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The Role of Proteins On Colloidal Formation in Italian Red Wine

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Abstract

Until recently, the presence of protein colloids in red wines has been denied due to the belief that they precipitate during maceration. There is a noticeable gap in literature on this topic due to the challenges associated with red wine analysis. The current research aims to investigate the role of proteins in colloidal formation as well as their interaction with other macromolecules in red wine. The proposed method for studying protein aggregation is fluorescence correlation spectroscopy (FCS). Purified grape proteins will be labelled with fluorescent dye and added to deproteinated red wine samples for analysis. Colloidal aggregation kinetics are to be followed before, during and post fermentation using SDS-PAGE and FCS. The changes induced in the wine colloidal structures should affect the colloidal properties, thus increasing solubility of particles in the wine matrix. Research findings on the mechanism of protein aggregation will edify our knowledge of colloidal formation in red wines. These notions will be used to propose solutions to prevent and manage colloidal instabilities in red wines. Grape variety and ethanol concentration are expected to influence the aggregation activity and protein content in wine. Wine proteins may participate in the colloidal matrix as protein-phenol sub-aggregates, linked non-covalently to polysaccharides. The other colloidal entities expected to be present in red wine are of polysaccharides and phenols. These macromolecules are expected to change in quantity, pre and post fermentation. Findings will help in the better understanding of the mechanism of colloid formation in Italian red wines and allow new solutions to be proposed in the future for managing colloidal instability in wines. The main focus of the research is on red wine proteins, details on the involvement of tannins in colloidal formation will not be investigated.

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CONTENTS

1 CHAPTER 1: INTRODUCTION

Colloids in wine originate from mainly proteins, polysaccharides, and condensed tannins. Colloidal development in wines happens in two stages. Firstly, the formation of small invisible colloidal particles followed by the aggregation and formation of large particles which can diffuse light and precipitate as they grow bigger. Two groups of colloids have been identified in oenology, associated colloids, and macromolecular colloids (Moreno & Peinado, 2012). The first group is naturally present in wine (phenols and coloured colloids), composed of small molecules bound by Van de Waals, hydrogen bonds and hydrophobic interactions. The second group of colloids is composed of high molecular mass particles which form covalent bonds, such as polysaccharides and proteins (Moreno & Peinado, 2012). Polysaccharides can form protective colloids by coating the weak associative colloids and preventing their precipitation in a liquid medium. The coating mechanism of protective colloids occurs at different concentrations, below the destabilisation point. Protective colloids are important in wine making because of their stabilising effect in preventing the precipitation of tartrates (Moreno & Peinado, 2012).

Macromolecules in wine involved in colloidal formation can be heterogenous. Some are obtained from the winemaking practices using additives or fining agents while others originate from yeast and bacteria Marassi et al., (2021). The colloid sources vary greatly in type, quantity, and macromolecular concentration in each wine. Additionally, proteins and polysaccharides in wine interact differently with phenolics (Kassara et al.,2019). Wine proteins have been reported to interact with tannins, forming insoluble complexes such that an increase in tannin concentration also increased the size of colloidal aggregates (Mierczynska-Vasilev et al.,2021).

Previously there have been controversies regarding the degree of glycosylation of wine proteins (Waters, et al., 1993), for example a red wine colloid fraction (4.3 – 5.2%) made up of arabinogalactans, had a protein content of less than 10% (Pellerin P, Waters E, Brillouet JM, 1993). These molecules were previously considered as polysaccharides and hence are not listed in the class of wine proteins. Proteins are the most important colloids in musts and wines because they have been noticed to play a major role in haze and colloidal formation in wines in the last decade (van Sluyter et al., 2009; Vincenzi et al., 2011 & Ndlovu et al., 2019).

The interest in the study of proteins in wine came about because of their role in the formation of turbidity during storage, and sedimentation. Previous research focused mostly on white wine colloidal stability (Versari et al., 2011 and Romanini et al., 2021) and the development of protein haze during storage (Van Sluyter et al., 2015). It was believed that red wine did not have the protein haze problem because proteins are supposed to precipitate with phenolics extracted during the maceration process, but this is not the case. Recently there has been ongoing research on the understanding of colloidal formation in red wines (Sommer et al., 2016; Marassi et al., 2021), which will be further discussed in the next chapter. However, there is more research knowledge needed on the subject matter to better understand the participation of proteins in colloidal formation.

This review is going to give an overview on the subject, incorporating relevant content from previous and ongoing research in red wine colloids. In addition, some knowledge gaps identified during the review motivated the planning and designing of an experiment to identify the protein activity in red wine and monitor their aggregation prior to the formation of colloids. Here, an experiment is proposed on the analysis of protein aggregation activity within a red wine matrix. Analysing the protein aggregation activity in red wines will give us a better understanding of the protein behaviour under wine conditions and create an opportunity for the invention of fining and clarifying methods that specifically target red wine proteins with less to no side effects on the organoleptic properties of red wine.

Secondary data was obtained from reputable journals and textbooks online using the university library services and institutional access for paid journal articles. The chosen method of research and data collection was convenient for the research timeline, cheap and in line with Covid-19 health restrictions and regulations concerning laboratory space. Having secondary data allowed a large scope of research material to compare and review. It gave a clear trend on the progress in the research area and the methods applied over time. There has been a shift from the focus on white wine colloidal stability to red wine stability in the last decade. The use of comparable secondary data helped to identify the gaps in research and the relevance of the proposed methodology to the current existing literature on wines.

2 CHAPTER 2: LITERATURE REVIEW

2.1 Colloid forming molecules.

2.1.1 Proteins

Grape derived proteins, thaumatin like and chitinases (PR proteins) participate in colloidal formation in wines (Marangon et al., 2019). Wine proteins represent a small number of a larger group of proteins in grapes which remain soluble in wine. During storage, wine proteins undergo certain changes which lead to haze formation/ turbidity and colloidal instability. This phenomenon has been highly linked to thaumatin-like proteins and chitinases. Wine turbidity formation is due to spontaneous denaturation and flocculation of proteins that are sensitive to different temperature changes, hence occurring as colloidal suspensions (Mercurio et al., 2010). The precipitate is called protein Casse which is highly related to storage temperature and changes in wine pH (Mesquita et al.,2001). The protein bound to the Casse is derived from grapes and naturally produced by the plant as a protection mechanism against pathogens (Hayasaka et al., 2001). Some proteins are more stable in a particular wine than in others, depending on pH, temperature, ionic strength, tannins, concentration of ethanol, phenolics, and cations such as copper. It was previously believed that protein instability is less likely to occur in reds because the proteins are expected to precipitate out with polyphenols during maceration, but recent findings have proven otherwise (Marassi et al., 2021). Proteins derived from the must are thermally unstable and may cause protein Casse in poor storage conditions, while proteins released during yeast autolysis are thermally stable, particularly mannoproteins.

Grape variety plays a role in the protein content of wine. It was discovered that grape varieties that are not of viniferous origin have a high protein content than *Vitis vinifera* cultivars. Hence a high protein concentration is observed in the red wine after fermentation and can precipitate oenological tannins present during the winemaking process (Springer et al., 2016). This discovery brought about more questions of research regarding the effect of proteins on the wine matrix, macromolecular extraction, and colloidal stability in red wines. Apart from quantitative changes in the protein content during fermentation, qualitative changes have also been reported by other studies (Bayly & Berg 1967; Hsu et al. 1987; Canals et al. 1998; Ferreira et al. 2000) with the appearance and disappearance of some protein components. These proteins have been reported to show resilience to winemaking process (Ferreira et al., 2002; Falconer et al., 2010; Marangon et al., 2010). The concentrations in which they exist in wines range from an undetectable level to over 700mg/L. These concentrations depend on the viticultural, vintage, region, oenological practices, and cultivar (Vincenzi et al., 2011).

2.1.2 Polysaccharides

Grape polysaccharides have been observed to have minimal effect on the physical sensation of the wine mouthfeel (Vidal et al., 2004) but they influence wine turbidity. A small amount of pectic substances is extracted during fermentation, mainly rhamnogalacturonans (RGs) and arabinogalactan proteins (AGPs). They are soluble and resistant to acids and enzymatic degradation during

fermentation hence they remain in the finished wine. Flash release (flash détente), a modern maceration technique whereby the must is heated and released into a vacuum causing the grape tissue to rupture, has been noted to increase arabinose and galactose rich polysaccharides in the finished wine (Doco et al., 2007). Polysaccharides can exist as a wine colloid independent of other macromolecules, but they are able interact with proteins and phenols to form bigger colloids (Bindon et al., 2016). Polysaccharides are believed to give a stabilising effect to the wine colloidal matrix through noncovalent interactions (Marassi et al., 2021) as in the figure below:

However, the effect of polysaccharides on protein aggregation is quite distinct with the rhamnogalacturonan II-enriched fraction inducing aggregation, and a mannoprotein-enriched fraction showing a protective effect (Mierczynska-Vasilev et al., 2021). Low and medium concentration of rhamnogalacturonan I-enriched fraction showed a protective effect against protein aggregation while high rhamnogalacturonan I concentration encouraged aggregation. An interaction between proteins and tannins has been reported to cause precipitation of protein tannin complexes. But polysaccharides in the wine like medium partially prevented this precipitation, resulting in the encapsulation of the model protein (bovine serum albumin) BSA in well dispersed aggregates.

2.1.3 Phenolics

Colour stability is essential in red wine making; hence it is important to understand the role of colouring compounds in colloidal formation. Phenolic compounds (anthocyanin and flavanols) can form colloids (Moreno & Peinado, 2012). Flavanols polymerise to give condensed tannins, which highly react with proteins hence increasing the tannin content of the wine. Flavanols either polymerise through acetaldehyde bridges or condense with anthocyanins, yielding molecules that are less reactive with proteins because of their bulk structure. Flavanol condensation with anthocyanins stabilizes the color of wine. Polymerisation of flavanols occurs during aging of red wine. These reactions increase the molecular weight of the colouring compounds, for example in aged wines they are about four times higher than in young wines. In red wines, phenolic compounds are of great importance because they are the chemical substances responsible for the organoleptic quality of wine, mainly colour and astringency. The colour attributes of a red wine are an important parameter in defining quality of the wine as well as influencing the consumer's purchasing decision. Anthocyanin composition and its related interaction with other compounds in the wine matrix determines the resulting colour of the wine product. The role of colourless phenolics by co-pigmentation, contributes to the evolution and stability of the colour (Gordillo et al., 2013).

2.2 Types of colloids in wine

It has been reported by Marassi and colleagues (2021) that two colloidal entities exist in red wines with one consisting of proteins that are covalently linked to phenolics interacting with polysaccharides non covalently.

