White Wine Protein Instability: Mechanism, Quality Control and Technological Alternatives for Wine Stabilisation—An Overview

Fernanda Cosme [1,*] , **Conceição Fernandes** [2], **Tânia Ribeiro** [1], **Luís Filipe-Ribeiro** [1] and **Fernando M. Nunes** [1]

[1] CQ-VR, Chemistry Research Centre-Vila Real, Food and Wine Chemistry Lab., School of Life Sciences and Environment, University of Trás-os-Montes and Alto Douro, 5000-801 Vila Real, Portugal; tania.im.ribeiro@gmail.com (T.R.); fmota@utad.pt (L.F.-R.); fnunes@utad.pt (F.M.N.)

[2] Centro de Investigação de Montanha (CIMO), ESA-Polytechnic Institute of Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal; conceicao.fernandes@ipb.pt

* Correspondence: fcosme@utad.pt; Tel.: +351-259-350-000

Abstract: Wine protein instability depends on several factors, but wine grape proteins are the main haze factors, being mainly caused by pathogenesis-related proteins (thaumatin-like proteins and chitinases) with a molecular weight between 10~40 kDa and an isoelectric point below six. Wine protein stability tests are needed for the routine control of this wine instability, and to select the best technological approach to remove the unstable proteins. The heat test is the most used, with good correlation with the natural proteins' precipitations and because high temperatures are the main protein instability factor after wine bottling. Many products and technological solutions have been studied in recent years; however, sodium bentonite is still the most efficient and used treatment to remove unstable proteins from white wines. This overview resumes and discusses the different aspects involved in wine protein instability, from the wine protein instability mechanisms, the protein stability tests used, and technological alternatives available to stabilise wines with protein instability problems.

1. Introduction

Proteins exist in wine at low levels, related to the protein content and composition of the grapes, which are also dependent on the grape variety and maturation conditions, as well as on the winemaking process [1]. Proteins can be responsible for a wine colloidal instability, forming amorphous sediment or flocculate, and produce a suspended and undesirable haze before or after bottling [1–5] that can cause serious economic losses to the wine producers. This instability is more important in white wines, as white wine limpidity is an essential sensory quality parameter. Wine protein haze formation can occur under high temperatures, throughout storage or wine transportation due to the protein self-aggregation phenomena, resulting in light-dispersing particles [6,7]. The formation of wine protein haze is a multifactorial process with several factors known to influence the process, such as storage or wine ageing temperature, pH, ionic strength, wine protein composition, organic acids, ethanol, phenolic compounds, metals and sulphate content; however, other important factors remain unidentified, such as the non-proteinaceous component(s) usually named X factor [8–12]. Protein instability can also occur via the blending of stable wines. The haze formation does not constitute a health risk to consumers [13] or affect the olfactory and gustatory wine characteristics [9,14]; however,

the formation of haze or deposits in bottled wines can affect their commercial presentation, making them unacceptable for consumers due to the visual aspect [1,15].

The most important proteins that have been related to wine protein instability are pathogenesis-related proteins of *Vitis vinifera* that include the chitinases and thaumatin-like proteins [2,16]. These proteins can be slowly denatured and aggregate throughout wine storage, forming a light-dispersing haze [17]; therefore, this phenomenon needs to be prevented by removing them from the wine, usually by fining, before wine bottling [13].

Protein instability is presently prevented by the elimination of unstable proteins from the wine using specific fining agents. Fining agents are substances that usually present an electric charge (negative and/or positive) that are put in contact with the wine, flocculating and precipitating the particles/compounds with an opposite electrical charge implicated in wine turbidity [18,19]. Bentonite fining is the most-used process to avoid protein instability in white wine, with the dose used being preferentially determined previously by stability tests [20]. However, bentonite fining can affect wine quality, for example, by removal of colour and aroma compounds [7,21] and, therefore, can affect wine sensory characteristics [22]. Therefore, alternative techniques to bentonite fining for this goal have been studied, such as ultrafiltration [6,23,24], addition of proteolytic enzymes [25,26], flash pasteurisation [27,28], other adsorbents (silica gel, hydroxyapatite and alumina) [29], zirconium oxide [30–32], natural zeolites [33,34], chitin and chitosan [35,36], carrageenan [5,37] and the use of some mannoproteins [19,38].

2. Profiling and Characterisation of Wine Proteins

Wine proteins are composed of grape proteins of *Vitis vinifera* and, in lower levels, by proteins from the autolysis of *Saccharomyces cerevisiae* [2,39]. Soluble proteins in grapes and wine have a molecular weight (MW) over a wide range, from 6 to 200 kDa [40] and isoelectric point (pI) from 2.5 to 8.7 [41–43] (Table 1). The concentration of each fraction depends, among other factors, on grape variety [44–46], the state of maturity of the grape berry at harvest (the protein content increases as the berry matures) [47], region climate conditions, soil, vineyard management [48], and winemaking conditions [47], that intensely affect the final protein levels [30,49]. The methods used by the different authors for protein determination can also be determinants for the described range of wine protein content found in the literature. Their levels, in untreated wine, usually range from 15 to 300 mg/L [2,4,9,50,51]; however, contents as high as 700 mg/L have been described [52]. Moreover, the levels of specific proteins can vary widely. Mierczynska-Vasilev et al. [53] determined levels of 182 and 11 mg/L of thaumatin-like proteins and chitinases for Semillon and Sauvignon Blanc, respectively. The proteins implicated in wine protein instability resist the winemaking process, since they are highly resistant to proteolytic activities of grape and yeast proteases and are stable under high ethanol conditions and at the low pH values of must and wines [54].

For the characterisation of wine proteins, due to their very low concentration in wines and the presence of other compounds in the wine matrix, a mandatory first step is in most of the protocols concentration and purification by dialysis [55,56]. Ultrafiltration [26] and precipitation with an organic solvent, such as ethanol, methanol, or acetone [40], or ammonium sulphate precipitation [57,58] have also been used (Figure 1). After its separation from the wine, and before its characterisation, the protein content is quantified by traditional methods that used to fail in wines due to the existence of interfering compounds, such as polyphenols in Lowry's method or peptides in Biuret's method [59]. For this reason, the proteins are quantified after purification by chromatography using different approaches, such as (i) summing the total amino acid content [41] or (ii) using the area of the peak obtained by ultraviolet (UV) absorbance at 280 nm [60]. Another method commonly used to quantify proteins is Bradford's method, which has been widely used in the quantification of wine proteins [23,61,62]. Wine proteins can be further characterised by native-polyacrylamide gel electrophoresis (PAGE) [40,61–66] and PAGE with denaturating conditions (SDS-PAGE) [45,61,65–72]. The use of isoelectric focusing (IEF) in the analysis of proteins from grape must or wine [1,41,61,65,71], based on agarose gels [41], or polyacrylamide either

in a tube [23] or on a plate [55], or PAGE with denaturing conditions (SDS-PAGE) [73,74] or lithium dodecyl sulphate polyacrylamide gel electrophoresis (LDS-PAGE) [23,75,76], or after fast protein liquid chromatography (FPLC) protein fractionation [72,77], has also been employed (Figure 1). More recently, the powerful two-dimensional gel electrophoresis (2D-GE) has been applied with great success [57], allowing the separation of more than 300 proteins or polypeptide fractions from a Chardonnay wine. In sparkling wine and in aged white wine, to a lesser extent, capillary electrophoresis (CE) has been used successfully [56,78] and high-performance capillary electrophoresis (HPEC) was used to create a wine protein profile [79]. Moreover, chromatography has been extensively used to separate and characterise wine proteins, such as, for example, molecular exclusion [80], reverse phased [40], or affinity interactions [81]. Fast protein liquid chromatography (LC) with ion exchange has been also used for wine characterisation, either using cation exchange [45,82,83] or anion exchange [45] (Figure 1). More recently, nano-high-performance liquid chromatography (nano-HPLC) [84] and nanoscale liquid chromatography coupled to tandem mass spectrometry (nano-LCMS/MS) [58] were used for protein separation and, in the case of the use of mass spectrometric techniques, also their identification. Mass spectrometry has become a central technique for protein identification, characterisation and quantification with its increasing sensitivity and applicability to complex samples. Mass spectrometric techniques are based on the separation of ionised molecules according to their mass-to-charge (*m/z*) ratios. Mass spectrometry (MS) can be used as an off-line technique but, nowadays, is more frequently coupled to liquid chromatography and capillary electrophoresis. Mass spectrometers can be used for the simple measure of the polypeptide molecular weight or for the determination of other important structural features like the amino acid sequence, by subjecting the selected ions to fragmentation through collision (tandem mass spectrometry (MS/MS)), allowing us to obtain detailed structural characteristics of the peptides from the analysis of the masses of the obtained fragment ions (Figure 1). A range of mass spectrometry-based analytical platforms and experimental strategies have emerged in the last years. Generally, all mass spectrometry–based proteomic workflows comprises three stages: (i) proteins are isolated from their source and can be further fractionated, the protein sample is digested, and the resulting peptides can also be further fractionated. (ii) The peptides after digestion are analysed qualitatively and quantitatively by mass-spectrometry. (iii) This approach generates a large amount of data that are analysed by appropriate software tools to deduce the amino acid sequence and, if applicable, the quantity of the proteins in a sample. The peptide identity is obtained through the tandem mass spectra by database searching [85], according to established guidelines to generate consistent results [86]. Additionally, a suitable statistical analysis of the search results is critical to ensure confidence in the identification [87].
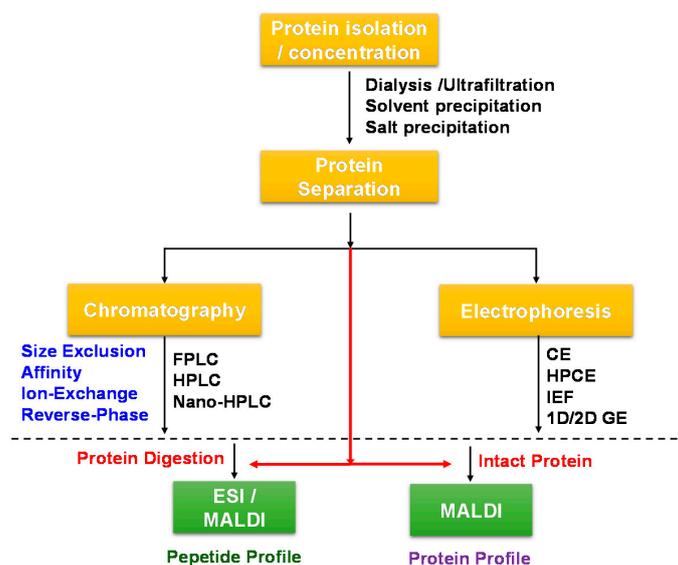

