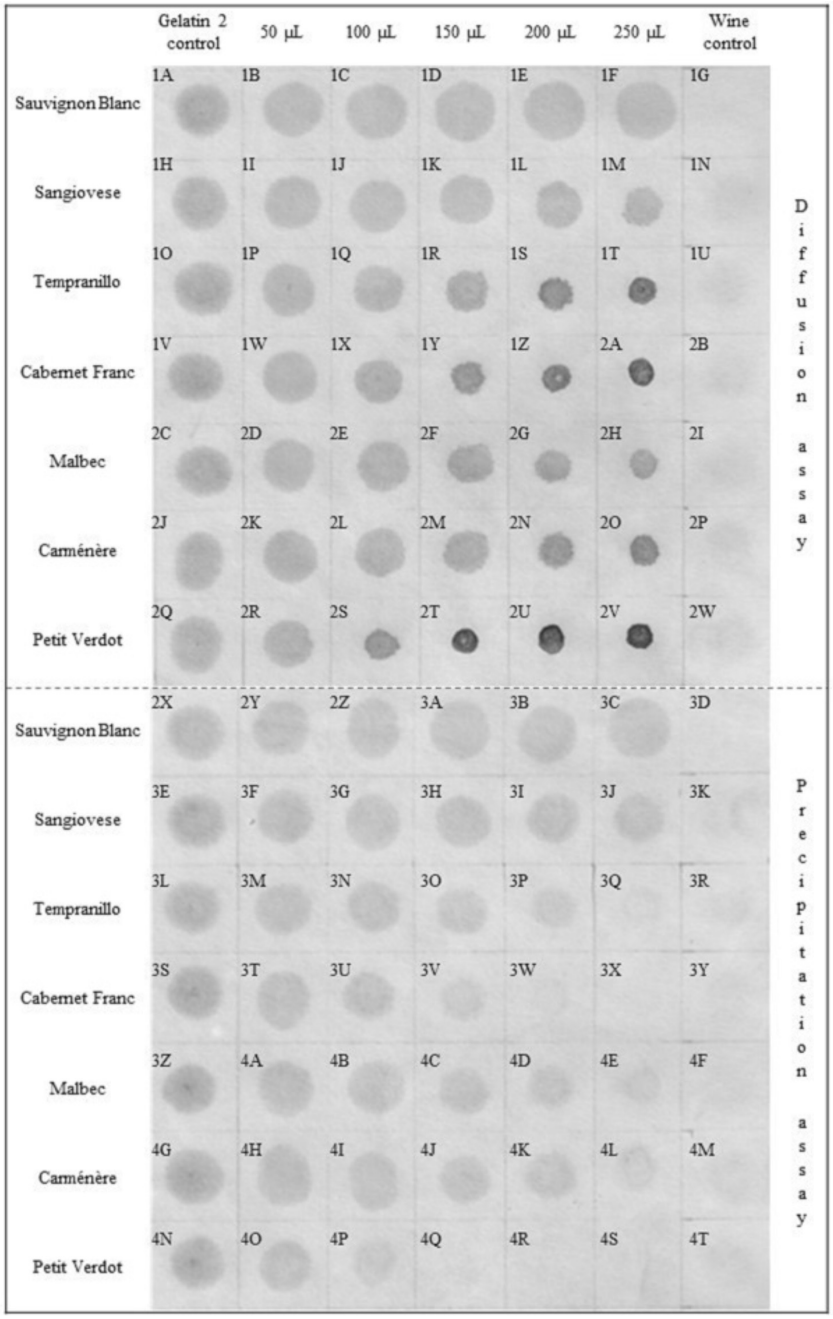


FIGURE 6. Effect of growing volumes of different wines on the diffusion (upper panel) and precipitation (lower panel) of a fix amount of gelatin 2 on cellulose membranes. Numbers on top of the panel represent volumes of wine mixed with a constant volume of a stock solution of gelatin 2.