The second colloidal structure reported did not involve proteins, instead it consisted of polysaccharides and phenols only and appeared less compact as compared to the first colloid (Marassi et al., 2021). This showed that colloidal matter in wine can exist with or without proteins as in the figure below:

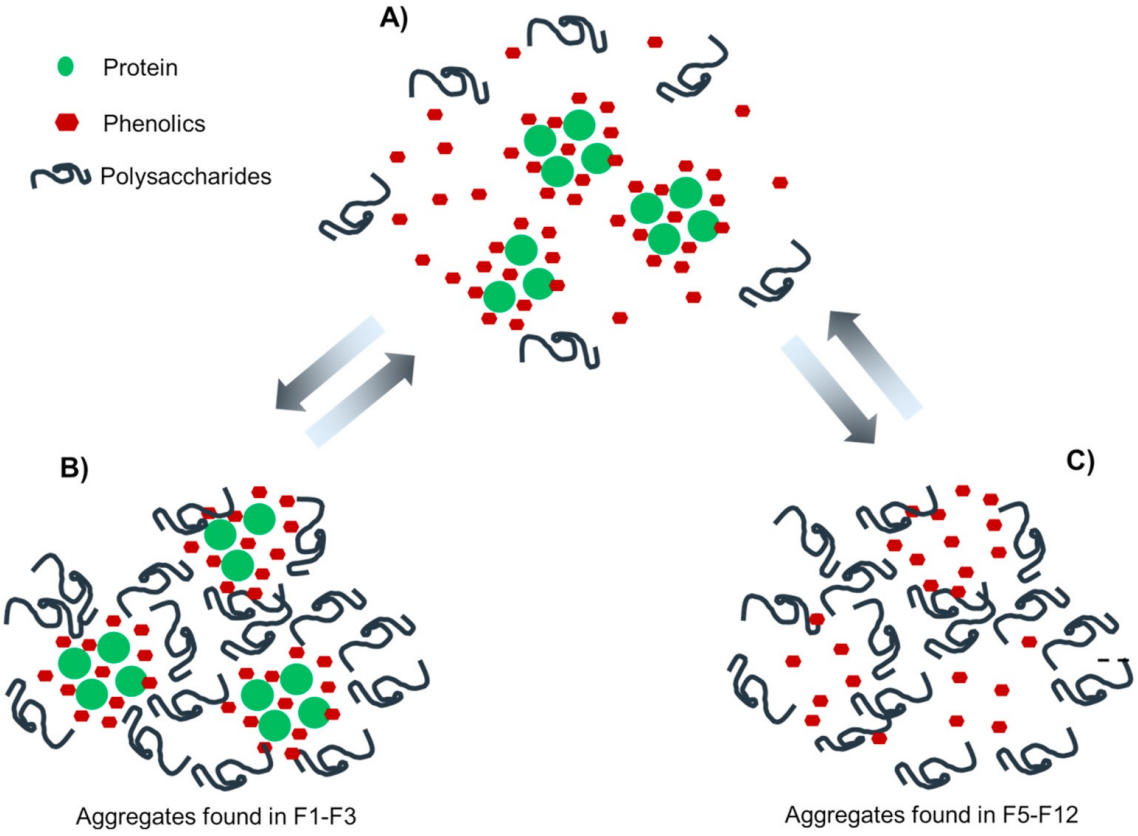

Figure 2.1. *"Proposed mechanisms for colloidal aggregation in red wine. A) Covalently linked protein-phenolics sub-aggregates and free polysaccharides and phenolics; B) Aggregates with low gyration radius (more compact); C) Aggregates with high gyration radius (less compact)."* Marassi et al., (2021).

2.2.1 Wine Protein Colloids

Though it has been previously reported that grape related proteins, chitinases and thaumatin-like proteins, are the main wine components associated with haze in white wine; greater protein concentration in wine does not necessarily indicate a greater haze forming potential (Van Sluyter et al. 2013). Variabilities associated with haze formation and colloidal stability need to be further investigated since instability can occur in low protein wines too (Cosme et al., 2020). Since the grape proteins are similar in both red and white wines, their influence in red wine colloidal stability might not be related to their quantity. For instance, a huge body of literature claimed that proteins did not exist in red wine in the past because they were believed to precipitate during maceration, unlike in white wines where haze was reported. However, despite the belief that proteins were not present or available in very small amounts in red wine, colloidal stability issues were still reported (Alcade-

Eon et al., 2014; Ningen et al., 2019). This may suggest that it is not solely proteins that cause the haze or instability in the wine colloidal matrix, but other macromolecules in the wine play a significant role. Despite that another study by Smith (2017) to investigate the impact of the wine matrix on haze formation in real wines found protein concentration and pH to be statistically significant in causing protein haze, a prediction model could not be developed based on these two factors due to a weak correlation ($R^2 = 0.56$). This discovery highly suggested that other factors play a major role in haze formation and collectively have an impact on colloidal stability.

Wine protein has been widely reported in rosé and white wine research, due to their haze problem. But minimal research has been done on red wines as it was previously believed that proteins were absent in wine because they are supposed to precipitate as tannin-protein complexes during the winemaking process. Researches at the Australian Wine Research Institute have proven that there is a significant amount of protein in red grapes that is capable of precipitating tannins that should be extracted in wine (Bindon et al., 2016).

2.2.2 Colloidal formation mechanism

The impact of different winemaking practices on colloidal formation

Colloids are generally larger than wine proteins and polysaccharides, hence proving that there is more than one macromolecular species in the colloid particles, of which the dimensions are modulated by different proportions of macromolecules. The maceration step in red wine making leads to the extraction of a lot of phenolic compounds and macromolecules from grape skins. The process of maceration determines the proportions because the kinetics of extraction of components are different. This results in a different composition of colloidal particles, size, and reactivity. The consequence of this determines the wine stability and wine quality output (Bindon et al., 2016). Hence, it's important to determine the colloids in red wines and their sizes.

During pre-fermentation treatment, solid grape residues in must quickly sink to the bottom of the tank. Suspended chemical material and colloids such as proteins, phenolics, crystalline compounds and microorganisms such as yeast, settle out slowly during and after alcoholic fermentation.
Indigenous colloids are naturally found in grapes and their concentration during wine making depends on the variety and maturity of the grape, as well as the pre-fermentative steps such as maceration (Nunan et al., 2001). After fermentation, the wine colloidal composition is rearranged by the metabolic activity of microorganisms, mainly *Saccharomyces cerevisiae* and *Lactobacillus*. The type of ageing used in red wine (lees ageing or wood ageing) also affects the ultimate colloidal fraction, thus affecting the organoleptic perception of wines and their age-ability (Dubourdieu et al., 1998).

The various macromolecular complexes that result with different winemaking techniques have been noticed to alter the extractability of tannins, polysaccharides, and protein (AWRI, 2017). Reconstituted wines were

used to study the role of changes in the macromolecular concentration and composition, to form colloids using NTA. Particle size was noted to increase (200nm) when the precipitate was recombined with tannins, while the addition of a monomeric fraction increased the concentration of smaller particles (90nm). A small difference in particle size was noted to be invoked by the microwave treatments and standard maceration techniques. However, microwave treatment increased the concentration of particles as the number of macromolecules increased in the wine samples. Hence suggesting that different wine colloidal behaviour may exist, but their functional impact is yet to be discovered (Bindon et al., 2016).

For sedimentation to occur, the density of the particles must be higher than that of the wine. The density range being, 0.992 (dry wines) to 1.050 (sweet wines). Particle size is also a factor to consider as they must not be too small because Brownian motion counteracts the sedimentation process (Cavicchi et al., 2018). This is crucial for wines that are not stirred and left to rest completely. Sediment deposits in wine are usually observed after the ageing period. This is due to the presence of previously suspended haze forming particles, colloids. When particles are suspended in a stable liquid medium for some time, turbidity is observed. A criterion that can be used to classify particles that cause haze formation in wine is the rate at which the suspended particles settle out. Particles that quickly settle at the bottom of the container do not cause haze, but particles that settle out slowly in suspension are responsible for haze formation (Moreno & Peinado, 2012). However, protective colloids limit flocculation and reduces sedimentation rate. Therefore, wines made from grapes high in polysaccharides take years to clarify naturally. Air is considered favourable to clarification as well as wood tannins. When the desired level of clarification is reached, the wine must be devatted (delestage) carefully to not disturb the lees at the bottom of the tank. Leaving wine in contact with lees for too long during natural clarification can cause odour and flavour problems due to anaerobic processes that produce hydrogen sulphide (H_2S) and other undesirable compounds. Some odours can be eliminated by aeration, but flavours are difficult to remove.

Different temperatures in the liquid mass cause different local densities and convection movements which slowly agitate the wine and interfere with sedimentation. These movements are greater in stainless steel tanks because they are good conductors of heat. However thermal fluctuations cause haze formation and colloidal instability, thus the reduction in disulphide bonds trigger the start of protein aggregation in wine systems (P Ruzza et al., 2019).

The slow denaturation of wine proteins is believed to lead to protein aggregation, then flocculate into a turbid suspension, forming precipitates (Waters et al., 2008). Most haze forming wine proteins have low isoelectric points and low molecular weight. PR proteins are mainly expressed during the ripening period after version. They are highly resistant to low pH, enzymatic and non-enzymatic proteolysis. Unfined white wine has been reported to contain about 300mg/L protein. Hence fining practices during wine making are relevant to lower the protein levels in wine and give it a better appearance sensorially. During transportation of grapes or

mechanical harvest, pathogen infection of the fruit may occur through skin contact or grapevine pathogens. This contributes to the increase in juice protein content. However an ultimately high protein content in wine results from machine harvesting grapes, causing an increased protein extraction from skins. Hence oenological control of proteins which may cause instability in wines is important.

In model wines it has been reported that low pH and high salt concentrations reduce the stability of proteins, hence contributing to aggregation (Smith P, 2017). This further proved the complex nature of wine interactions and haze/colloidal formation. AWRI (2017) also studied the interactions between proteins and polysaccharides in model wines. It was discovered that when proteins were in their native state, the interactions between macromolecules were limited as compared to unfolded proteins after heat exposure. This supports the predictions that fermentation is a causative step of protein colloidal formation and macromolecular interactions, with temperature as a driving force. There is minimal evidence on proteins having a solo impact on the organoleptic quality of wine especially red wine. They usually work together with other macromolecules to cause a significant impact on the organoleptic and sensory effect of wine. It was proved that proteins mainly interact with tannins to form insoluble complexes, and this is mainly applied in fining treatments to remove some of the most reactive tannins from wines (Marangon et al., 2019). Proteins in red wine are strongly linked to tannins, which makes their extraction and analysis difficult.

Microbial spoilage also plays a marginal role in colloidal formation. *Botrytis cinerea*, produces B-glucan which causes filtration problems in wine due to clogging. Ropiness, the viscous and oily appearance in wine can also occur due to lactic acid bacterial infection. Affected wines must be racked off, thoroughly stirred, and filtered at an intermediate pore size to retain the colloidal particles. Sterilisation must be employed to eliminate the causative bacteria. Streptococcal fungi can also cause ropiness by converting malic acid to lactic acid. It can also secrete highly viscous heteropolysaccharides formed by arabinose, mannose, galactose and galacturonic acid units. All these microorganisms can contribute to wine turbidity (Moreno & Peinado, 2012).