Figure 1. Typical workflow for wine protein characterisation.

MS-based techniques have been applied to study the wine proteins and these applications have been reviewed by Flamini and Rosso [88] and Nunes-Miranda et al. [89], including the use of direct MS analysis after MALDI ionisation [90,91] or after previous protein cleavage with trypsin [92]. Direct infusion electrospray ionisation mass spectrometry (ESI-MS) has also been used to obtain the fingerprint of wines by Cooper and Marshall [93] and Catharino et al. [94].

Table 1. Isoelectric point (pI) and molecular weight (kDa) identified in different protein fraction from grape and wine.

Isoelectric Point (pI)		Molecular Weight (MW)		References
Grape	Wine	Grape	Wine	
	3.1–8.3			[61]
	4.0–8.2		10.0–70.0	[72]
			15.5–69.0	[45]
	4.1–5.8	11.2–190.0		[75]
5.6–7.6		19.0–100.0		[73]
	4.6–8.8		12.0–41.0	[74]
			18.0–23.0	[64]
	3.1–9.2		11.0–88.1	[47]
	3.0–5.6		14.0–94.0	[65]
	3.2–9.0			[68]
	3.6–9.0		6.0–200.0	[40]
			10.0–50.0	[95]
			10.0–64.0	[70]
			21.0–65.0	[76]
	2.5–9.7			[41,42]

3. Proteins Responsible for Wine Haze

The first studies done concerning wine protein instability were performed by Koch and Sajak [96] who found that the heat-formed sediments contained two main electrophoretic fractions with different heat sensitivities. Moretti and Berg [64] and Bayly and Berg [66] fractionated and analysed wine proteins, concluding that, among grape and wine proteins, those protein fractions with low isoelectric points and low molecular weights were more sensitive to heat treatment and were responsible for wine protein instability. Furthermore, Hsu et al. [23], using ultrafiltration followed by two-dimensional electrophoresis to separate wine proteins, came to the same conclusion, showing that the principal proteins responsible for the white wine protein instability have a low molecular weight (12.6–30 kDa) and pI (4.1–5.8) and also contain glycoproteins. In muscadine wines, Lamikanra and Inyang [74] proposed that proteins with a molecular weight higher than 32 kDa were also unstable. This was later confirmed by Waters et al. [97], who separated and fractionated wine proteins using a combination of salting out with ammonium sulphate and ultrafiltration, showing that the protein fractions with those characteristics (24 and 32 kDa) were more sensitive to high temperatures and contribute to wine instability and haze formation. The same author also confirmed [6] that the lower molecular weight protein fractions may be more important to wine haze, showing that the protein with 24 kDa caused nearly 50% more haze, at the same concentration than the protein fraction with 32 kDa.

Some authors stated that wine protein haze is associated to the total wine protein content, with wines containing higher total protein concentration, showing also more predisposition to becoming unstable [2,51]. However, other authors argue that wine protein instability is not associated to wine total protein content [2,64,98] as each individual wine protein fraction behaves differently [66,69,75,99].

Subsequent works indicated that proteins responsible for wine instability are pathogenesis-related proteins [3,21,100]. Pathogenesis-related proteins are essential for plant performance, such as disease resistance, development, and general adaptation to stressful environments [101]. Due to their high quality, *Vitis vinifera* is the most extensively cultivated species for winemaking; however, it is mainly vulnerable to pathogens, particularly fungi and oomycetes, such as *Botrytis cinerea* and *Plasmopara*

viticola, respectively [54]. As a defence mechanism against several pathogens, pathogenesis-related proteins are synthesised in response to infection by pathogens, although they are constitutively expressed during berry ripening [4], for repairing the damage caused to the plant [102]. In *Vitis vinifera* grapes, the two major pathogenesis-related (PR) proteins isolated from wine, with a globular structure and a positive charge at the normal wine pH, are thaumatin-like (PR-5 type, proteins fraction with 24 kDa) [21] and chitinases (PR-3 type, protein fraction with 28 kDa) [21,103]. Other minor PR proteins (e.g., osmotins, invertases, β-1,3-glucanases, lipid transfer proteins) are also present in wine [90,104]. Different isoforms of thaumatin-like proteins and chitinases have been identified in grape juices of different *Vitis vinifera* varieties, with a wide range of molecular weight 20–30 kDa, and an isoelectric point between three and five [1,2,105,106]. These proteins are also the main soluble proteins from *Vitis vinifera* [50,100] and have been considered responsible for haze development in bottled white wine during storage and transportation [9,54,77,100,103,107–109]. They are synthesised during development regardless of variety [46], region and year [21,54,90,110] and increase during ripening; consequently, riper grapes are also more susceptive to protein haze [50]. These proteins (chitinases and thaumatin-like protein) have a high number of disulphide bonds that contribute to their highly stable structures and resistance throughout the winemaking process (that means, resistance to proteolysis and stable at wine acid pH 3.0–3.8) [2,21,111].

Chitinase proteins are low molecular weight proteins and are sensitive to temperature variations [100] and pH [112]. Thaumatin-like proteins are characterised mainly by their higher thermostability and by presenting no significant conformational variations or aggregation when exposed to pH variations [112]. This unequal behaviour appears to be associated to the differences in the secondary structure of both protein families, globular for thaumatin-like proteins and elliptical for chitinases [112,113]. Pocock et al. [50] additionally studied the variation in pathogenesis-related proteins concentrations among grape varieties for Muscat of Alexandria, Sultana, Sauvignon Blanc, Pinot noir and Shiraz grape juices with thaumatin-like proteins/chitinases of 119/118, 119/76, 35/21, 23/44 and 18/9 being observed, respectively. These authors have also observed that berry damage during mechanical harvesting, associated with a long transport time, will also induce the production of pathogenesis-related proteins as a result of a grape defence mechanism before pressing [114]. Thaumatin-like protein and chitinase differences in heat stability support the idea that protein composition may impact haze formation in wines [13,115,116]. In fact, the thaumatin-like protein (24 kDa fraction) contributed to twice as much haze as the chitinase (32 kDa fraction) [6,12,97]. However, chitinases have been shown to have a major importance in wine haze as they are the most sensitive to precipitation, and a linear correlation was found between chitinase wine concentration and wine haze formed [13,32,108]. The evidence that chitinases are primary contributing proteins to wine haze formation is based on thermal unfolding studies. Falconer et al. [100] showed that chitinases have a low melting temperature, with denaturation in minutes at temperatures >40 °C, compared to weeks needed for the thaumatin-like protein under the same circumstances. Chitinases have a denaturation half-life of 6 min at 55 °C, thus extrapolating down to a denaturation half-life of 3 days at 35 °C or 2 years at 25 °C compared to the thaumatin-like protein, which had a melting temperature of 62 °C, with a calculated denaturation half-life of 300 years at 25 °C. On the other hand, Sauvage et al. [1] (2010) showed that vacuolar invertase (GIN1), from the grapes and β-(1–3)-glucanases (32 kDa fraction), also considering pathogenesis-related proteins, can influence haze formation [107,110]. Besides the presence of *Vitis vinifera* thaumatin-like protein bands, β-(1,3)-glucanase and ripening-related protein-like (27.4 kDa) Grip22 precursor have also been identified in the natural protein haze of white wines [21,107,117]. Additionally, no correlation between the total amount of protein existing in wine and protein instability has been observed [107]. These authors showed that the protein proportion of the natural precipitates was only 10.3% (*w/w*), using a direct protein analysis (Bradford dye-binding assay) while phenolic compounds and polysaccharides represented 7.2% and 4.4%, respectively.