assay. Furthermore, in this correlation, some spots in the precipitation assay disappeared (e.g., Figure 3, spots 4R, 4S, 4T from binary mixes containing PV), thus indicating that full precipitation had occurred. In addition, the precipitation test showed a common reduced diffusion of the spots from the binary mixtures containing any of the wines in the study. Such reduced diffusion, representing the occurrence of soluble ovalbumin-wine compound complexes, was significant, even in the spots from the binary mixtures containing the lowest volumes of wine (50 uL). The results of the precipitation test to assess the interaction between casein and the group of study wines (Figure 4, 2X-4T) showed that the fining agent had completely disappeared from the supernatants (i.e., precipitation had occurred), even for the lowest doses of all the red wines in the assay (Figure 4, spots 3F, 3M, 3T, 4A, 4H, 4O). By contrast, the spots from the binary mixtures containing SB white wine and casein remained mostly in solution (Figure 4, spots 2Y-3D). However, as can be seen in Figure 4, the reduced area of protein diffusion is indicative of soluble complexes comprising casein and some wine compounds. These complexes formed even with the lowest volumes (50 µL) of white wine in the assay (Figure 4, spots 2Y, 2Z, 3A, 3B and 3C). In the precipitation assay on gelatin 1 (Figure 5, 2X-4T), the complete disappearance of the

protein fining agent from the supernatant was observed with 150 µL (or more) of PV wine (Figure 5, spot 4Q), 200 µL (or more) of CF wine (Figure 5, spot 3W) and 250 µL of CR, MB or TP wines (Figure 5, spots 4L, 4E, and 3Q, respectively). Full precipitation of gelatin 1 was not achieved, even with the highest doses of SB or SG wines in the assay. In the assay, the significant presence of gelatin 1/wine soluble complexes (restricted diffusion of samples taken from supernatants) was observed even for the lowest doses of any of the wines in the assay (Figure 5, spots 2Y, 3F, 3M, 3T, 4A, 4H and 4O). In the precipitation test for gelatin 2 (Figure 6, 2X-4T), complete precipitation of the fining agent was observed with 150 µL of PV wine (Figure 6, spot 4Q), 200 µL of CF wine (Figure 6, spot 3V) and 250 µL of TP wine (Figure 6, spot 3Q) whereas 250-µL of CR and MB wines resulted in an almost complete precipitation of the fining agent (Figure 6, spots 4L and 4E, respectively). The same maximum volume of SG and SB (250 µL) failed to produce any precipitation of gelatin 2 (Figure 6, spots 3J and 3C, respectively). Likewise, the apparent lower reactivity of these latter wines with gelatin 2 was confirmed by the complementary measurement of the diffusion area of the spots produced by the supernatants of the corresponding binary mixtures. Thus, a significant decrease in diffusion (soluble protein fining agent/wine compounds) was observed only after the addition of at least 100 µL of any of the red wines. Moreover, in this assay the SB white wine showed no significant decrease in the diffusion area of gelatin 2 over the whole range of volumes (Figure 6, spots 2Y-3C).