2.3 Oenological relevance of colloids: stability and organoleptic impact

Colloidal particles in wine impact its physicochemical properties including stability, taste and mouthfeel, and their content can vary greatly depending on the grape characteristics and vinification practices (Del Barrio-Gal´an et al., 2015; Martínez-Lapuente et al., 2019; Mierczynska-Vasilev & Smith, 2015; Vernhet, 2019). A study on tannin colloidal state in a wine-like medium was performed using Dynamic Light Scattering (DLS) (Poncet-Legrand et al., 2003) and showed that tannin solubility increases with ethanol content and decreases with an increase in ionic strength. Self-aggregation of tannins increased with their concentration and % gal while mDP had a more complex effect. There was an increase in aggregation first with mDP for relatively low molecular weight polymers but later a decrease was observed for higher molecular weight fractions. Hence higher molecular weight polymers can adopt a conformation in solution, enhancing their solubility. Another sensory study showed that astringency increases with tannin concentration while a high ethanol containing

medium decreases astringency (S Vidal et al., 2004). Polysaccharides can bind to tannins, causing a diminished astringency perception in wine (Carvalho et al., 2006; McRae & Kennedy, 2011; Riou et al., 2002; Watrelot et al., 2017). These studies prove that tannin aggregates have an impact on astringency in wine.

The texture of wine gives a clear distinction for a wine style and product value on the market. Clarity and colour stability being of great importance in the success of the red wine marketing industry. Research conducted by The Australian Wine Research Institute (2016) identified the key drivers of texture, bitterness, clarity, protein, and colour stability, to control and manage them during winemaking. The bitterness of red wine is mainly due to flavan-3-ols, flavanols, hydroxycinnamates and derivatives of benzoic acid (Gallego et al., 2016). Low molecular weight flavanols and their polymeric derivatives (procyanidins and tannins) are the main phenolics which contribute to astringency. These have been reported to decrease in red wine during maturation and ageing period due to polymerisation and precipitation reactions (Pineda et at., 2017). To obtain high quality full bodied red wines with stable deep colours and a balanced taste of bitterness and stringency, it is vital to control and monitor the phenolic composition during winemaking. Previous research has shown that wines produced from less clarified juices had high concentrations of total polysaccharides due to a high concentration of mannoproteins. When polysaccharides bind to tannins, a decrease in the astringency perceived in wine is observed (Watrelot et al., 2017).

Juice clarity affects the flavour and mouthfeel of wine (AWRI, 2017). Sauvignon Blanc and Chardonnay juice samples with a high content of solids produced bitter wines with high viscosity as compared to juice with less solids. The observed phenomenon was related to the concentration of wine phenolics and their composition. Fermenting the juice on solids causes a high production of esters, higher alcohols, fatty acids, and thiols (AWRI,2017). The composition of fermentation lees proves that chemical particles contribute more to haze as compared to plant particles and microorganisms which settle out at a higher sedimentation rate (Moreno & Peinado, 2012). There are two types of chemical-based haze forming particles: organic and inorganic compounds. Organic compounds include crystalline precipitates formed by potassium bitartrate, tartaric acid, mucic acid and calcium salts. Amorphous precipitates are formed by proteins related to tannins and heavy metals of colouring matter condensates. Particles originating from inorganic compounds are white casse (iron + phosphate ion) and copper casse (Copper + sulfide ion) as well as a combination of tin and proteins (Moreno & Peinado,2012).

Various particles responsible for haze formation and deposits in wine aggregate the colouring matter and the colourless phenolics, hence affecting the sensory quality of red wines (Etaio et al., 2008). These colloidal forming particles need to be removed or stabilised to prevent alterations of taste, flavour and colour prior to bottling and consumption. Knowledge on the grape and wine composition of polyphenols, polysaccharides and proteins is needed to control the colloidal matrix in wine better to prevent any organoleptic changes in the product during storage.

2.4 Colloid-related instabilities in wines

2.4.1 White wines

Haze colloidal proteins, thaumatin like and chitinases (PR proteins) are grape derived. At the normal pH of wine proteins exist as positively charged colloids responsible for the protein casse (Moreno & Peinado, 2012). The protein casse is the haze that is observed in white wines and rosé wines during storage at inappropriate temperatures. The other cause of this haze is the reaction between the proteins in wine and the tannins from the cork (Tian & Harrison, 2021). Studies on wine protein research which analysed the change in berry derived proteins during wine making showed a decrease in the concentration of total proteins **(**Manteau et al. 2003, Luguera et al. 1998, Hsu et al. 1987, Dizy and Bisson 2000, Canals et al. 1998). On the other hand, some studies, (Fukui and Yokotsuka 2003, Dizy and Polo 1996, Bayly and Berg 1967) reported an increase in soluble protein content post alcoholic fermentation. An explanation for this increase could be that proteins were released from yeast cells during alcoholic fermentation. However, most of the previous studies proved the difference in the protein content mainly before and after fermentation, between the grape must and the wine but they did not highlight the changes in the protein itself (type and quantity) during the fermentation process. Knowing and monitoring the changes in protein at different wine making steps would help us have a deeper understanding on the subject matter.

A study on matrix effects on protein haze formation (McRae, et al., 2018) proved that protein haze formation in white wine is due to ionic strength, sulfate, phenolic compounds, and polysaccharides. The protein polysaccharide interaction studied using isothermal titration calorimetry showed limited interaction in the macromolecules when proteins were in their native state. However, after the protein unfolded due to heat exposure, weak interactions were observed between proteins and polysaccharides. Matrix parameters' influence on the stability of protein in wine were studied. Factors explored were pH, ethanol, and salts. Their influence on the stability of four proteins was investigated and the results suggested that ethanol did not influence protein stability, but pH proved otherwise. This encouraged more research on the complex wine interactions and colloidal haze formation. Another study proved that the effect of protein fining treatments in white wines depends on the chemical nature, quantity, concentration, and the nature of the proteins present in a wine (Lambri yet al.,2010).

2.4.2 Red wines

In red wines, colloidal related instabilities can be seen through wine turbidity and sedimentation. Proteins found in red wines have similar properties to those found in whites, mainly composed of grape pathogenesis-related proteins (PRPs) with the same properties across all *Vitis vinifera* cultivars (Wigand et al., 2009). However, a study on red wine proteins using 2D electrophoresis and mass spectroscopy analysis using Cabernet samples did not show the presence of chitinases (Mainente et al., 2014), which is usually reported in white wines (Marangon et al., 2011). The absence of chitinases maybe due to their loss during the maceration step as these proteins interact with polyphenols, giving complexes removed by sedimentation. Some red wines

have been seen to show a lipid transfer protein (LTP) in their protein profile, of which the occurrence and distribution is dependent on the wine variety (Wignand et al., 2009).

LTPs were identified in Portugieser red wine, Chardonnay and grapes (Wigand et al., 2009: Okuda et al., 2006 & Vassilopoulou et al., 2007). Wigand reported that these proteins may exist in wines as hydrolysis products. Some wines (Shiraz, Cabernet Sauvignon, Chianti, Bordeaux) did not have LTPs. It is possible that variety, growing conditions and vinification procedure may influence the protein composition of the resulting wine. LTPs are found in the skin of grapes, hence time of contact during maceration may influence their extraction. In plants, LTPs exist as a defence mechanism for adverse conditions, hence the climatic conditions have an influence on the concentration and occurrence of these proteins. LTPs are resistant to proteolytic activity and acidic conditions, hence they can survive the vinification process and still be present in the finished wine (Wigand et al., 2009). Other proteins reported in red wine from *vitis vinifera* cultivar are vacuolar invertase and endochitinase. (Pastorello et al., 2003 & Okuda et al., 2006), but the main suspects involved in instability are thaumatin-like proteins and chitinases (Wigand et al., 2006).

2.5 Prevention of colloid-related instabilities in wines

Haze formation is difficult to predict hence it poses a high risk to the winemaker's product because precipitation may become visible after the wine has already been bottled. This results in a great loss for the wine maker due to product recall of the entire line and rebottling of the wine. The winemaker's reputation and product image may be ruined if consumers purchase a hazy product. Necessary preventative measures must be taken by the winemaker to prevent turbidity, including the use of additives, stabilisers, filters, and fining treatments.

Stephan Sommer and colleagues (2016) conducted a study to identify the source of haze in red wine after caboxymethyl cellulose (CMC) addition (a stabilizer against tartrate precipitation) and characterised the dynamics of precipitation. Of the ninety commercial wines analysed from eight grape varieties and tested with commercial CMC products, seventy-four samples showed precipitation withing two weeks. The precipitates of four representative samples were then analysed for elemental composition (CHNS analysis) and the solubility was checked to determine the nature of the solids. All precipitates contained 50% proteins and 50% CMC and polyphenols. Interactions between CMC and bovine serum albumin are pH dependent in wine-like model solution. The objectives of this study with real wines and model systems were to identify the source of turbidity in red wine treated with CMC.

Carboxymethyl cellulose (CMC) is a common additive to foods and beverages. It is a heterogenous ingredient with a complex mixture of polysaccharides of different molecular sizes (Hoogendam et al., 1998). Uses of CMC as a stabilising agent for tartrate crystals in wine (Bosso et al., 2010 & Gerbaud et al., 2010). CMC inhibits crystal growth of potassium bitartrate and is added to wine immediately before bottling. Other

stabilising agents (metatartaric acid or mannoproteins) have been seen to provide short term wine stability for a few months but with no absolute protection from crystal precipitation (Guise et al.,2014 & Lasanta and Gomez, 2012). CMC was discovered to remain in solution while preventing crystal precipitation in wine and not altering any sensorial qualities (Salagoity et al., 2011).

Among the factors that influence potassium bitartrate super saturation in a wine solution and end up causing the instability of tartaric salts in wine, is an increase in ethanol during fermentation is highly significant (Low et al., 2008). The use of Cold stabilisation in red wines is due to higher ethanol concentrations, lower acidity, increased potassium associated with longer ripening periods and higher pH values (Boulton et al.,1996). This leads to tartrate precipitation in wines, hence the need for stabilization.

2.5.1 Role of fining in colloidal removal

The maximum recommended protein content of wine is 275mg/L (Bayly and Berg, 1967; Moretti and Berg, 1965; Moio and Addeo, 1989), hence fining methods are employed during wine making to remove excess proteins. The main purpose of stabilising agents or fining agents, irrespective of the protein type, is to remove the excess protein from the wine without altering the quality of the final wine product. Protein sizes and their high solubility make it difficult to remove them by simple filtration. (Eisenhour and Brown, 2009). This issue has been tackled over the years using bentonite (Lambri et al., 2010), and there has been great success in its use in white wines.

Organic and inorganic fining agents can be used to clarify wines, especially white wines, to prevent haze formation. During fining, a floccular precipitate is formed in wine to absorb the haze forming particles and colloidal properties during settling. A rapid fining process is required as well as minimal loss of wine in the sediment or lees. The fining process used must not cause undesirable effects to the wine such as removal of desired flavour or addition of undesirable flavour compounds.