4. Factors That Affect Wine Protein Stabilisation

Although the denaturation of some wine proteins results in aggregation and flocculation, rendering a turbid suspension and eventually the formation of precipitates [4], other non-proteinaceous wine compounds and factors can also be implicated in wine protein haze formation. It has been observed that wines with very similar protein fractions can have different haze potential [118]. However, wine ethanol concentration showed no significant effect on wine protein turbidity formation [119]. The most studied mechanism for wine protein instability and haze development is the protein–polyphenol interaction [67,77,95]. Wine proteins, to form turbidity, needed the presence of procyanidins, as wine proteins only (isolated and added back into a model wine) did not provoke turbidity [120,121]. A model for the interaction between haze-active polyphenol and haze-active protein was proposed by Siebert et al. [122] and showed that the quantity of haze developed depended on both protein and polyphenol content and on their ratio. In addition, wine pH has been implicated in protein haze formation, Yokotsuka and Singleton [123], showed that the turbidity of a protein–polyphenol complex is enhanced with a pH increase from 2.5 to 3.7 (model wine solution with 10% ethanol). Later Batista et al. [9] postulated that the protein haze formation is an isoelectric precipitation mechanism. Marangon et al. [108] verified that haze development in white wines is associated to hydrophobic interactions between proteins and tannins occurring on the hydrophobic tannin-binding sites of proteins that can be exposed depending on heating and reduction. Esteruelas et al. [99] found numerous phenolic compounds in protein haze, such as tyrosol, *trans-p*-coumaric, *trans*-caffeic, vanillic, protocatechuic, syringic, gallic, ferulic, shikimic acids, (+)-catechin and ethyl coumaric acid ester; quercetin and cyanidin were also detected after acid hydrolysis showing the existence of procyanidins. Phenolic compounds can also rise haze development by crosslinking denatured proteins to stimulate aggregate development [115], and the elimination of phenolic compounds from wines has been observed to reduce haze development [12].

Media conditions such as pH, ionic strength, metal ions [124], polysaccharides [125], organic acid concentration [9,11,12], polyphenols/phenolic compounds [4,10,12,126] and sulphate anions, formerly designated as the X factors essential for protein turbidity [12,13,127], affect protein haze formation. Wine pH is a serious inducing factor in protein haze formation, with model wines at pH 4.0 inducing higher protein aggregation and haze after heating than model wines of pH 3.0 [9,11,51]. Increasing electrical conductivity and ionic strength, such as by the addition of sulphate anions or sodium cations, also rise haze development after heating, by decreasing the electrostatic repulsion of proteins [11–13]. Other ions, comprising chloride, tartrate, $Fe^{2+/3+}$ and $Cu^{+/2+}$, do not affect haze development in model wines [12]. Increasing protein concentrations (9 and 25 mg/L) and electrical conductivity (0.134 and 0.163 S/m) result in more visible haze formation, while low iron concentrations (0.3 and 0.9 mg/L) seem to improve the protein stability of white wines, so haze was observed to be negatively correlated with iron levels [128]. In contrast, McRae [129], in a study using several white wines, showed a negative correlation between electrical conductivity and white wine haze formation, probably related to the lower sulphate concentration of these wines (150–550 mg/L) compared to the sulphate concentration of the model wines (500–4000 mg/L) used by Marangon et al. [13]. Polysaccharides may potentially decrease protein aggregation in wines by forming a protective layer around unfolded proteins [17], while the role of polysaccharides in haze development in reconstituted wines is variable [116,130,131].

Organic acids are recognised to interact with phenolic acids, free amino acids, pectic compounds, tannins and sulphate ions preventing, in this way, their interaction with proteins [10]. These authors showed that organic acids presented a stabilising effect on the wine potential to the formation of protein haze. This effect has been attributed to electrostatic interactions that depend upon the organic acid pKa and protein pI values and on the medium pH.

Pocock et al. [12] suggested that sulphate ions could be the non-proteinaceous factor for protein haze formation, as they promote protein–protein hydrophobic interactions, as well as the suppression of electrostatic repulsion between proteins by the increase in the ionic strength of the medium [13]. In a study dedicated to the characterisation of some wine haze-forming

thaumatin-like proteins, it was shown that potassium hydrogen sulphate can modulate the haze formation [106]. Van Sluyter et al. [115] reviewed the mechanism of protein haze formation and proposed a three-stage process that included protein unfolding, protein self-aggregation, and aggregate cross-linking, highlighting the role of sulphate ions in all steps. More recently, Chagas et al. [127] showed the importance of sulphur dioxide present in wines in the irreversible denaturation and aggregation phenomena of thaumatin-like proteins and their contribution to wine protein haze formation. The presence of HSO3⁻ induces the cleavage of the thaumatin-like proteins' intra-disulphide bonds, enhanced by the high temperature-induced unfold of these proteins. The exposed hydrophobic surfaces and buried cysteine/cystine residues contribute to the inter-protein interactions. The formation of proteins S-thiosulfonates by the reaction of HSO_3^- and disulphide bonds promotes the formation of inter-disulphide bonds between thaumatin-like proteins that are responsible for wine protein aggregation following a nucleation-growth kinetic model.

5. Protein Stability Tests for Wine Quality Control—Advantages and Disadvantages

To decrease the risk of turbidity and/or formation of organic deposits in white wine related to the thermolabile wine protein or colloidal suspensions precipitating over wine before bottling, diverse protein stability tests have been developed and are used today in the wine industry [69,77,119,132]. These tests are also performed to define the dose of fining agent necessary in the fining treatments for stabilising wine concerning the protein haze formation [133,134]. In general, protein stability tests include the determination of wine total protein content or methods involving a reduction in wine protein solubility by applying heat [135–137] or chemicals. As protein chemical precipitants, trichloroacetic acid [8,136], phosphomolybdic acid, also called bentotest [138], and the increase in the ethanol [8] or tannin content [139], have been described. In agreement with Esteruelas et al. [69], these tests originate dissimilar precipitates, as well as differences from the natural precipitate formed in protein-unstable wines and, therefore, they do not perfectly reproduce the natural phenomenon. Forced precipitation leads to the formation of precipitates with increased protein content, polysaccharides and polyphenols in relation to the precipitates obtained naturally; they contain proteins that otherwise would not appear in the natural precipitate [69]. The heat test using lower temperatures (60 °C/4 days followed by 6 h at 4 °C) does not precipitate thaumatin-like proteins and the ethanol test precipitates a great number of polysaccharides, making neither test suitable. Esteruelas et al. [69] showed that the higher temperature (90 °C/1 h followed by 6 h at 4 °C) heat test is more like the natural precipitate concerning its chemical composition and, therefore, appears to be the most suitable stability test. Additionally, different tests yield different instability indexes and, therefore, different doses of fining agents are needed in order to achieve stability according to their results. Protein stability tests do not correlate well with wine total protein concentration since individual protein fractions act differently [69] and, therefore, total protein assays are limited concerning the prediction of wine protein stability and do not take into account the role that other wine components play in protein instability [12].

5.1. Heat Test

The heat test is the most-used method in the industry for predicting wine haze-forming potential and determining the relative protein instability, being probably the most reliable method to predict the haze/sediment formation in the bottle during storage. There is no standard protocol to perform the heat test, as numerous researchers and the wine industries use different heating times and temperatures (Table 2) [9,140]. This test is supported by wine sample heating at a high temperature over a period of time and, therefore, relies on protein heat denaturation to induce precipitation. This test is applied to simulate the formation of protein turbidity and is considered suitable to establish the adequate doses of fining agent necessary to eliminate heat-unstable proteins, being less affected by other wine compounds and conditions, such as metal cations and pH [119]. Wine proteins present different heat sensibilities to form precipitates [23,75,76] and, therefore, the most-used test is that described by Pocock and Rankine [137], where wine is heated at 80 °C for 6 h. Ribéreau-Gayon et al. [135] suggested that

wine must be heated at 80 °C for 10 min. Instead, Esteruelas et al. [69] indicated that heating wine at 90 °C for 1 h, when compared to the other tests, forms a precipitate with a composition similar to the natural precipitate.

One of the described drawbacks of these tests is the increase in phenolic compound oxidation and condensation with proteins at high temperatures [119], which can cause protein precipitation and, therefore, interfere with the test results. However, Sauvage et al. [1] determined that the heat test can induce the precipitation of almost all wine proteins, leading to an overestimation of the fining agent doses required for stabilisation.

Protein aggregation mechanisms changes between long-term storage at lower temperatures and heating and can be influenced by the protein's isoelectric point and the pH of the solution [11]. At a lower temperature (25 °C), protein aggregation is improved by a low pH (pH 2.5).