DISCUSSION

Protein-based fining agents have traditionally been used in the wine industry with the aim of improving wine stability, eliminating some sensory defects and increasing the performance of clarification and filtration operations (Delanoe *et al*., 1988). Additionally, these products have been widely used to analyse interactions between polyphenols and proteins and hence their effects on astringency (Llaudy *et al*., 2004; Bajec and Pickering, 2008; Ma *et al*., 2014; Obreque-Slier *et al*., 2010a; Obreque-Slier *et al*., 2010b; Olatujoye *et al*., 2020). In this study, the quantitative interactions between approximately equivalent masses of some widely used protein fining agents (ovalbumin, casein and two gelatins) and growing volumes of stock solutions of three polyphenol-rich agents (tannic acid, a grape seed extract and seven varietal wines) were compared. Single-volume spots on cellulose membranes (15 uL), which had been revealed using a protein dye in all of the experimental conditions (volume-to-volume binary mixtures) were examined with the naked eye and their digital images characterised morphometrically (Image J software). The physicochemical interaction between a protein fining agent kept in solution (stock solution) and a given phenolic compound also in solution was associated with the reduced ability of the protein to diffuse on a cellulose membrane when in contact with a series of increasing amounts of the phenolic compound (diffusion test). The physicochemical interaction between both types of compounds was also associated with a reduced ability of the protein to remain in solution when in contact with a phenolic compound (precipitation test). Overall, marked differences between the protein fining agents in the study were observed. For instance, in decreasing order, the volumes of a single stock solution of tannic acid found to be necessary to produce first a significant reduction in the diffusion area of the protein and then the appearance of a non-diffusible fraction were ovalbumin > casein > gelatin 2 = gelatin 1. Likewise, also in decreasing order, casein > ovalbumin > gelatin 2 > gelatin 1 was the observed order in terms of volume of stock solution of tannic acid necessary for the complete precipitation of the standard amount of protein fining agent in the assay. Thus, the effectiveness of the interaction between the fining agent and tannic acid in reducing diffusion on the cellulose membrane can be arranged in the following decreasing order: gelatin 1 = gelatin 2 > casein > ovalbumin. Likewise, the effectiveness of tannic acid in terms of producing precipitation of the fining agent was found to be (in decreasing order): gelatin 1 > gelatin 2 > ovalbumin > casein. Such putative differential reactivities between the various protein fining agents with the same gallotannin may be due to differences in the size of the proteins (gelatins are usually much bigger and thus easier to precipitate), or in their affinities for the polyphenol under the experimental conditions of the study. In this regard, gelatins are a mixture of relatively large fibrilar proline/glycine/glutamine-rich protein products that are derived from the partial hydrolysis of collagen. Their aminoacidic primary structure (high content of reactive proline-residues and minor steric hindrance by glycine) resembles that of the major family of salivary proline-rich proteins that display high affinities for polyphenols (Harrington and Von Hippel, 1962; Bennick, 2002; Marchal and Waters, 2010). The findings of the diffusion assay in the present study match those of the precipitation assay, indicating that gelatins are clearly more effective as reactants for tannic acid compared to ovalbumin and casein. An identical comparative analysis of the interaction of the same four protein fining agents with a single grape seed phenolic extract by means of the diffusion assay showed that their ranked effectiveness was gelatin 2 > gelatin 1 > casein > ovalbumin. In addition, a similar outcome was obtained in the precipitation assay carried out to assess the effectiveness of the protein fining agents when interacting with the phenolic extract: gelatin 2 > gelatin 1 > casein = ovalbumin. In the study, all four protein fining agents were also compared with one another in terms of their effectiveness when interacting with seven different varietal wines by carrying out both a diffusion and a precipitation test. In these assays, which were identical to the ones on tannic acid and the seed extract, some highly relevant observations were made. First, ovalbumin was found to be highly reactive, because even the lowest concentrations of wine in the binary mixtures showed a marked reduction in diffusion of the protein component on the cellulose membrane. Furthermore, this anti-diffusive effect was not intensified by the higher volumes of wine in the binary