The purpose of fining agents in wine is to soften its texture and reduce astringency or bitter flavours. Fining also aims to remove proteins which cause turbidity. In some cases, it is used by winemakers to reduce the colour intensity of the wine by adsorption and precipitation of tannins and polymeric phenols. The fining agent reacts with the compounds on wine chemically or physically, forming a new complex that separates from the wine medium through precipitation (Puig-Deu et al., 1999; Ribreau-Gayon et al. 2000 & Marangon et al, 2019).

The fining agents that are mostly used in the wine industry are gelatine, isinglass, egg white, casein, skim milk, bentonite, carbon and polyvinylpolypyrrolidone (PVPP). There are factors that determine which fining agent to use for a certain wine. An important factor to consider is the destined market of the wine and the winemaker's wine style. Trials usually take place in the laboratory before the use of a chosen agent in the

cellar. Laboratory replicates of the identical components used in the cellar for the finished wine must be obtained. The sensory effect of the fining addition can be assessed by setting up a control sample with no fining agent. It is advised that fining agents must be tested for taints or off flavours before use (Lambri et al., 2016).

Table 2.5.1: Different fining agents and their efficiency, reproduced from Zoecklein (1987).

	Fining agents Comparison			
Decreasing efficiency ↓	**Colour removal**	**Tannin removal**	**Tendency to over fine**	**Clarity and stability**
	Carbon	Gelatine	Carbon	Bentonite
	Gelatine	Egg white	Gelatine	Carbon
	Casein	Isinglass	Egg white	Isinglass
	Egg white	Casein	Isinglass	Casein
	Isinglass	Bentonite	Casein	Gelatine
	Bentonite	Carbon		Egg white

Table 2.5.2: Typical rates of additions of fining agents in red wine reproduced from Ribreau-Gayon et al. (2000).

Type of product	Typical doses used (mg/L)	Characteristics
Gelatine	30 – 240	Very good fining agent for tannic wines. Affects only the most aggressive tannins. May make wine softer or thinner.
Egg white (egg albumen)	30 – 150	Very good fining agent for tannic wines with some age. Tends not to remove protective colloids.
Casein	50 – 250	Good clarification. Treats and prevents oxidation. No over fining.
Bentonite	200 – 500	Clarification of young wines. Eliminates colloidal colouring matter. Facilitates sedimentation of protein fining agents.
Carbon	50 – 2000	Removes off-odours. Effective in colour reduction (browning and pinking).
PVPP	100 – 450	Reduces bitterness. Brightens colour. At high rates can result in colour and flavour stripping.

2.5.2 Effect of stabilisation methods on the sensory of wine

Organoleptic quality of the resulting wine is greatly influenced by the method of stabilisation (Mecurio et al.,2016). During protein adsorption, significant interactions may occur with other compounds linked to the wine matrix, causing an enhancement or degradation of aroma compounds in the wine. Due to this reason, there is still ongoing research on the use and development of appropriate wine stabilisation methods.

During winemaking, bentonite is used to improve the limpidity and stability of wine. Bentonite has exchangeable cations in its lamellar structure which contribute to its surface characters, adsorption behaviour and exchange capacity. The use of bentonite in juice or wine fining has detrimental side effects on the colloidal and protein stability, aroma compounds, as well as the sensory profile. The impact of commercial bentonite on juice and wine fining was investigated on the colloids, phenolic compounds, proteins, and aroma

compounds in red wines (Lambri et al., 2012). Parameters assessed to check the effects of bentonite in wine were heat stability, total proteins and haze forming proteins concentration and aroma content.
The main objective in the use of bentonite in oenology is to reduce the protein content through fining to induce precipitation of suspended particles, causing them to form sediments at the bottom. Bentonite can be added to the must to help in the precipitation of suspended solids (Puig-Deu et al., 1999). Must clarification cause a reduction in the protein content of the grape juice, but it's not related to the final colloidal instability in a wine because other compounds come to play during the different winemaking steps such as, phenols and polysaccharides (Lambri et al., 2012).

While bentonite is mainly used in white wines, it is also used in red wines as a softening method during fining. This is to achieve the removal of tannins and polyphenols with the aim of improving the astringency of the product. Other fining agents which are mainly used in red wine production are gelatine, caseins, albumin, and isinglass (Ribéreau-Gayon et al., 2000). Despite the side effects of bentonite in the reduction of colour by absorbing anthocyanins, some winemakers still use it in limited amounts because its affordable, highly effective, and easily removed through sedimentation, and its readily available (Lambri et al., 2016).

The use of proteolytic enzyme during short term heat exposure to induce PR protein denaturation was suggested as an alternative method to bentonite fining. Yeast mannoproteins can be added to wine to decrease the haze particle size. They do this by competing with wine proteins for non-proteinaceous wine components necessary for the formation of insoluble protein aggregates (Waters, 2005). Use of bentonite in red wines should be limited because of its ability to reduce colour by adsorption of anthocyanins.

2.6 Protein stabilisation strategies

Several methods have been explored in the attempt to stabilise wine colloidal proteins, but most research (Lambri et al., 2010; Versari et al., 2011; Mierczynska-Vasilev et al., 2017; Cosme et al., 2020) was mainly focused on white wines unlike red wine. Here we review the strategies that have been employed so far to stabilise wine proteins.

Magnetic separation technology for selective removal of pathogenesis-related proteins from wines was developed (Mierczynska-Vasilev et al., 2017 & Mierczynska-Vasilev et al., 2019) as a rapid selective method to separate pathogenesis-related proteins from white wines that did not go through fining. In the past, magnetic nanoparticles (MNPs) have been used in biotechnology (Seenuvasan et al., 2017) to separate products or in the removal of immobilised enzymes from reaction mixtures, and in separating cells, microorganisms, and yeast. It was hypothesised that careful tuning of the surface functionality of magnetic particles could be used to selectively capture pathogenesis-related proteins from wines. These would then be separated from the system by applying an external magnetic force (Mierczynska-Vasilev et al., 2017). The method was used in

protein removal in white wine, where controlled functional groups coated onto magnetic particles were used to bind protein molecules. Firstly, the magnetic beads were coated by plasma deposits of acrylic acid vapour to generate COOH-rich surfaces. In the second step, the coated magnetic nanoparticles were added to white wine, then the final step involved the use of an external magnet to separate the particles from the wine.

Another investigation was done on proteases with the potential to degrade haze forming proteins (Van Sluyter et al., 2013). The main protease that was studied was BcAP8 which is a *Botrytis cinerea* enzyme. In the presence of heat, more haze formed with the addition of BcAP8 but in the absence of heat the proteins were successfully degraded. Protein stability is better achieved using an enzyme together with another technique, for example a combination of aspergillopepsin (AGP) enzyme with flash pasteurisation (Marangon et al., 2012).

The use of flash pasteurisation without AGP enzyme was also investigated as an alternative stabilising strategy. It was found that heating grape juice for a minute at 75 °C was enough to produce a stable wine using Semillon and Sauvignon Blanc. However, Muscat Gordo had to be heated longer (three minutes) to obtain a stable wine because it contained a higher protein content. Pasteurization proved to be a sufficient technique to stabilise wines and can be an alternative stabilising strategy for the wine industry.

However, there are issues associated with flash pasteurisation in the wine industry (AWRI, 2017) hence an alternative protein unfolding stabilising method that uses a vortex fluidic device with sheer force instead of elevated temperature to unfold proteins has been exploited. It proved to be effective for protein folding and unfolding of enzymes (Britton et al.,2017). When applied in wine research, change in protein concentration was observed in the treated and untreated grape musts (AWRI, 2017). This suggests that protein unfolding had not occurred since unfolding would cause a notable precipitation and hence a change in soluble protein concentration would result.

A lot of previous research has been done on white wine clarification and stabilisation. This is because white wines had a lot of sensorial problems linked to haze formation and this would affect the wine market due to consumer dissatisfaction. Several fining methods and other clarifying methods applicable to white wine were invented. However, some of the existing methods cannot be applied to red wine colloidal instability and hence more work need to be done in this area. Protein stabilisation in white wines has been mostly targeting haze formation, which is not a major factor in red wines, hence some fining methods used in white wines only clarify when applied to red wine but do not stabilise it.

The use of Acacia gum in protein stabilisation of wine has also been explored (Nigen et al., 2019). Arabinogalactan-proteins (AGPs) from *Acacia senegal* gum prevent precipitation of colouring matter in red

wine. These AGPs are used as a protective colloid in young red wines. Acacia Senegal gum have been proven to be efficient in stabilizing iron hexacyanoferrate salts in hydro-alcoholic solution (Nigen et al., 2019). A study on the protective activity of Acacia Senegal gum and its fractioned macromolecules showed that the successful prevention of colloidal instability of iron hexacynoferrate salts and polyphenols in model solutions and young red wine. Salts precipitation was prevented by the electrostatic binding of Ca^{2+} with the negative charge of amino acids, and uronic acid monosaccharides, all associated with the protein backbone of AGPs. The protein content of AGPs determine the protective activity and thus, the more the protein content the more efficient the colloidal stability is. A difference was noted in the protective activity between AGPs from three HIC fractions due to their rate of glycosylation which changes the accessibility of the protein to its environment, consequently the physicochemical properties with a hydrophobic behaviour (Nigen et al., 2019).

2.7 Analytical methods used in wine protein analysis.

Most reviewed papers have a lot of information on white wine colloidal formation and the macromolecules involved. Research performed on red wine so far, shows a lot of progress in the development of analytical methods and the study of polysaccharides, tannins, and macromolecules, but there is missing information on how proteins play a role in colloidal haze formation. The applicable methods from past research can be used in combination, to investigate how proteins in red wine affect or influence the colloidal system.

The effect of proteins on red wine quality is not clear since red wine colloids have been difficult to study. A few techniques have been used to quantify and measure the size of the different colloids, but they fail to characterise their chemical composition and behaviour. Dynamic Light Scattering (DLS) has been used by several researchers (Riou et al., 2002 ; Legrand et al., 2003; Pascal et al., 2008; Nguela et al., 2016) to find the size of different colloids but not their chemical properties. DLS has been widely used because it is easily accessible. However, it has its drawbacks regarding its principle of technique. DLS is time consuming and does not do well for non- transparent samples like red wine because small aggregates or even dust can be a hindrance to size determination. DLS is not selective for the component of interest (proteins) and gives a low signal strength.

Protein quantification methods have evolved over time. The KDS quantification assay method was proven to be more effective than Bradford assay (Vincenzi et al., 2005) which had been previously used in wine protein quantification. KDS was able to quantify proteins in the presence of interfering compounds (phenolics and sugars). A relatively higher protein content was detected using KDS, mainly due to the Manzoni Bianco wine which already has a high protein content as compared to other varieties. It was observed that Bradford assay may have previously underestimated the true value of the concentration of protein and hence the lack of long backdating evidence on wine protein research.