Over time, there is a variation in protein conformation, possibly exposing hydrophobic groups, which triggers aggregation. Proteins are completely unfolded [100] (at higher temperatures (70 °C) and are more disposed to aggregation at a higher pH (pH 4.0) [11]. In model wines, haze formation and protein aggregation can rise upon cooling with more haze development after extensive cooling times [11]. Cooling time may, consequently, be essential for the heat test results. Variations in heat test conditions are, therefore, likely to compromise the accuracy and repeatability of the results.

McRae et al. [129] showed that in white wines heated at 80 °C for 0.5 to 6.0 h and then cooled for 0.5–18 h at either 0, 4 or 20 °C, prolonged heating times, prolonged cooling times and a lower cooling temperature all increased the quantity of haze formed in the heat test. For example, heating for 6 h and cooling for 18 h at 4 °C (24 h test) commonly yielded an increased predicted bentonite dose by up to 0.3 g/L compared to heating for 2 h. These authors concluded that wines heated for 2 h at 80 °C and then cooled for 3 h at 20 °C (5 h heat test) resulted in a repeatable production of haze and needed a bentonite fining dose for wine stabilisation. The wine protein haze formation in these tests is explained by the thermal treatments that expose more protein-active sites for haze-active polyphenol binding [122] and the cooling process could decrease the solubility of protein–polyphenol complexes [123].

Although this is one of the most used tests, the greatest disadvantage is the time needed for its execution.

Table 2. Heat test conditions recommended for heating and cooling.

Heating	Cooling	Reference
60 °C for 4 days	4 °C for 6 h	[29]
80 °C for 2 h	4 °C for 16 h	[134]
80 °C for 2 h	0 °C for 2 h	[5,127,141]
80 °C for 2 h	4 °C for 2 h	[128]
80 °C for 2 h	20 °C for 3 h	[37]
80 °C for 3 h	20 °C for 30 min	[131,142]
80 °C for 6 h	4 °C for 16 h	[9,137,143,144]
80 °C for 30 min	No specified cooling time	[145,146]
90 °C for 1 h	4 °C for 18 h	[147]
90 °C for 1 h	4 °C for 6 h	[29]

5.2. Trichloroacetic Acid Test

The trichloroacetic acid (TCA) test is established on the ability of this acid to precipitate all proteins present in wine, yielding results close to those obtained by the determination of the wine total protein content [8].

The TCA test consists of the addition of 1 mL of a TCA solution at 55% (*v/v*) to 10 mL of wine followed by heating in a water bath at 100 °C for 5 min, and cooling and standing at room temperature for 15 min before measuring haze formation [23,76,77]. In agreement with Berg and Akiyoshi [136],

this test can be correlated with protein stability; however, at the industrial scale it does not yield satisfactory results, because it overestimates the fining agent dose needed to stabilise the wine.

5.3. Tannin Test

The tannin precipitation test is based on the hypothesis that wine proteins could precipitate during wine storage by binding with phenolic compounds and, therefore, yield information about the amount of wine proteins able to be precipitated by tannins [119]. A previous work performed by Yokotsuka et al. [148] showed that the ability of phenolic compounds to bind to proteins increased with their degree of polymerisation. This test is influenced by numerous intrinsic wine factors, namely pH, total protein content, iron content, copper and potassium, and, therefore, is not a good predictor of the fining agent dose needed to stabilise wines [69,119].

5.4. Bentotest

The bentotest uses a solution of phosphomolybdic acid in HCl which precipitates wine protein by neutralising the protein charge, leading to aggregation with the molybdenum ion [23,76,77]. This procedure can precipitate all the proteins in the wine sample, being principally used to determine the bentonite dose. However, this test has the disadvantage of overestimating the fining agent used to stabilise the wine [23,76,77].

5.5. Ethanol Test

The ethanol test is established on the reduction in the medium dielectric constant, resulting in a reduction in protein solubility [149], leading to the precipitation of the least soluble protein fractions at wine pH. This test is significantly influenced by the total protein concentration, pH, pectin, tartaric acid and calcium content, which may result in differences in the formation of wine turbidity [29]. Esteruelas et al. [69] also showed that polysaccharides are the key compounds precipitated by the ethanol addition to wines, followed by proteins and polyphenols. Therefore, the fining agent needed for wine protein stabilisation, based on the ethanol test, would be done in excess.

5.6. Spectroscopic Methods

The conventional stability tests previously described are time consuming. Therefore, the use of near-infrared spectroscopy (NIR) to predict wine protein instability seems to be promising. Moreover, the feasibility of using infrared spectroscopy for the estimation of protein instability haze development in white wines resulting from heat and colloidal stability tests has been explored for the first time by Versari et al. [150]. The authors analysed one hundred and eleven white wines using the near and mid-infrared spectral region and, simultaneously, the heat and colloidal (ethanol addition) stability tests were done on the same wines. Partial-least squares (PLS) regression analysis was used to construct predictive models of the turbidity-acquired spectra. These authors proved that the turbidity obtained from the ethanol addition to the wine could be expected from the short wavelength NIR spectra.

6. Wine Protein Stabilisation—Strategies and Treatments

Wine protein stabilisation consists of the removal of unstable proteins. This stabilisation process is usually achieved by bentonite fining [4]. However, bentonite fining treatment presents some disadvantages, resulting in a loss of economic value [7]. Decreasing bentonite applications and/or finding alternative technologies or products, such as the use of yeast mannoproteins [19,38,140], the use of regenerable adsorbents [29–34], carrageenan, chitin, chitosan, ultrafiltration and flash pasteurisation or the use of proteolytic enzymes [5,6,23–28,35–37], are of utmost interest for the wine industry.

6.1. Winemaking Practices to Prevent or Reduce Wine Protein Instability

The climate conditions and grape variety can significantly influence the wine protein composition and wine protein stability. Intense grape maturation in white grape varieties must be avoided due to the higher concentration of proteins and phenolic compounds in the grape juice [135]. The application of pectolytic enzymes contaminated with proteolytic activity can decrease the level of proteins in the grape juice. In protein-rich grape varieties, the pre-fermentation maceration operation with enzyme extraction must be avoided due to the high levels of proteins and phenolic compounds that can be extracted, increasing the wine protein instability. In the Bordeaux region, in grape varieties like Sauvignon Blanc and Semillon, the skin contact can increase by up to more than 50% of the levels of unstable proteins. The oxidative protection of the grapes with SO_2 can increase the level of proteins; in protein rich grape varieties, the SO_2 levels must be minimised and the oxidative protection should be performed using inert gases like CO_2 [135].

Tannins from the grape stalks have a special tendency for interacting with grape juice proteins when the grapes are pressed [151]. Thus, mechanical grape-harvesters that eliminate stalks may be considered one contributing factor in the protein instability of wines made from certain grape varieties [152].

In white grape varieties, like Moscatel, that consistently contain high levels of unstable proteins, the use of bentonite in juice clarification or during the grape juice fermentation can be useful to decrease the level of proteins [51].

6.2. Bentonite Fining

Bentonite has been used to improve wine limpidity and stability for many years and is still, today, the most used method for wine protein stabilisation, being one of the most efficient treatments widely used at the industrial scale to prevent haze formation after bottling and storage [2,4,115].

Bentonites are complex hydrated aluminium silicates, mostly composed of at least 75% of montmorillonite. Montmorillonite has a multilayer structure of aluminium hydrosilicates, forming platelets [153] with exchangeable cations [154]. The exchangeable cations can be Na^+, Ca^{2+}, and Mg^{2+}, and determine the bentonite type [22], namely sodium, calcium and magnesium bentonites, respectively [154] (Figure 2). Other cations are present, such as K^+ and Fe^{2+}, but in minor amounts [155]. The different cations are complexed in the interlayer region and can influence the interlayer distance throughout the swelling and the adsorbing performance [156].

The predominant forms are calcium and sodium bentonites, but sodium bentonite is still the most extensively applied, as they swell more than calcium bentonites [154] and swelling can potentially increase the surface area-accessible adsorption for wine protein [29,39,132,157]. The processing time of sodium bentonite is less than calcium bentonite, but the quantity of sediment is greater for sodium bentonite, with calcium bentonite yielding more compact sediment. To enhance the adsorption properties of calcium bentonites, they are activated with sodium carbonate (Na_2CO_3) at 80 °C [157], resulting in calcium-activated bentonites with similar or even better adsorption properties than sodium bentonites [154].

The bentonite wine fining involves the dispersion of the adsorbent and the adsorption of the solutes and sedimentation of the complex formed between the adsorbent and adsorbate [157]. Bentonite removes proteins by electrostatic interaction, forming complexes which can be removed by filtration. Proteins with a pI above the wine pH have an overall positive charge and can be adsorbed by bentonite via an exchange of sodium, calcium and magnesium ions [132]. Bentonite treatment efficiency depends, therefore, on bentonite type and dose, temperature, pH and wine composition [135].