mixtures, and ovalbumin was very rarely fully precipitated, even in the presence of the highest volumes of wine in the assay; in this regard, Petit Verdot was the exception. On the other hand, in the assay on the red wines treated with ovalbumin, the precipitation of ovalbumin was most commonly found to be partially dependent on the concentration of the wine. Moreover, in the same assay with ovalbumin, no major differences between any of the six red wines and the single white wine were found, as was the case with tannic acid or the seed extract. The presence of alcohol in the binary mixtures with wine and its absence in those with tannic acid and the seed extract may account for this particular behaviour of ovalbumin, given the well-known enhancing influence of alcohol on polyphenol-protein interactions (Obreque-Slier *et al*., 2010c; McRae *et al*., 2015). In contrast to ovalbumin, in the assay with the wines, casein showed marked differences. Thus, casein showed high reactivity with all the red wines (even with the lowest volumes of wine in the assay) represented by the occurrence of intense staining with non-diffusible spots in the diffusion test. This high reactivity may be due to the abundance of proline residues in the aminoacidic composition of casein and the high ability of these residues to form hydrogen bonds with red wine phenolics and, therefore, a potentially high number of binding sites (Eskin and Goff, 2012). However, no differences were observed between the red wines, but a clear difference between red wines and the white wine were found. Indeed, the interaction of white wine and casein resulted simply in the reduced diffusion of the binary mixture on the cellulose membrane. All the complexes of casein and red wine components, shown to be non-diffusible in the diffusion assay, were almost completely removed from the supernatants by means of centrifugation, thus revealing their additional insoluble character. By contrast, the precipitation test revealed the fully soluble character of the complexes of casein and white wine components on the basis of the noteworthy similarity of the corresponding spots in the diffusion and precipitation tests. In the assay on the interactions between the protein fining agents gelatin 1 and gelatin 2 and seven different wines, some noteworthy observations were also made. At variance with the results obtained for ovalbumin and casein, gelatins 1 and 2 distinctively showed a broad spectrum of different reactions to each wine in the assay. Firstly, the white wine/gelatin1 or white wine/gelatin 2 mixtures showed just minor changes in diffusivity in comparison to the corresponding gelatins alone. No white wine-induced precipitation was observed. In addition, the progressive decrease in area of diffusion of the spots with the progressive increase in red wine in the mixtures was commonly observed irrespective of the series of red wine/gelatin mixtures. Furthermore, for some wines these series progressively increased up to produce intensely stained spots of non-diffusible complexes. Finally, two different commercial gelatins, such as those used in the study, can produce similar results when in contact with the same wine. This is particularly important, because gelatins are highly diverse commercial multi-origin collagen-hydrolysates (Harrington and Von Hippel, 1962). Indeed, when ranked from minus upwards based on their reactivity with gelatin 1 in the diffusion assay the order of the group of wines in our study was SB-SG-MA-(TM/CR)-CF-PV; with gelatin 2 the order was nearly the same, with the two wines in brackets possibly having opposite positions in the sequence. Accordingly, the diffusion assay of wine/gelatin binary mixtures could be useful for ranking wines according to their physicochemical reactivities with a single gelatin or, vice versa, for ranking gelatins according to their physicochemical reactivities with a single wine. Finally, placing side-by-side the corresponding series of spots of the diffusion and precipitation tests of the gelatin/wine binary mixtures allows to associate the degree of reactivity, density or condensation of the non-diffusible complexes with their precipitability. For instance, the 2T, 2U and 2V spots (but not the 2R and 2S spots) in the upper panel of Figure 5 completely disappeared after centrifugation, as shown in the corresponding positions of the precipitation test; i.e., 4Q, 4R and 4S (lower panel, Figure 5). It can be concluded, then, that different protein fining agents can interact very differently with the same phenolic solutions, including complex wines. This suggests that fining should be considered as a selective process in which the purpose of the operation and the selection and characterisation of the fining agent to achieve the purpose need to be equally taken into account.

As well as comparing the reactivities of four protein fining agents to a specific phenolic solution, the interactions of different phenolic solutions with single protein fining agents were also analysed. The apparently "simplest" phenolic in the study was tannic acid, a commercial hydrolisable gallotannin, which comprises indeed a mixture of molecules each with a glucose backbone that is diversely esterified with up to 5 galloyl residues, of which some are in turn esterified with additional galloyl residues. In the study, these relatively small-sized phenolics (despite major intermolecular networks being able to form by means of hydrogen bonding) were shown to interact with all four protein fining agents by displaying changes in both diffusivity and solubility in the diffusion and precipitation tests, respectively (Labieniec and Gabryelak, 2006). In addition, the seed extract used in the study comprised a mixture of free and conjugated monomers, oligomers and polymers of proanthocyanidins together with some free gallic acid. A number of these components have been widely reported as being highly interactive with proteins and as being protein precipitants (Hufnagel and Hofmann, 2008; Chira *et al*., 2009; Obreque-Slier *et al*., 2012a; Ren *et al*., 2016). As with tannic acid, the individual compounds in a phenolic extract may well be part of complex supramolecular structures interlinked by hydrophobic interactions and hydrogen bonding (Bennick, 2002; Bertelli *et al*., 2021; Obreque-Slier *et al*., 2022). Wine also comprises a complex structure, including a continuous hydroalcoholic matrix in which a diversity of flavonoids and non-flavonoid phenolic and non-phenolic compounds are in solution or suspension. Varietal wines, such as the ones used in this study, exhibit diverse molecular profiles linked to the genetic background of the grape varieties, the wine producing practices and the seasonal edaphoclimatic