Proteins are usually found in low amounts in fermented beverages and their study is hindered by the presence of interfering compounds such as phenols (D' Amato et al., 2010 and Tolin et al., 2012). This was found to be true for red wines due to their composition hence making it difficult to study red wine proteins in the past years (Wigand et al., 2009). To overcome this problem, a KDS precipitation method was utilized for wine protein recovery and quantification (Vicenzi et al.,2005; Fusi et al., 2010 ; Tolin et al., 2012). Federica Mainente and colleages (2014) modified the protocol by adding PVPP to red wine before protein precipitation to remove the phenols that interfere with protein analysis. This method allowed them to obtain pellets suitable for colorimetric protein quantification.

The isoelectric point of proteins is affected by bound DS, hence they had to remove the ionic detergent to carry out the first dimensional separation for two-dimensional electrophoresis analyses. Proteins were precipitated according to their experimental protocol, but 20% TCA treated samples hardly showed any solubility probably due to irreversible protein aggregation and denaturation (Chen & Liang, 2011). However, for the remaining samples that proceeded for further analysis, only AU-PAGE and IEF analyses showed a good protein separation, and the gels were tested in SDS–PAGE. The presence of 10% saccharose in the DS removal step associated with the effective buffer solubilisation was identified as the best choice for protein solubilisation since a well resolved proteomic profile of red wine was obtained (Mainente et al., 2014).

IEF separations using a narrow pH gradient (pH 3.9 – 5.1) were performed to obtain a good resolution of Cabernet wine proteins. A smeared signal in the more acid area was noticed. This was probably due to the traces of interfering molecules which were still present in the protein prepared sample. The protein spots cut out from 2-D Coomassie maps were further analysed by nano-HPLC ChipMS/MS. Most of the identified wine proteins originate from grape, mostly classified as the pathogenesis-related proteins (PRPs), such as thaumatin-like and osmotin-like proteins (Wigand et al. 2009). This 2-D electrophoretic approach to map out red wine proteins and analyse with mass spectrometry can be used as a starting point for a deeper analytical study of red wine proteins. The same method can be used to analyse and compare the PRPs from different wines obtained from other grape varieties to see how the different cultivars derived proteins relate or differ; to check if the protocol is specific to one grape variety or universal; how the pH gradient changes with each variety since they all differ in acidity naturally; to compare the protein profile of each wine variety and use it to predict the chances of haze formation during ageing and storage.

Considering that colloidal aggregates in wine have a potential to be stabilised by weak interactions, special purification methods that preserve their nature are needed. Provisionally, size exclusion chromatography (SEC) method has been used to achieve this (Watrelot et al., 2017). The downside of this method is that colloids separated by chromatography can interact with the stationary phases (Coelho et al., 2017), they can also be destructured when placed in a physico-chemical condition different from wine conditions (Scollary et al., 2012). This led to other techniques being applied, such as Nanoparticle Tracking Analysis (NTA). The impact of

matrix components on the stability of chitinase protein was investigated using NTA (Smith P, 2017). The study confirmed that protein haze formation in white wine is a multifactorial process where ionic strength, sulfate and other macromolecules such as phenolics and polysaccharides all contribute to white wine protein haze potential and colloidal instability.

This technique was used recently to assess the hydrodynamic diameter and concentration of nanoparticles in liquids and for analysing sizes of simulated wine supramolecular structures. Reconstitution experiments have been used so far to obtain progressive results in this area by fractioning wine macromolecules and adding them back in a model system (Bindon et al., 2016); and separating colloids from wines in harsh conditions which inevitably affect their structure (Mierczynska-Vasilev & Bindon, 2019).

It has been widely reported that protein analysis of red wines is extremely complicated because of the presence of interfering compounds such as polyphenols and polysaccharides (Moreno-Arribas, Pueyo, & Polo, 2002). This limited the number of studies on red wine protein analysis in the past years but recently there has been a great development in the research on red wine and the development of analytical methods that deal with some problems mentioned in the past. For example, the recent use of field flow fractionation in the fractionation and characterisation of polyphenolic compounds in red wine (Pascotto et al., 2020) and in the analysis of native red wine colloids (Marassi et al,2021).

The complexity of red wine mainly comes from its macromolecules, condensed tannins, and polysaccharides. Macromolecular components in wine have been reported to affect astringency but there is minimal information on the colloidal systems involved. For characterisation of this colloidal environment, careful analytical methods need to be applied to preserve the sample structure and limit the denaturation of macromolecular complexes. A method of Asymmetric Flow Field-Flow Fractionation (AF4) was used (Pascotto et al., 2020), coupled with UV detection, multi-angle light scattering (MALS), and differential refractometer index (dRI) (AF4-UV-MALS-dRI). This method separates objects according to their hydrodynamic radius and does not require calibration to determine molecular weight. AF4 allows separation of wine colloidal particles at the same time using simulated wine as a mobile phase. They used generic wine to investigate several parameters of crossflow rate, optimizing separation conditions. Polysaccharide and tannin fractions were then purified and further analysed using the selected AF4 parameters. Peaks obtained for these fractions were then compared with those of the wine sample to determine the retention time of the macromolecules. The AF4 fractogram of wine was divided into four fractions. The first three were assigned to higher molecular weight tannins coeluted with lower molecular weight polysaccharides such as rhamnogalacturonan II (F1), to intermediate molecular weight polysaccharides (F2), and to higher molecular weight mannoproteins (F3) whereas the last fraction (F4) was not identified. Their results showed that AF4-UV-MALS-dRI could be an efficient technique to separate large size tannins as well as polysaccharides and macromolecular complexes. The Gap of this study is that it mainly focused on red wine polyphenols, polysaccharides, tannins and macromolecular complexes in general

but not proteins in red wine. This method can be used to analyse proteins in wine and understand how they participate in colloidal haze formation.

Marassi and colleagues (2021) suggested the use of Field Flow Fractionation to isolate and characterize red wine colloids in native state. Field Flow Fractionation has been seen to not have the above-mentioned methodological issues and is regarded suitable enough for the characterisation of nanosized and micro sized systems, in this case wine (Roda et al., 2009). Of all the Field Flow Fractionation sub techniques, Asymmetrical Flow-Field Flow Fractionation (AF4) was found to be a gentle separation mechanism capable of not altering particles in the process. This is due the lack of stationary phase in its operational technique (Rigaux et al., 2017). AF4 has been seen to have a wide application range in terms of analytes size and choice of carrier fluid (Zattoni et al., 2014) which makes it more convenient and efficient for analysing different samples.

Previous research showed that AF4 was widely used in hyphenation with several detection systems to characterise the macromolecular and colloidal fractions in different foods and ingredients (Nilsson, 2013) but its application in white wine colloidal fractionation has been limited. The fractionated chromophoric colloids were further studied offline by UV and fluorescence analysis (Coelho et al., 2017). However, the mobile phase composition used for the AF4 separation was different from wine. Field Flow Fractionation was first applied in red wine analysis to isolate and characterise colloids by Marassi and colleagues (2021).

An advantage of their research method was that it worked well in physicochemical conditions such as wine, hence preventing colloidal modifications during separation and analysis. The AF4 multidetector platform was equipped with UV, Fluorescence and Multi-Angle Light Scattering (MALS) and used to analyse colloids in native state. It is a recent successful method of evaluating red wine colloidal structure and composition. Kevin Pascotto and colleagues (2020) also proved the successful application of asymmetrical flow field fractionation in the fractionation and characterisation of polyphenolic compounds and macromolecules in red wine. This method and approach can be used to analyse the proteins in red wine to see how these are involved in colloidal formation.

However, drawbacks were also noted in the application of the AF4 technique. It is a tiresome method to conduct especially on the analysis of AF4 fractions with SDS PAGE. This usually leads to a small number in the sample sizes used in the analysis. However, to speed up the fraction analysis, another instrument can be suggested, Size Exclusion HPLC. This can be used in the quantification of the macromolecules (polysaccharides and proteins). The plate reader analyses the colour and fluorescence of a large sample size (96 samples) in one go, to quantify proteins, phenolics and polysaccharides in wines and wine fractions in high throughput mode.

Despite the progressive research in red wine achieved by AF4 technique, the method is not applicable to this current research because the objective is not to characterise the proteins in red wine but to study their movement and aggregation activity within the wine matrix. AF4 can however be proposed a better method to fractionate and analyse protein colloids in red wines, coupled with fluorescence detection.

Fluorescence correlation spectroscopy (FCS) has been deemed relevant in the study of wine systems. It can be a useful tool in the investigation of the complex interactions between wine macromolecules. A. Mierczynska-Vasilev et al. (2021) demonstrated the use of Fluorescence Correlation Spectroscopy (FCS) in providing valuable information on protein conformational changes in wine. FCS also showed the dynamics of protein interaction with other wine macromolecules. Using this technique, protein haze formation and turbidity can be studied in real wines. Their model protein, BSA has been widely reported to be a good model protein for studying protein stability, folding and denaturation (Carrotta et al., 2009). BSA turned into a more compact globular structure but with no sign of aggregation under wine like conditions.

It is here proposed that FCS will be used in our research to monitor the aggregation activity of proteins in red wine samples. The difference in our method is that we are analysing real wine systems and grape proteins instead of using generic wine and BSA. We will also label the target grape proteins with fluorescence dye before analysis.

3 CHAPTER 3: METHODOLOGY

After comparing the different methods used in wine fractionation and analysis previously, an intermediate approach is here suggested to study protein aggregation activity in red wine, without destroying the wine matrix. This approach involves the extraction of white grape juice proteins, according to the protocol by (Van Sluyter et al., 2009), labelling them with a fluorescent dye then adding them to a deproteinated red wine for observation.

The experimental objectives are:

- Isolate and purify grape proteins.
- Visualise protein aggregation behaviour in wine.
- Assess the impact of wine composition (e.g.., ethanol content) on colloidal aggregation.

Hypothesis: Red wine proteins contribute to the colloidal instability of red wines.