The method used to prepare bentonite affects significantly its capacity to eliminate wine proteins [39]. Hydrated bentonite (with wine or water), can swell, increasing their surface area and forming a gel with a strong negative charge at the wine pH. These negatively charged bentonites interact electrostatically with positively charged wine colloids, proteins, leading to flocculation [1,22]. However, high doses of bentonite produce lees that can contain 5% to 20% of the wine volume,

resulting in wine losses [4,113,158]; the used bentonite cannot be recycled, having, therefore, a high environmental impact [31].
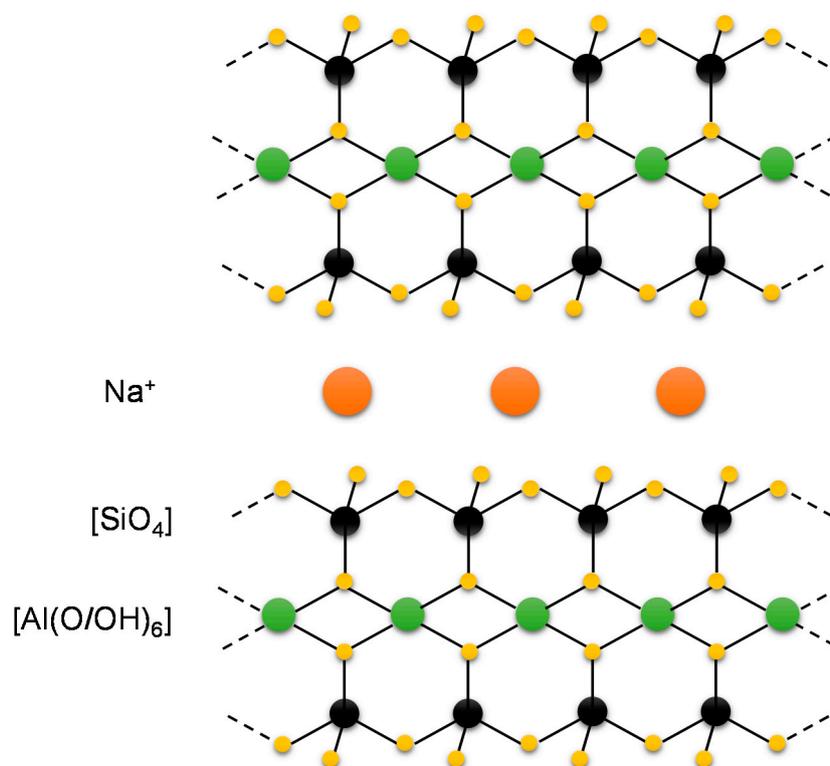

Figure 2. Schematic representation of the three-layer platelet formed by two layers of silicon oxygen tetrahedra and aluminium hydroxide sheet in sodium bentonite montmorillonite layer and sodium exchangeable cations, between adjacent platelets.

The competition with the cations (Ca^{2+}, K^+, Na^+, and Mg^{2+}), pH and ethanol concentration of the matrix can influence the protein adsorption [157]. According to Hsu et al. [23], pre-hydrated bentonite at a high temperature, low pH, high alcohol concentration and low tannin, improved the clarification results. Lambri et al. [22] showed that the efficiency of bentonite adsorption changed with the different pH values, with more protein being removed at 3.60 than at pH 3.30, this probably being related to the competition of hydrogen ions for adsorption.

Bayly and Berg [66] conclude that the elimination of the different wine protein fractions by bentonite addition did not happen in equal proportion. Bentonite first eliminates proteins with high pI (5.8–8.0) and intermediate MW (32–45 kDa). However, by 2D-GE, Hsu et al. [23], observed that, to stabilise wine, it is essential to eliminate proteins with lower pI (4.1–5.8) and lower MW (12.6 and 20–30 kDa), including glycoproteins, which represent a major fraction of proteins. This hypothesis is sustained by Lambri et al. [118] who, using five different types of activated sodium bentonite (with different labels), could selectively remove specific proteins implicated in the turbidity after heating. More recently, Jaeckels et al. [159] analysed the influence of a NaCa-combined bentonite on the protein concentration and composition of different wines showing a partial selectivity on protein adsorption. Notably, glycosylated proteins were not eliminated in considerable levels by bentonite. Using mass spectrometry, these authors showed that 96% of class IV chitinase was adsorbed by NaCa-combined bentonite, although changing adsorption behaviours were observed for different thaumatin-like protein isoforms, ranging from no removal to 98% elimination. The surface hydrophobicity of the proteins can explain these behaviour differences.

Dawes et al. [77] found that bentonite was not selective based on protein pI; therefore, bentonite fining could eliminate all protein fractions. Ferreira et al. [2] and Lambri et al. [22] stated that bentonite

is not specific for proteins and may also eliminate other charged species or aggregates. However, the existence of certain wine colloids is positive, as they confer structure and volume to the wine, and contribute to the retention of aromatic compounds [160]; therefore, bentonite can also interact with wine aroma compounds [161], resulting in a loss of wine aroma and flavour [20,22,162,163]. Moreover, most odour-active molecules are indirectly eliminated via deproteinisation, and only a few odour-active molecules are directly eliminated through adsorption [22]. The impact of bentonite on wine aroma depends more on the aroma chemical nature (hydrophilic or hydrophobic characteristics) than on the direct bentonite impact. In wine with more hydrophilic aroma compounds, with a good protein affinity, a more negative impact on wine aroma by the bentonite treatment is observed [22]. Therefore, an excessive quantity of bentonite may have a negative effect on wine sensory characteristics.

On the other hand, it has been described that fining grape juice with bentonite can decrease the total dose of bentonite needed for wine stabilisation compared to wine fining [20]; however, contradictory results have been obtained [164,165]. Must fining negatively affects the amount of available nitrogen, the varietal and fermentation aromas, and wine quality [20,22,162,166]. Another approach is the application of bentonite during wine fermentation, first described by Ewart et al. [167], and more recently by other authors [164,168], showing the potential to decrease the bentonite dose required for wine stabilisation and, therefore, improve wine quality.

Besides the described negative effects when using bentonite for wine fining, it also shows a cation exchange effect and can increase wine cation levels, especially sodium ions, above the maximum limits [169].

Due to the negative impact on wine quality and wine volume losses from wine protein stabilisation by bentonite, alternative solutions to wine protein stabilisation have been studied and explored and are still under research today.

6.3. Other Adsorbents

The application of other adsorbents beside bentonite has been studied to access their efficiency, concerning protein instability, in stabilising wine. Zirconium dioxide, a metal oxide usually known as zirconia, is a material characterised by low thermal conductivity, low corrosion potential, hardness and high thermal and mechanical resistance [170]. Zirconium oxide has demonstrated the ability to adsorb wine-unstable proteins [30], stabilising the wine and eliminating, preferentially, the wine protein fractions between 20–30 kDa. This adsorbent allowed the stabilisation of the wine via a continuous stabilisation process with minor negative impacts [31,171] on the wine's physicochemical and sensory characteristics [30,31]. Marangon et al. [32] showed that, although white wines are stabilised by eliminating unstable proteins through adsorption using zirconium oxide pellets enclosed into metallic cage submerged in wine at 25 g/L for 72 h, the authors observed a slight decrease in fruit aroma and flavour intensity. This work also showed that the regeneration of this material can be relatively simple.

Mercurio et al. [33] and Mierczynska-Vasilev et al. [34] also proposed natural zeolites as alternative adsorbents for wine protein stabilisation. Natural zeolites have a highly negatively charged external surface allowing their interactions with other cations, or polar molecules High zeolite/wine ratios allowed wine protein stabilisation, and treatment with zeolite-rich powder significantly reduced potassium ion concentration, improving the tartaric stability [33,34]. It was determined that zeolite particle size in the range 20–50 μm and treatment for 3 h were enough to reach complete heat stability using 4 or 6 g/L for Semillion and Sauvignon Blanc or Chardonnay wine, respectively. Furthermore, it did not significantly affect the content of the most representative phenolic compounds related to taste, and not significantly affect the wine aroma quality after treatment [172]. Additionally, in contrast to bentonite, zeolites did not cause significant wine volume losses and can be reused as soil improvers in agriculture [34].

The adsorption of wine proteins by phenolic compounds (tannic acid derivatives), immobilised on agarose chromatography resins, has also been applied to stabilise wine concerning wine-unstable

proteins [173]. Tannic acid derivatives revealed the capacity to eliminate wine proteins but suffered from a reduction in protein binding ability after a small number of regeneration cycles [173].

Sarmento et al. [29] studied the ability of diverse materials, such as swelling clays, low-swelling clay, ion-exchange resins, alumina, hydroxyapatite and silica gel as alternative adsorbents to eliminate wine proteins. The results show that packed columns with ion-exchange resins have good potential to adsorb proteins, but the colour and aroma of the wines were negatively affected.