conditions (Obreque-Slier *et al*., 2021; Pantelic *et al*., 2016). In this study on wine reactivity, despite the broad diversity of both the phenolic profiles and the four protein fining agents may give rise to numerous confounding factors, it can also serve to throw some common light on the general process. For instance, under the experimental conditions in the study, the protein fining agent ovalbumin was found to be highly interactive with some of the phenolic solutions in the study, but hard to precipitate. By contrast, casein was precipitated by tannic acid, the seed extract and all red wines in the study, including the Sangiovese wine that exhibited the lowest content of phenolics. Notably, none of the protein fining agents in the study precipitated when in contact with the SB white wine despite them all interacting and showing reduced diffusion on the cellulose membrane as evidenced by the diffusion and precipitation tests. Studies aiming to characterise protein fining agents by analysing or separating the sediments produced by binary mixtures comprising oenological inputs in solution or phenolic solutions, including wine, may be missing relevant data concerning the lack of precipitation of soluble complexes (Cosme *et al*., 2008; Maury *et al*., 2016). For instance, the just mentioned case regarding SB white wine is consistent with the organisation of small diffusible and soluble protein aggregates and might be accounted for by the low content of phenolics in the SB wine (Cosme *et al*., 2008). However, the interactions between the protein fining agents and all the chemical components of the white wine, including alcohol and organic acids among others, could substantially influence the contribution of the fining operation to the sensory attributes of wine (Cosme *et al*., 2008; Fontoin *et al*., 2008; Kallithraka *et al*., 2011; Obreque-Slier *et al*., 2012b). Another noteworthy observation in the study was the invariability of the patterns of behaviour shown by ovalbumin and casein when in contact with any of the six red wines in the study, but not for the white wine. This observation indicates that ovalbumin and casein do not distinguish between the phenolic profiles of the red wines under consideration. Nevertheless, what could be regarded as a negative result due to lack or paucity of specificity could also be considered as positive, as it sheds some light on the other two protein fining agents in the study. Indeed, gelatin 1 and gelatin 2 exhibit diverse interactions with the whole group of wines in the study (including the white variety), all of which have different phenolic profiles that can be associated with the intrinsic characteristics of the corresponding *V. vinifera* L. varieties (Chira *et al*., 2009; Obreque-Slier *et al*., 2012b; Obreque-Slier *et al*., 2021). Thus, it follows that the red wine in which both gelatins 1 and 2 showed the highest reactivity (Petit Verdot) stood out for displaying, among other parameters, the highest concentrations of monomeric, oligomeric and polymeric proanthocyanidins, total flavanols, total anthocyanins, total flavonoids and total phenols. By contrast, the lowest values of most of these parameters were shown by Sangiovese, the red wine in which both gelatins showed the least reactivity. This compositional data mostly agrees with reports from other laboratories (Fernández *et al*., 2007; Cejudo-Bastante *et al*., 2011; Medel-Maraboli *et al*., 2021; Ramazzotti *et al*., 2008; Tessarin *et al*., 2016). The second main general conclusion that can thus be drawn from the study is that each protein fining agent may display a specific way of interacting with solutions that have a complex phenolic profile, including wine.

On the whole, this reciprocal comparative study on the physicochemical interactions between widely used protein fining agents and several phenolic solutions (including red and white wines) as a reference, shows that the interactions depend highly on both the biochemical properties of the fining agent and the matricial properties of the polyphenol solution. This double look complements the traditional view that the characteristics of the protein fining agent is a differentiating parameter in the fining of red wines. Accordingly, in addition to characterising and acquiring in-depth compositional and functional knowledge about these inputs, the wine industry should continue taking into particular account a number of other parameters and conditions that have a decisive influence on the complexities of the wine matrix. This broader view will facilitate on a technical level both the task of the oenologist when undertaking particular decision-making processes, as well as modern precision wine-making practices oriented towards fulfilling specific needs.

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