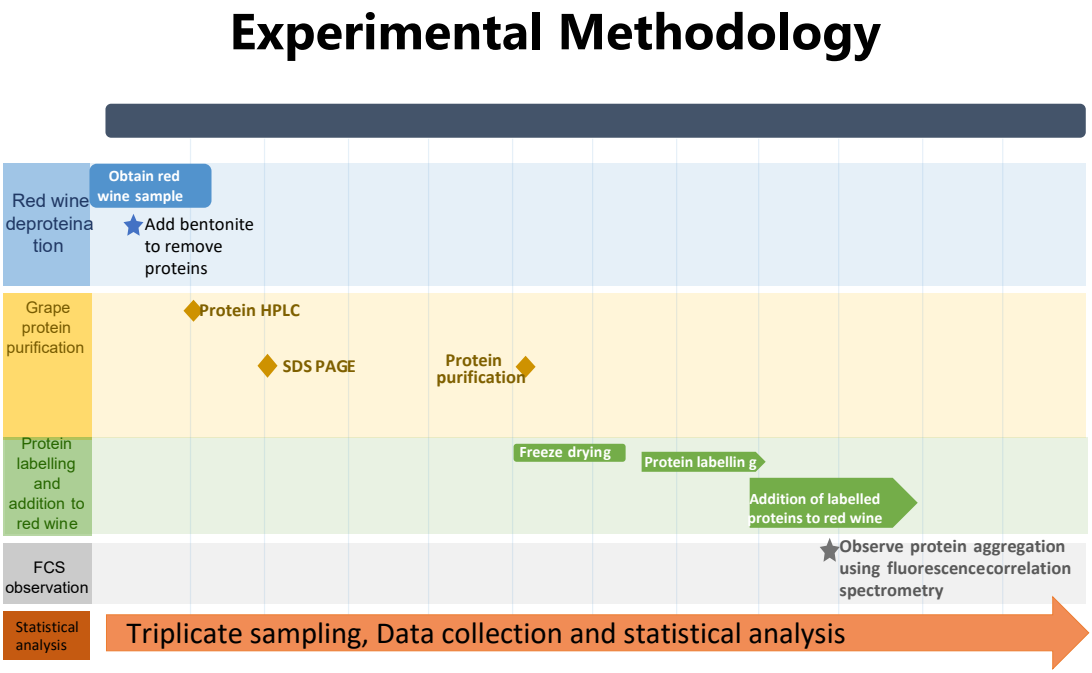

Figure 3.1. Methodology Gantt chart

3.1.1 Model solutions and Wine samples

Obtain two commercial red wines from Veneto region from the Raboso Piave grape variety and Corvina. The red wine samples are deproteinated using Bentonite then set aside for later use.

The model wine solution will be prepared as follows: 12 or 15% ethanol, 4 g/L of tartaric acid, pH 3.20. pH and ionic strength adjustment can be performed, using NaCl and NaOH respectively. This model wine is later used as a medium to dissolve labelled proteins.

3.1.2 Obtain Purified wine proteins from white wines using a method proposed by Van Sluyter and colleagues (2009).

- Protein purification

Two clarified juices (e.g., Pinot Grigio and Manzoni Bianco, will be obtained from commercial wineries in Veneto and stored at -20 °C until use. Juices are then adjusted to pH3.0 with HCL and treated with 30g/L polyvinylpolypyrrolidone overnight at 4 °C. Juices must then be filtered by vacuum filtration.

All chromatographic steps are performed at room temperature. Filtered juice is loaded at 8−18 mL/min after column equilibration with 30 mM sodium citrate at pH 3.0. The column is then connected to a chromatography system with UV detector and washed at 20 mL/min with 1.7 L of 30 mM sodium citrate, pH 3.0. Bound proteins are then eluted at 20ml/min with 30mM MES/1 NaCl at pH 6. Strong cation exchange (SCX) fractions are then grouped based on elution profiles at 280 nm absorbance. When the ammonium sulfate addition to SCX fractions produces precipitate, more ammonium sulphate is added to reach 80% saturation and stirred overnight at 0 ºC. The precipitate is then collected by centrifugation and the resulting pellets are dissolved in 50mM sodium citrate containing 1.25M ammonium sulfate at pH 5 for loading on the HIC column. The column is then washed with 0.2L of 50mM sodium citrate containing 1.25M ammonium sulfate at pH 5 and eluted with a 110min gradient at 10mL/min to 50mMsodium citrate at pH5. HIC fractions are to be grouped according to their elution profiles A_{280} and reverse phase HPLC analysis. Ammonium sulfate concentrations of the pooled HIC fractions are then calculated based on elution times and adjusted to 90% saturation at 0 °C. Proteins are collected by centrifugation, dissolved in 0.1 M malic acid, pH 3.5 (KOH), and desalted. Desalted fractions are then pooled based on conductivity and A_{280} and stored at −80 °C.

- Protein quantification by Reverse-phase HPLC

Reverse-phase HPLC is used to measure the total amount of proteins in wine samples and will be performed according to a previously published protocol by Marangon et al., (2009). Briefly, the separation will be done using a C8 HPLC column, and by applying a gradient of acetonitrile (CH_3CN) buffer containing 0.1% trifluoroacetic acid. The flow rate for the column must be set to 0.25ml/min. Samples must be adjusted to 10% CH_3CN and 0.1% tifluoroacetic acid. The injection volumes must be between 15 and 30 µL. After 32 mins, the eluent composition is to be corrected back to equilibrium conditions.

- Electrophoresis (SDS-PAGE)

Protein samples will be analysed by Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) as suggested by Van Sluyter and colleagues (2009). The purified proteins are diluted into sample loading buffer containing 5% 2-mercaptoethanol as a reducing agent and then boiled for 5min. Precision protein standards that are unstained must be used to indicate known molecular weights. The separation will be performed in gels with acrylamide percentage of at least 10%. After the separation, gels must be fixed for 10 minutes in 50% methanol/5% acetic acid and stained with a Coomassie protein stain. An extended incubation period in stain is recommended to increase sensitivity.

3.1.3 Freeze drying

The extracted protein is then freeze dried and labelled with fluorescent dye according to Merck fluorescent dye specifications.

3.1.4 Addition of labelled proteins to red wine from step 1 above

Labelled proteins are dissolved in the previously prepared model wines in step 3.1 then added to the deproteinated red wine samples.

The labelled proteins are also added to the prepared wine model solutions, at different concentrations of ethanol.

A control wine sample without labelled proteins will be analysed by FCS

3.1.5 Observation of the protein activity

Observe and monitor the protein aggregation process using Fluorescence Correlation Spectroscopy according to the method by A. Mierczynska-Vasilev et al. (2021) As illustrated in figure 2, with the FCS setup. A) Light is emitted from the argon laser then focused through a water immersion microscope objective. This excites the fluorescent species in the wine sample being studied. The fluorescence light emitted is then collected by the same objective and transmitted through a confocal pinhole to the photo detector, PC unit. In part B of the diagram, there are fluctuations in the fluorescent signal dictated F(t), caused by species diffusing through the confocal detection volume. These are recorded and evaluated by autocorrelation analysis in part C to obtain the species' residence time and consequently their diffusion coefficient and hydrodynamic radius.

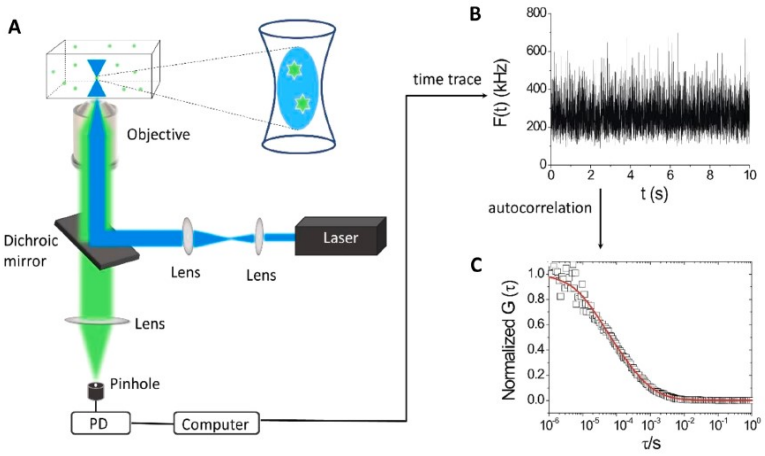

Figure 2.0 Graphical representation of FCS analysis (Image from - Mierczynska-Vasilev et al.,2021)

3.1.6 Phenolic quantification

Phenolic content of wines and AF4-fractions will be evaluated by the Folin-Ciocalteu (FC) method (Singleton et al.,1999). Briefly, sample (200 µL) or blank (model wine alone) is mixed with FC reagent (Sigma-Aldrich) and 800 µL of Na_2CO_3 (7.5% w/v). After 30 minutes of incubation at 40°C, the absorbance is measured at 725 nm in a UV-VIS spectrophotometer. A calibration curve will be prepared using gallic acid solution (Sigma-Aldrich) in a concentration range between 0.025 and 0.2 mg/mL.

3.1.7 Polysaccharides quantification

The quantification of the total polysaccharides will be performed using a previously proposed method (Dubois, et al., 1956; Segarra, Lao et al.,1995) as reported by Marassi and colleagues (2021). Briefly, 20 µL of filtered and PVPP-treated wine is added with 500 µL of absolute ethanol, stored at 4°C for 16 h and centrifuged at 14000 g for 30 minutes. The obtained pellets are dried by placing the open vials on a heating mantle set at 65°C for 30 minutes. Pellets are then solubilized with 1 mL of a water/phenol solution prepared by dissolving phenol at 2% (v/v) in distilled water. Then, 400 µL of the samples will be transferred into a new vial and added with 1 mL of pure sulphuric acid. After 30 minutes, the absorbance is measured at 490 nm. A calibration curve will be prepared using a serial dilution of glucose (0-100 mg/L,) prepared in the water/phenol solution.

3.1.8 Statistical analysis

All samples will be prepared in triplicates, data is collected in each step and Statistical analysis of the results is done using Anova and Excel. T- test is used to assess the significance of the results. The level of significance is set to $p< 0.05$.

3.2 Limitations of the experimental method

Deproteination with bentonite will successfully remove the proteins from the red wine, but it does not come without its faults. The previously reported side effects of Bentonite use in red wine can be anticipated (Donovan et al., 1998; Dordoni et al., 2015) by binding to phenols and anthocyanins, hence reducing the colour intensity of the wine. As a result, the wine to be used post the bentonite fining will not resemble the usual full bodied red wine with a deep red hue. The absence of some colour compounds from our deproteinated wine may influenced the expected results because less colour compounds will be present to interact with the labelled proteins. In this context, we cannot replicate the exact real wine model system which has all the colouring compounds, proteins and other macromolecules interacting together in a colloidal system.

However, the bentonite problem can be avoided in the future by using a different method to cleave the red wine proteins while in the wine matrix using enzymes. Research by Marangon and colleagues (2012) reported the use of Aspergillus I and II (AGP) in degrading wine proteins in situ. AGP is a cheap food grade protease enzyme active at wine pH and tolerant to high temperatures (60 – 80^{O}C). During fermentation, AGP can degrade about 20% wine proteins and when combined with grape juice heating technique, a 90% protein reduction can be achieved, as reported. This is a very significant reduction if the method is to be applied in this protein analysis research. The advantage of the targeted protein cleavage method is that all other wine properties are preserved, especially colouring compounds. Hence the wine samples will be much closer to a real wine model system as compared to that fined with bentonite. Physicochemical parameters and sensorial characteristics of wine are not expected to change.