6.4. Mannoproteins

In agreement with Gonçalves et al. [174], 32.2% of the total polysaccharides existing in white wine are mannoproteins. They originate from the outer layer of the yeast cell wall, specifically from *Saccharomyces cerevisiae*, since they make up 35–40% of the cell wall. These polysaccharides are glycoproteins, highly glycosylated and are covalently linked to an amorphous matrix of β-1,3-glucan [175] and containing 10–20% of protein and 80% of D-mannose, with lower amounts of D-glucose and *N*-acetyglucosamine [176,177]. Mannoproteins contain *N*- and *O*-linked carbohydrates bound to asparagine or serine or threonine residues, respectively. The *O*-glycosylated carbohydrates in mannoproteins are composed of short mannose chains linked to the hydroxyl groups of serine or threonine residue side chains through α-glycosidic linkages. These short chains are normally formed by mannose residues, in which the inner first two are 1,2-α-linked, while the outer mannose residues contain 1,3-α-D linkages [178] (Figure 3). The *N*-linked glycans can be extended with an outer chain of 50 or more α1,6-linked Man residues that are widely substituted with short α1,2-Man side branches terminated in α1,3-linked mannose residues. Phosphodiester-linked mannose residues can also be attached to the α1,2-linked residues (Figure 3). Mannoproteins present in wine are heterogeneous with a molecular weight between 5–400 kDa; however, Waters et al. [179] identified a mannoprotein with 420 kDa which was composed of 30% polypeptide and 70% carbohydrate, of which 98% was mannan. However, it is usually considered that the amount of mannoproteins released under winemaking conditions is too low to be effective in protein stabilisation [130].
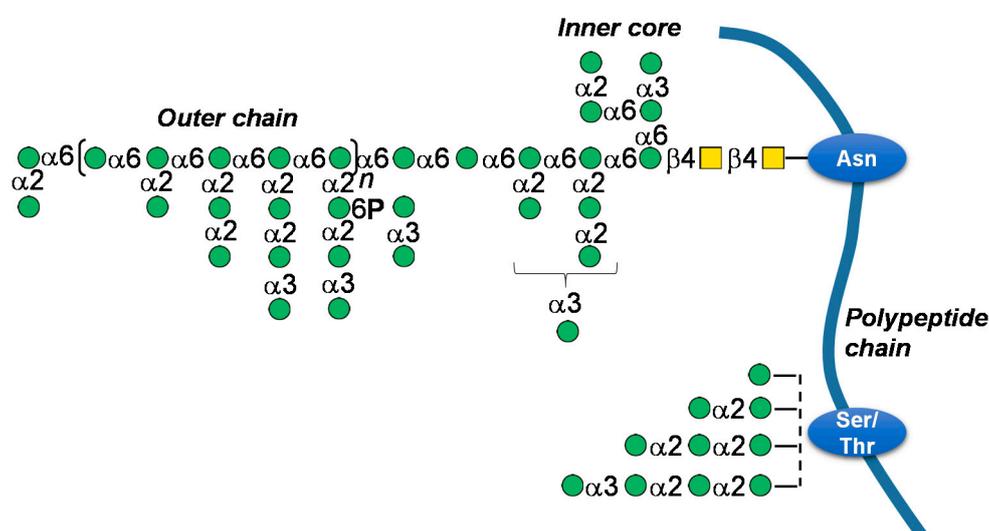

Figure 3. Some structural details of *Saccharomyces cerevisiae* mannan, adapted from Hernández et al. [180]. Structure was draw using the Symbol Nomenclature for Graphical Representation of Glycans, [181].

The application of mannoproteins in oenology has been suggested to decrease or even eliminate the use of bentonite and other treatments used for wine protein stabilisation. The treatment of wines with yeast mannoproteins was authorised by the International Organisation of Vine and Wine (OIV) in 2005, resolution: Oeno 4/01; 15/05 [182].

Mannoproteins for oenological application are extracted from the purified yeast cell wall, by enzymatic extraction, using exo-β (1→3)-glucosidase (EC 3.2.1.58) for glucan hydrolysis, or by

physicochemical processes, such as the heat treatment of the yeast wall using a citrate buffer at pH 7 [78,183,184]. Biotechnological tools, such as engineered yeast strains (*S. cerevisiae*), for increasing mannoproteins levels have been applied to increase commercial mannoprotein production [140].

Mannoproteins are often selected considering their useful behaviour in protein stabilisation and haze decrease in white wines; furthermore, they could also exert a positive effect on wine quality [4,17,19,38,179,185–190].

Waters et al. [17] have shown that wine mannoproteins protect unstable proteins, preventing wine turbidity when wine is exposed to high temperatures; these authors showed that this action does not prevent the precipitation of the proteins. Instead, they observed a decrease in particle size, justifying, in this way, the wine stabilisation observed when measured by turbidimetry.

Other glycoproteins have shown a protective effect, avoiding haze formation and conferring wine protein stability, including a fragment of yeast invertase (32 kDa) [130,191], wine arabinogalactan proteins with 210 kDa [186], arabic gum and arabinogalactan proteins from apples [125].

6.5. Polysaccharides from Seaweeds

Agar, carrageenan and alginic acid, polysaccharides extracted from seaweeds, due to their negative charge at low pH, can electrostatically flocculate and precipitate positively charged proteins and be a potential alternative for wine protein stabilisation [5,37,192,193].

Carrageenans are a family of water-soluble, linear, sulphated galactans that are extracted from red seaweeds, called carrageenophytes, where they are the most abundant cell wall constituents. Carrageenans are composed of α-(1→3)-D-galactose units and β-(1→4)-3,6-anhydro-D-galactose units (Figure 4). The galactose and 3,6-anhydro-D-galactose units of carrageenan can be sulphated with 15–40% of ester sulphate content and an average relative molecular mass well above 100 kDa. Several isomers of carrageenan are identified (κ-, ι-, and λ-carrageenans) and they differ in the number and position of the ester sulphate groups on the repeating galactose units (Figure 4) [194]. The primary differences that effect the properties of kappa, iota, and lambda carrageenan are the number and position of the ester sulphate groups on the repeating galactose units. The structure of κ-carrageenan is composed of alternating 3-linked-D-galactose residues and 4-linked 3,6 anhydro-galactose residues, with an ester sulphate content of about 25–30% and 4-linked 3,6-anhydro-galactose content of about 28–35%. Iota carrageenan has an additional sulphate group on C-2 of the 3,6-4-linked anhydro-galactose residue, resulting in two sulphates per disaccharide repeating unit. It has an ester sulphate content of 28–30% and about a 25–30% 3,6-anhydro-galactose content. Lambda carrageenan contains three sulphate groups per disaccharide unit, with the third sulphate group being linked at the C-6 position of the 4-linked residue, with an ester sulphate content of about 32–39% and no 3,6-anhydro-galactose content [195].

Agar is, like carrageenan, composed of heterogeneous populations of molecules differing in their physicochemical properties, composed mostly of two types of polysaccharides, including agarose, composed of a linear chain of 3-*O*-substituted β-D-galactopyranosyl residues joined by (1→4) linkages to 3,6-anhydro-α-L-galactopyranosyl residues (structure 1 in Figure 5, agarobiose). Agaropectin, the other agar polysaccharide, is a branched polysaccharide. Small amounts of sulphate ester groups and pyruvate 3,6-cyclic acetal groups may be present in agarose. The presence of L-galactose containing agarobiose have been described (structure 2 in Figure 5). These agarobioses can be produced in several variable forms by the different agarophytes depending on the gender and species which depend on their genetic characteristics. Several factors, such as substrate composition, nutrient availability, and hydrodynamic conditions can influence agarobiose production. However, an important factor is the harvesting period, since plants mature with the summer season. Considerable amounts of methyl ether groups are also present. These structural characteristics make agar the least hydrophilic and least water-soluble of the red seaweed polysaccharides, usually only being dissolved in water at 100 °C or higher temperatures, although preparations that hydrate and dissolve at about 80 °C are available. Agaroses are the fractions of agar that are, essentially, gel-forming polysaccharides, with

high molecular weights above 100,000 Daltons and frequently surpass 150,000 Daltons, as well as a low sulphate content usually below 0.15%. The remain agar fractions are essentially agaropectin with lower molecular weight, usually below 20,000 Daltons, typically 14,000 Daltons, containing higher levels of sulphate groups that can sometimes achieve 5% to 8%. This is far below carrageenan's, which range from 24% to 53%.
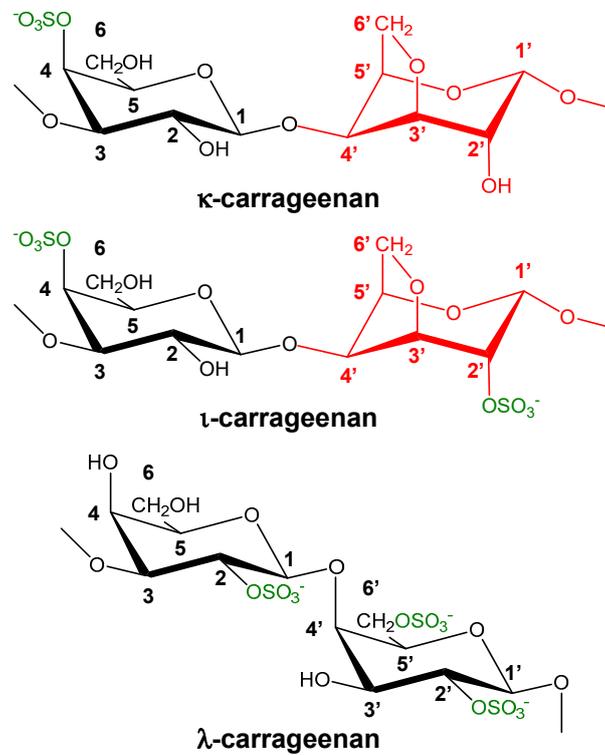
Figure 4. Schematic representation of the structural disaccharide repeating units of κ, ι and λ-carrageenans.
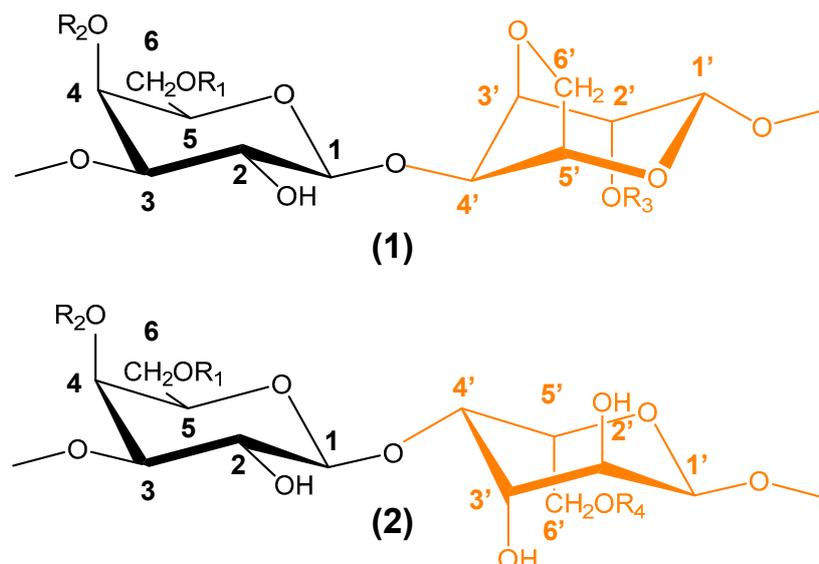
Figure 5. Schematic structures of the agarobiose disaccharides present in agar, (1) 3,6-anhydro-α-ʟ-galactopyranosyl containing agarobiose; (2) ʟ-galactose containing agarobiose. R_1 = H, CH_3 or SO_3^-; R_2 = H, CH_3 or SO_3^-; or R_1 and R_2 = $C(CH_3)CO_2H$; R_3 = H or CH_3; R_4 = H or SO_3^-.

Alginate is another natural algal hydrophilic linear polysaccharide family present in the cell walls of algae, typically obtained from brown seaweed, containing blocks of (1,4)-β-linked-D-mannuronate (M) and (1,4)-α-L-guluronate (G) residues, being also negatively charged at a low pH [196]. The blocks are composed of consecutive M residues (MMMMM), consecutive G residues (GGGGG), and alternating M and G residues (GMGMGM) (Figure 6). Alginates extracted from different sources differ in M and G contents, as well as the length of each block, although alginates with high G content have far more industrial significance [197]. The molecular weights of commercially available sodium alginates range between 32,000 and 400,000 g/mol. The viscosity of alginate solutions increases as pH decreases, and reach a maximum around pH 3–3.5, as carboxylate groups in the alginate backbone become protonated and form hydrogen bonds.
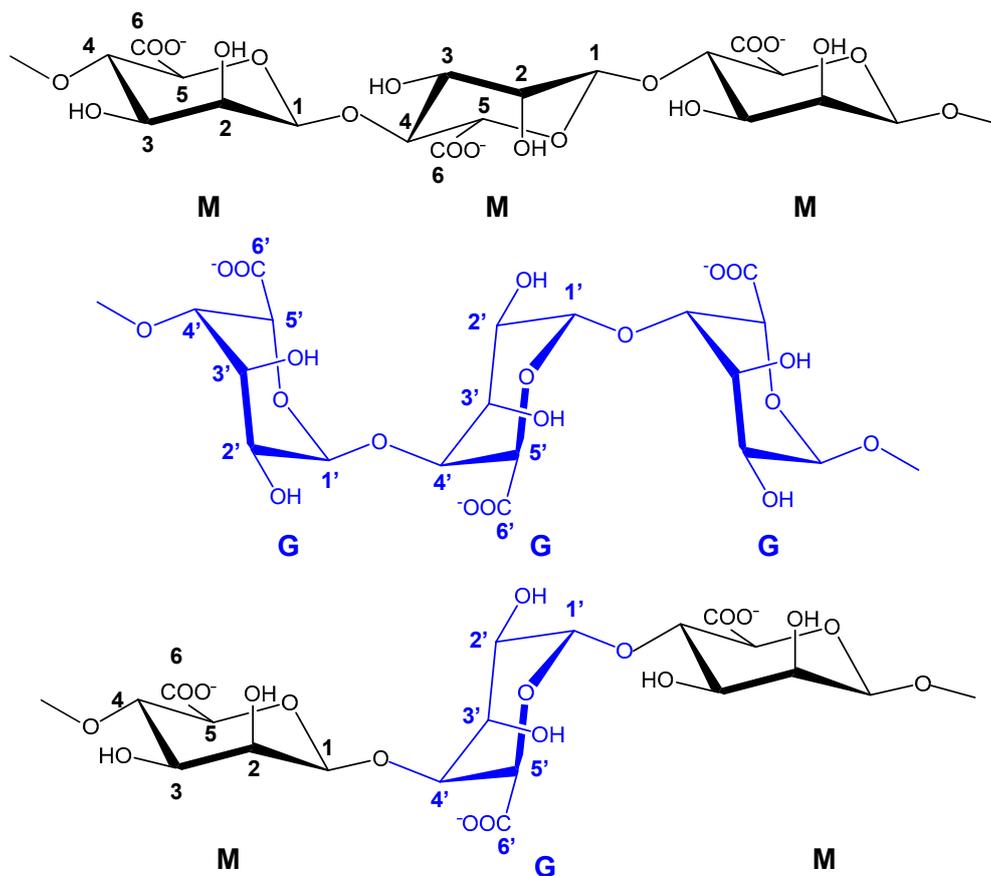

Figure 6. Schematic representation of the M-block, G-block and alternating block in alginate.

Purified carrageenan, dried carrageenophytes, purified alginic acid, and dried alginophytes showed a two-fold higher ability to flocculate and precipitate proteins compared to agar and agarophytes [193]. The better flocculation ability of carrageenan and alginic acid is related to their higher quantity of free negative charges relative to those of agar [193]. Carrageenan applications at different stages of winemaking were studied and the application time proved to be very important for its efficiency. Carrageenan addition before or during fermentation resulted in stable wines, comparable to the wines fined with bentonite [5,192]. However, although less carrageenan is required when applied after fermentation, there is the risk of obtaining wines that fail the heat test due to carrageenan remaining in wine, contributing to haze formation in the heat test and also the wine presenting a lower filterability. It was shown that carrageenan removed the same protein fractions adsorbed by bentonite [193], demonstrating that these polysaccharides might have a greater wine stabilisation capacity without modifying the tannin composition of wines when compared to bentonite [31]. The removal capacity of alginic acid was maximum at protein concentrations less than 50 mg/L; however, carrageenans

removed proteins at concentrations surpassing 400 mg/L [193]. Marangon et al. [192], also showed that carrageenan has no deleterious sensory impacts compared to bentonite-treated wines. More recently, Ratnayake et al. [37] studied commercially available carrageenans at different winemaking stages—in grape juice, during fermentation and in wine—to know the efficacy of protein removal and impact on the wine sensory properties. Three different types of carrageenans that were commercially available, κ, ι, and λ-carrageenans, were used. κ- and κ-/ι-carrageenans were effective in stabilising wines in relation to haze formation when measured by the heat test (80 °C/2 h, 20 °C/3 h) without negative impacts on the wine's sensory characteristics. Wine filterability and metal ion concentration changed and were dependent on the stage of carrageenan addition in the winemaking process and on the carrageenan structure.