4 CHAPTER 4: EXPECTED RESULTS

The use of bentonite in deproteination of red wine is expected to significantly reduce the protein as reported by Chagas, Feirreira and Monteiro (2012). This step is necessary in our research because we aim to study a labelled protein sample we can control. Hence, we must remove the pre-existing proteins in the red wine and add back the labelled proteins to monitor their interaction with other macromolecules in the red wine matrix. An advantage of using bentonite in this experiment is that it reduces the anthocyanin compounds that have been previously reported (Silva et al., 2019), to fluoresce naturally in red wine hence preventing any interference during FCS analysis. Alimelli and colleagues (2007) also observed a red fluorescence during red wine analysis but there was no explanation as to the origin of emission. Previously reported fluorescent compounds in red wine are polyphenols, flavonoids, stilbenes vitamins, tannins, and amino acids (Arnaoutakis, Georgios, 2015), hence any of these compounds could influence fluorescence activity in wine if they are not controlled.

In our proposed experiment, grape varieties from Veneto will be selected depending on availability and on protein content, but most likely they will be Pinot Grigio and Manzoni bianco as previous studies have shown that they contain high amounts of proteins (Vincenzi et al.,2010). The chosen purification method is quick and efficient enough to generate many proteins under non denaturing conditions. Cation exchange (SCX) captures and fractionates proteins at the pH conditions of grape juice while HIC complements the first SCX step by resolving PR proteins. This protein purification method is a significant improvement as compared to the previous methods which denatured proteins during analysis, for example anion exchange and chitin affinity methods. Apart from being a non-denaturing method it also purifies Thaumatin like proteins and chitinases. The purification overall will be fast and separating isoforms of TL proteins and Chitinases within the same juice matrix, giving about 97% purity (Marangon, Van Sluyter, Haynes & Waters, 2009).

Freeze drying, also known as lyophilization involves the removal of water from a material under high pressure in the form of ice by sublimation has been widely used in the stabilisation of proteins, vaccines, and biological components (Nowak D and Jakubczyk E, 2020). In our research, the protein fraction will be desalted using desalting columns before being frozen and freeze dried. The quality of the product is retained, i.e., the biological component, nutritional value, and organoleptic properties. the native properties of wine proteins will be maintained because the freezing process inhibits any chemical, biochemical and microbiological changes prior to lyophilisation. This method was proposed because freeze dried products are highly porous such that rehydration of the lyophisates, in this case grape protein, is easier when adding back to the red wine (Meda and Ratti, 2005 ; Jia Yet al., 2019).

Different protein staining techniques have been applied in the past, such as Coomassie stains and silver stains which are used to visualise protein separation on gel electrophoresis. However fluorescent dye will be used in

this research because it offers unique advantages and its applicable to the spectrometric analytical method chosen. Advantages of fluorescent dye are low interference, high sensitivity, wide linear dynamic range, and high throughput (Agati et al., 2013; Arnaoutakis, 2015; Mierczynska-Vasilev et al., 2021; Silva et al., 2019). This method has no interference problems since the fluorometric technic targets the specific molecules for analysis which can fluoresce. Fluorometric measurements use a simple effective protocol which can be automated for high throughput applications.

Wine haze and colloidal formation can be influenced by ethanol concentration, pH, metal ions and phenolic compounds (Waters et al., 2005). It is expected that the alcohol content of the wine samples will have an impact on the protein activity and aggregation. Ethanol as a primary metabolite of fermentation is expected to play major role in the stability, ageing and sensory properties of wine. The alcohol content of red wine falls between 12 and 15.5% and hence it is necessary to use the same ethanol concentrations in this study. Partial aggregation of protein is expected to be influenced by ethanol and at 12 and 15% ethanol concentration the protein is expected to collapse into a compact globular structure but with no further aggregation beyond this (Mierczynska-Vasilev et al., 2021). The matrix effect of ethanol on tannin-protein interactions is related to the concentration of ethanol in red wine and the astringency perception. An increase in alcohol content was previously reported to cause a decrease in wine astringency, with a decreasing effect on the strength of the tannin-protein interaction (McRae et al., 2015).

PR proteins have been mainly linked to colloidal haze in wine, mainly chitinases and thaumatin-like proteins. When the persistence of these proteins is analysed during fermentation (Ndlovu T et al., 2019) an evolution of total grape proteins, chitinases and thaumatin-like proteins is expected, showing a decrease in the total protein content during fermentation. A decrease in protein levels is expected to occur throughout fermentation, influenced by temperature, yeast strain and grape variety (Ndlovu T et al., 2019). Protein instability is expected to increase during fermentation and then decrease upon wine storage for a month due to the stabilising effect of polysaccharides (Vincenzi et al., 2010). Grape thaumatin-like proteins are expected to show the largest increase during fermentation, accounting for about 40% of colloidal instability in the final wine. Thermal fluctuations lead to haze and colloidal instability in wines, while the reduction in disulphide bonds lead to the onset of protein aggregation in the wine matrix. Another study conducted by the Australian Wine Research Institute (2017) to study the mechanism of haze formation and obtain prevention strategies employed a similar method (Sluyter et al. 2009 & Culbert et al. 2017) to study aggregation behaviour in white wine using reconstitution experiments with wine proteins. The key components that were highly linked to haze formation were purified and added back to wines. However, nephelometry (Pocock & Waters,2006), differential scanning fluorimetry, nanoparticle tracking analysis (NTA) (McRae et al., 2017) and isothermal titration calorimetry (McRae et al. 2015), different from our suggested method, were used to study the aggregation behaviour of purified proteins and other wine components.

5 A HYPOTHESIS ON THE POTENTIAL FINDINGS.

The method can be successful in studying the aggregation activity of proteins in red wine. Thaumatin like and chitinases proteins have been noticed to play a major role in the colloidal instability phenomena. Thermal fluctuations may lead to haze and colloidal instability in wines, while the reduction in disulphide bonds may cause the onset of protein aggregation in the wine matrix.

This proposed methodology can be further applied to study the impact of ethanol, polysaccharides, tannins, and inorganic compounds on colloidal stability in red wines. Fluorescence correlation spectroscopy has been successfully demonstrated as a promising method in the study of complex interactions between wine macromolecules, especially red wine which has been difficult to study for a while now using other methods. It can be applied in the study of both model and real wines in situ as it is a highly sensitive and selective method. It is predicted that different concentrations of ethanol may cause wine proteins to unfold and aggregate. Protein haze and colloidal formation can be studied in real wines using this technique. This technique can also be applied in the future to monitor the colloidal activity and complex phenomena in red wines in situ. Furthermore, the FCS technique can be further applied to other scientific challenges in complex liquid matrices in food and beverage research.

6 REFERENCES:

A. Vernhet Red Wine Technology 2019 Elsevier 237 251 10.1016/B978-0-12-814399-5.00016-5 https://linkinghub.elsevier.com/retrieve/pii/B9780128143995000165.

A. D'Amato, A.V. Kravchuk, A. Bachi and P.G. Righetti. Journal of Proteomics, 73 (12) (2010), pp. 2370-2377. Noah's nectar: The proteome content of a glass of red wine

Advances in Protein Chemistry and Structural Biology, Academic Press, Volume 118, (2019), Pages 413-436, ISSN 1876-1623, ISBN 9780128177501, https://doi.org/10.1016/bs.apcsb.2019.08.004.

Agati, G., Matteini, P., Oliveira, J., de Freitas, V., & Mateus, N. (2013). Fluorescence approach for measuring anthocyanins and derived pigments in red wine. Journal of Agricultural and Food Chemistry, 61(42), 10156–10162. https://doi.org/10.1021/jf402398a

Alimelli, A.; Filippini, D.; Paolesse, R.; Moretti, S.; Ciolfi, G.; D'Amico, A.; Lundstrom, I.; Di Natale, C. Direct quantitative evaluation of complex substances using computer screen photo-assisted technology: the case of red wine Anal. Chim. Acta 2007, 597, 103– 112

Alvarez, I., Aleixandre, J.L., Garcia, M.J., Lizama, V. (2006) Impact of prefermentative maceration on the phenolic and volatile compounds in Monastrell red wines. Analytica Chimica Acta, 563 (1–2), 109–115.

Andrea Versari, Luca Laghi, John H. Thorngate, Roger B. Boulton, Prediction of colloidal stability in white wines using infrared spectroscopy, Journal of Food Engineering, Volume 104, Issue 2, 2011, Pages 239-245, ISSN 0260-8774

Apolinar-Valiente, R., Williams, P., Romero-Cascales, I., et al. (2013) Polysaccharide composition of Monastrell red wines from four different Spanish Terroirs: effect of wine-making techniques. Journal of Agricultural and Food Chemistry, 61 (10), 2538–2547.

Arnaoutakis, Georgios. (2015). Fluorescence Spectroscopy of Wine. 10.13140/RG.2.1.2220.3765/1.

Belén Gordillo, Francisco Chamizo-González, M. Lourdes González-Miret, Francisco J. Heredia,Impact of alternative protein fining agents on the phenolic composition and color of Syrah red wines from warm climate,Food Chemistry,Volume 342,2021,128297,

Bindon, K. A., Carew, A. L., Mierczynska-Vasilev, A., Kassara, S., Kerslake, F., & Smith, P. A. (2016). Characterization of macromolecular complexes in red wine: Composition, molecular mass distribution and particle size. Food Chemistry, 199, 838–846. https://doi.org/10.1016/J.FOODCHEM.2015.12.079.

Bosso, A.; Salmaso, D.; De Faveri, E.; Guaita, M.; Franceschi, D. The use of carboxymethylcellulose for the tartaric stabilization of white wines, in comparison with other oenological additives Vitis 2010, 49, 95– 99

Boulton, R. B.; Singleton, V. L.; Bisson, L. F.; Kunkee, R. E. Principles and Practices of Winemaking; Kluwer Academic/Plenum Publishers: New York, 1996.

C. Poncet-Legrand, D. Cartalade, J.L. Putaux, V. Cheynier, A. Vernhet, Flavan-3- ol aggregation in model ethanolic solutions: incidence of polyphenol structure, concentration, ethanol content, and ionic strength, Langmuir 19 (2003) 10563–10572 10.1021/la034927z .

Carrotta, R., Manno, M., Giordano, F. M., Longo, A., Portale, G., Martorana, V., & Biagio, P. L. (2009). Protein stability modulated by a conformational effector: Effects of trifluoroethanol on bovine serum albumin. *Phys Chem Chem Phys, 11*(20), 4007–4018. https://doi.org/10.1039/b818687a. Carvalho, E., Mateus, N., Plet, B., Pianet, I., Dufourc,

Cavicchi, R., King, J., & Ripple, D. (2018). Measurement of Average Aggregate Density by Sedimentation and Brownian Motion Analysis. *Journal Of Pharmaceutical Sciences*, *107*(5), 1304-1312. doi: 10.1016/j.xphs.2018.01.013

Cosme, F., Fernandes, C., Ribeiro, T., Filipe-Ribeiro, L., & Nunes, F. (2020). White Wine Protein Instability: Mechanism, Quality Control and Technological Alternatives for Wine Stabilisation—An Overview. *Beverages*, *6*(1), 19. doi: 10.3390/beverages6010019

Cutler, L. (2009) Industry Roundtable: Fermentation Techniques. Wine Communications Group [cited December 20, 2013]; Available from: *http://www.winebusiness.com/wbm/?go=getArticle&dataId=62843*.