6.6. Chitin/Chitosan

Chitin is the most abundant polysaccharide in nature after cellulose. Chitin is generally found as ordered crystalline microfibrils in the structural component of crustaceans and insects and is also found in the cells of fungi and microorganisms. Chitin is a linear polysaccharide composed of *N*-acetyl-ᴅ-glucosamine residues linked by β (1→4) linkages (Figure 7). Chitosan is produced commercially by the deacetylation of chitin. It is also a linear polysaccharide composed of randomly distributed β-(l,4)-linked ᴅ-glucosamine residues (deacetylated) and *N*-acetyl-ᴅ-glucosamine residues (acetylated). The degree of deacetylation in commercial chitosans is in the range of 60–100%. The amino group in chitosan has a pKa value of about 6.5. Therefore, chitosan is positively charged and soluble in acidic-to-neutral solutions with a charge density dependent on pH and the deacetylation extent. Chitin is insoluble in most organic solvents, such as water and dilute acids, due to the high inter- and intramolecular hydrogen bonding. Chitosan is soluble in dilute acidic solutions below pH 6.0, such as acetic, formic and lactic acids. Both chitin and chitosan are insoluble in neutral water. The chitosan solubility is controlled by the degree of deacetylation and the molecular weight. Although the majority of chitin and chitosan are produced commercially from shellfish waste through chemical treatment, for wine applications, only chitin (Oeno 367-2009 Chitin-Glucan) and chitosan (Oeno 368-2009 Chitosan) obtained from the cell walls of *Aspergillus niger* are allowed [182,198], with the same maximum limit of 500 g/hL. Although it shares the same structural features of crustacean chitins, most of the fungal chitin is present in cell walls linked to β1,3- and β1,6-glucans [199].
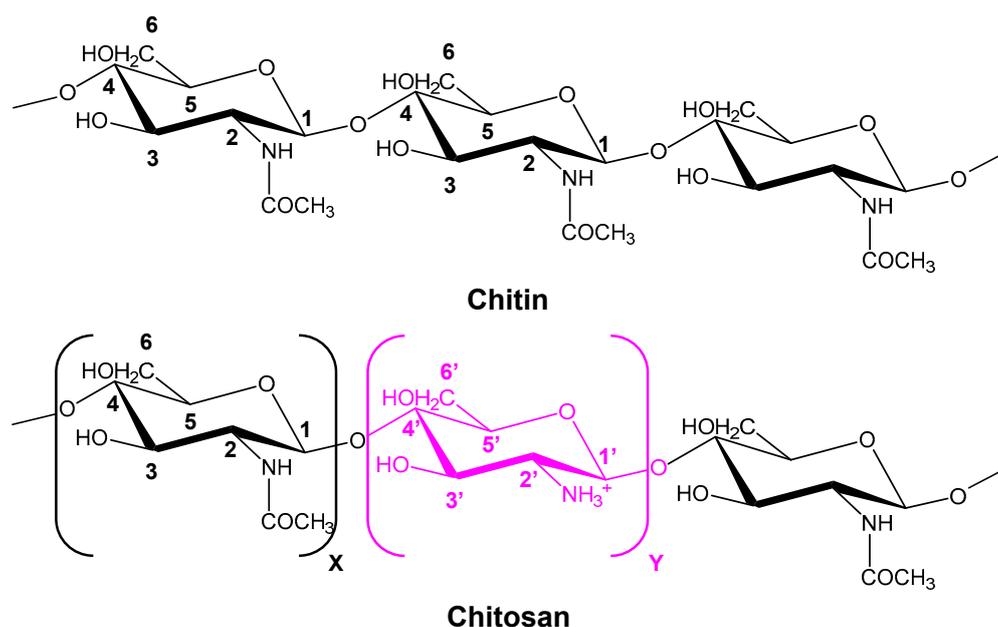

Figure 7. Schematic representation of chitin and chitosan. The relative values of X and Y determined the deamidation degree of the chitosan.

Studies carried out by Vincenzi et al. [35] showed that chitin can remove specific wine proteins, namely the grape class IV chitinases. The addition of 1 g/L of chitin to wine decreased the haze induced by the heat test by 50%, while the addition of 20 g/L of chitin decreased the wine haze by almost 80%. This haze reduction was directly related to the removal of the class IV grape chitinases. However, the impact on the wine sensory quality after chitin treatment was not studied. Interestingly, Ndlovu et al. [200] have shown that the use of yeast strains with high cell wall chitin levels can bind chitinases, offering a possibility for reducing wine protein haze formation. On the other hand, Colangelo et al. [36] showed that heat stability of the wines treated with 100 g/hL of fungal chitosan–glucan [201] improved in the 55–62 °C range and this was also due to the specific removal of chitinases.

6.7. Ultrafiltration

Membrane ultrafiltration using membranes with diverse molecular weight cut-offs has been studied, aiming to increase wine stability [2]. This technique is based on the ability of membranes with an MW cut-off, ranging between 1–100 kDa, to separate molecules according to their molecular weight. However, ultrafiltration to deal with the white wine turbidity problem has been relatively limited, since the potential of this method in the elimination of other high MW molecules, such as polysaccharides that could associated to the wine quality [202], is not known. Hsu et al. [23] have investigated the effect of ultrafiltration on wine protein stability by using membranes with different MW cut-offs, ranging between 10–50 kDa. The use of a membrane with an MW cut-off between 10–30 kDa cut-off removed 99% of wine proteins. However, proteins with an MW between 12.6–30.0 kDa were able to permeate the membrane [24]. These authors have also shown that the white wine treated by ultrafiltration has a high reduction in the yellow colour (A420), total phenols and in aromatic compounds, changing, in this way, the wine's aromatic profile [24,203,204]. Additionally, a decrease in the "body" and "mouthfeel" related to the removal of colloids was observed [205]. Gonçalves et al. [202] showed that using an ultrafiltration membrane with an MW cut-off of 100 kDa may be an alternative for wine clarification, in terms of wine quality. However, the efficiency of ultrafiltration depends on wine composition. Moreover, the high cost of equipment and operation and, eventually, the aroma losses associated with this operation, makes the membrane ultrafiltration process unattractive to the wine industry as an alternative for removing unstable proteins.

6.8. Proteases

The use of proteases for the hydrolysis of wine proteins [104,206,207], can be an interesting alternative to the previously mentioned stabilisation methods, including the use of bentonite, since it is expected that, comparatively, it has the potential to reduced wine volume loss and the aroma removal observed in the other treatments. However, although some proteases have been used in the beverage industry, papain from papaya [207–209] and bromelain from pineapple [206], they have been tested concerning their effectiveness in the degradation of heat-unstable proteins from white wine. Bromelain showed effectiveness in the degradation of wine proteins (approximately 70%) in the model solution as well as in wine, if immobilised in chitosan beads and used in a laboratory-scale stirred reactor [144,210]. Therefore, immobilised bromelain on chitosan beads seems an interesting treatment alternative to bentonite for white wine haze stabilisation.

On the other hand, the combination of heat treatment (90 °C for 1 min) and commercial proteases showed promising results in reducing the incidence of haze formation [28,104], by optimisation of the temperature and time needed for wine protein stabilisation, in an attempt to minimize the negative impact in wine quality. Protease application with flash pasteurisation has been shown to be effective at an industrial scale.

6.9. Acrylic Acid-Coated Magnetic Nanoparticles

Acrylic acid-coated magnetic nanoparticles were developed for the selective elimination of pathogenesis-related proteins from wines [211,212], by cation exchange mechanism due to the presence

of carboxylic acid groups in the modified surface. After these coated magnetic nanoparticles have been placed in heat-unstable wines, the proteins bind to the surface coating and can then be eliminated using a magnet. Even effective in removing most of the wine soluble proteins in the wines, these particles had to be applied to a concentration of 1.66% (*v/v*), which corresponds to 13.3 g/L. This can be associated with the pKa of the carboxylic group existing on the surface of the nanoparticles, which can be very close to the wine pH, affecting its cation exchange capacity. Recently Mierczynska-Vasilev et al. [53] studied the potential of these nanoparticles to be reused in multiple fining and regeneration cycles. The authors verified in the regeneration study that the acrylic acid plasma-coated magnetic nanoparticles, which underwent ten consecutive adsorption–desorption cycles, still retained close to the initial elimination ability for haze proteins from wines when 10% SDS solution and water were applied for surface regeneration.

7. Conclusions

White wine protein instability is linked with many wine external and intrinsic factors, such as wine exposition to high temperatures, wine pH, organic acids, metals, sulphur dioxide levels and phenolic composition and its degree of polymerisation, and some unknown factors (X factors) leading to wine protein haze formation and precipitation.

Technologies of white wine stabilisation require detailed knowledge about proteins and other wine compounds, as well the interactions that can happen between them. The principal proteins responsible for the haze formation present in wine are the pathogenesis-related proteins, thaumatin-like proteins and chitinases that are resistant to proteolysis during winemaking. Many works have been performed in the last years concerning wine protein stabilisation; however, sodium bentonite remains the most used and efficient method to remove unstable proteins from white wine, with its advantages and disadvantages. Nowadays, the methods to prevent protein haze are not specific enough and are not completely efficient. Controlling the wine haze-formation potential is critical in winemaking, with the heat test method being the most used, allowing for a better prediction.

It is, furthermore, essential to deepen our knowledge regarding the characteristics of wine colloids, where proteins have an important role. The wine protein characterisation, its interactions with other wine components and the effect of other instability factors, are necessary to mitigate this important wine industrial problem. More specific and accurate methods to predict protein instability are necessary for the wine industry. Finally, technology for protein stabilisation (concerning protein instability), with a lower sensory impact and higher specificity, allowing a reduction in the treatment dosage and, consequently, also decreasing the environmental impact of dealing with wine protein instability in the wine industry is still needed.

Conflicts of Interest: The authors declare no conflict of interest.

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