Des Gachons, C.P. and Kennedy, J.A. (2003) Direct method for determining seed and skin proanthocyanidin extraction into red wine. Journal of Agricultural and Food Chemistry, 51 (20), 5877–5881.

Doco, T., Williams, P., Cheynier, V. (2007) Effect of flash release and pectinolytic enzyme treatments on wine polysaccharide composition. Journal of Agricultural and Food Chemistry, 55 (16), 6643–6649.

Donovan, J. L., McCauley, J. C., Nieto, N. T., & Waterhouse, A. L. (1998). Effects of small-scale fining on the phenolic composition and antioxidant activity of merlot wine. In A. L. Waterhouse & S. E. Ebeler (Eds.), ACS symposium series. Washington: American Chemical Society. *http://dx.doi.org/10.1021/bk-1998-0714.ch011*. [*Links*]

Dordoni, R., Galasi, R., Colangelo, D., De Faveri, D. M., & Lambri, M. (2015). Effects of fining with different bentonite labels and doses on colloidal stability and colour of a Valpolicella red wine. *International Journal of Food Science & Technology*, 50(10), 2246-2254.

Dubourdieu, D., Moine-Ledoux, V., Lavigne-Cruège, V., Blanchard, L., and Tominaga, T. (2000) Recent advances in white wine aging: The key role of lees. In: Proceedings of the ASEV 50th Anniversary Annual Meeting, Seattle, WA, June 19–23, 2000, pp. 345–352. American Society for Enology and Viticulture, Davis, CA.

Elia Romanini, Jacqui M. McRae, Eleanor Bilogrevic, Donato Colangelo, Mario Gabrielli, Milena Lambri, Use of grape seeds to reduce haze formation in white wines, Food Chemistry, Volume 341, Part 1, 2021, 128250, ISSN 0308-8146, https://doi.org/10.1016/j.foodchem.2020.128250.

Etaio, I., Elortondo, F.J.P., Albisu, M., et al. (2008) Effect of winemaking process and addition of white grapes on the sensory and physicochemical characteristics of young red wines. Australian Journal of Grape and Wine Research, 14 (3), 211–222.

Falconer RJ, Marangon M, Van Sluyter SC, Neilson KA, Chan C, Waters EJ. Thermal stability of Thaumatin-like protein, Chitinase, and Invertase isolated from Sauvignon Blanc and Semillon juice and their role in haze formation in wine. Journal of Agricultural and Food Chemistry. 2010;58(2):975-980

Favre, G., Peña-Neira, Á., Baldi, C., et al. (2014) Low molecular-weight phenols in Tannat wines made by alternative winemaking procedures. Food Chemistry, 158 (0), 504–512

Gao, L., Girard, B., Mazza, G., Reynolds, A.G. (1997) Changes in anthocyanins and color characteristics of Pinot noir wines during different vinification processes. Journal of Agricultural and Food Chemistry, 45 (6), 2003–2008.

Gerbaud, V.; Gabas, N.; Blouin, J.; Crachereau, J. C. Study of wine tartaric acid salt stabilization by addition of carboxymethylcellulose (CMC): comparison with the ‘protective colloids’ effect J. Int. Sci. Vigne Vin 2010, 44, 231– 242

Giribaldi M, Perugini I, Sauvage FX, Schubert A. Analysis of protein changes during grape berry ripening by 2-DE and MALDI-TOF. Proteomics. 2007;7(17):3154-3170

Gomez-Miguez, M. and Heredia, F.J. (2004) Effect of the maceration technique on the relationships between anthocyanin composition and objective color of Syrah wines. Journal of Agricultural and Food Chemistry, 52 (16), 5117–5123.

Guise, R.; Filipe-Ribeiro, L.; Nascimento, D.; Bessa, O.; Nunes, F. M.; Cosme, F. Comparison between different types of carboxylmethylcellulose and other oenological additives used for white wine tartaric stabilization Food Chem. 2014, 156, 250– 257 DOI: 10.1016/j.foodchem.2014.01.081

Hayasaka, Y.; Adams, K. S.; Pocock, K. F.; Baldock, G. A.; Waters, E. J.; Høj, P. B. Use of electrospray mass spectrometry for mass determination of grape (Vitis vinifera) juice pathogenesis-related proteins: a potential tool for varietal differentiation J. Agric. Food Chem. 2001, 49, 1830– 1839

Hoogendam, C. W.; de Keizer, A.; Cohen Stuart, M. A.; Bijsterbosch, B. H.; Smit, J. A. M.; van Dijk, J. A. P. P.; van der Horst, P. M.; Batelaan, J. G. Persistence length of carboxymethyl cellulose as evaluated from size exclusion chromatography and potentiometric titrations Macromolecules 1998, 31, 6297– 6309 DOI: 10.1021/ma971032i

Jia, Y.; Khalifa, I.; Hu, L.; Zhu, W.; Li, J.; Li, K.; Li, C. Influence of three di_erent drying techniques on persimmon chips' characteristics: A comparison study among hot-air, combined hot-air-microwave, and vacuum-freeze drying techniques. Food Bioprod. Process. 2019, 118, 67–76.

Kevin Pascotto, Véronique Cheynier, Pascale Williams, Olivier Geffroy, Frédéric Violleau,Fractionation and characterization of polyphenolic compounds and macromolecules in red wine by asymmetrical flow field-flow fractionation,Journal of Chromatography A,Volume 1629,2020,461464,ISSN0021-9673,https://doi.org/10.1016/j.chroma.2020.461464. (https://www.sciencedirect.com/science/article/pii/S0021967320307408)

Koyama, K., Goto-Yamamoto, N., Hashizume, K. (2007) Influence of maceration temperature in red wine vinification on extraction of phenolics from berry skins and seeds of grape (Vitis vinifera). Bioscience Biotechnology and Biochemistry, 71 (4), 958–965.

Lambri M, Dordoni R, Silva A, De Faveri DM. Comparing the impact of bentonite addition for both must clarification and wine fining on the chemical profile of wine from Chambave Muscat grapes. International Journal of Food Science and Technology. 2012; 47:1–12.

Lambri M, Dordoni R, Silva A, De Faveri DM. Effect of bentonite fining on odor active compounds in two different white wine styles. American Journal of Enology and Viticulture. 2010; 61:225–233.

Lasanta, C.; Gómez, J. Tartrate stabilization of wines Trends Food Sci. Technol. 2012, 28, 52– 59 DOI: 10.1016/j.tifs.2012.06.005

López-Miranda S, Hernández-Sánchez P, Serrano-Martínez A, Hellín P, Fenoll J, Núñez-Delicado E. Effect of ripening on protein content and enzymatic activity of crimson seedless table grape. Food Chemistry. 2011;127(2):481-486

Low, L. L.; O'Neill, B.; Ford, C.; Godden, J.; Gishen, M.; Colby, C. Economic evaluation of alternative technologies for tartrate stabilisation of wines Int. J. Food Sci. Technol. 2008, 43, 1202– 1216 DOI: 10.1111/j.1365-2621.2007.01591

M. Marangon S. Vincenzi A. Curioni Wine Fining with Plant Proteins Molecules 24 11 2186 10.3390/molecules24112186 https://www.mdpi.com/1420-3049/24/11/2186.

M. Marangon, F.X. Sauvage, E.J. Waters and A. Vernhet. Journal of Agricultural and Food Chemistry, 59 (6) (2011), pp. 2652-2662

Mainente, F., Zoccatelli, G., Lorenzini, M., Cecconi, D., Vincenzi, S., Rizzi, C., et al. (2014). Red wine proteins: Two dimensional (2-D) electrophoresis and mass spectrometry analysis. Food Chemistry, 164, 413–417. https://doi.org/10.1016/j. foodchem.2014.05.051.

Marangon, M., Vincenzi, S., & Curioni, A. (2019). Wine Fining with Plant Proteins. *Molecules*, *24*(11), 2186. doi: 10.3390/molecules24112186

Marangon, M.; Van Sluyter, S. C.; Haynes, P. A.; Waters, E. J. Grape and wine proteins: their fractionation by hydrophobic interaction chromatography and identification by chromatographic and proteomic analysis J. Agric. Food Chem. 2009, 57, 4415– 442

Marsh E, Alvarez S, Hicks LM, Barbazuk WB, Qiu WP, Kovacs L, et al. Changes in protein abundance during powdery mildew infection of leaf tissues of Cabernet Sauvignon grapevine (Vitis vinifera L.). Proteomics. 2010;10(10):2057-2064

McRae, J.M., Ziora, Z.M., Kassara, S., Cooper, M.A., Smith, P.A. 2015. Ethanol concentration influences the mechanisms of wine tannin interactions with poly(L-proline) in model wine. J. Agric. Food Chem. 63(17): 4345-4352.

Meda, L.; Ratti, C. Rehydration of freeze-dried strawberries at varying temperatures. J. Food Process Eng. 2005, 28, 233–246.

Mekoue Nguela, J., Poncet-Legrand, C., Sieczkowski, N., & Vernhet, A. (2016). Interactions of grape tannins and wine polyphenols with a yeast protein extract, mannoproteins and β-glucan. Food Chemistry, 210. https://doi.org/10.1016/j.foodchem.2016.04.050

Mierczynska-Vasilev, A., Qi, G., Smith, P., Bindon, K., & Vasilev, K. (2019). Regeneration of Magnetic Nanoparticles Used in the Removal of Pathogenesis-Related Proteins from White Wines. *Foods*, *9*(1), 1. doi: 10.3390/foods9010001

Monteiro S, Piçarra-Pereira MA, Loureiro VB, Teixeira AR, Ferreira RB. The diversity of pathogenesis-related proteins decreases during grape maturation. Phytochemistry. 2007;68(4):416-425

Moreno, J., & Peinado, R. (2012). Enological chemistry. Amsterdam: Elsevier/AP.

Moreno-Perez, A., Vila-Lopez, R., Fernandez-Fernandez, J.I., et al. (2013) Influence of cold pre-fermentation treatments on the major volatile compounds of three wine varieties. Food Chemistry, 139 (1–4), 770–776.

Murphey JM, Spayd SE, Powers JR. Effect of grape maturation on soluble protein characteristics of Gewürztraminer and White Riesling juice and wine. American Journal of Enology and Viticulture. 1989;40(3):199-207

Ndlovu, T., Buica, A., & Bauer, F. F. (2019). Chitinases and thaumatin-like proteins in Sauvignon Blanc and Chardonnay musts during alcoholic fermentation. Food Microbiology, 78, 201–210. *https://doi.org/10.1016/j.fm.2018.10.018*

Nigen, M., Valiente, R., Iturmendi, N., Williams, P., Doco, T., & Moine, V. et al. (2019). The colloidal stabilization of young red wine by Acacia senegal gum: The involvement of the protein backbone from the protein-rich arabinogalactan-proteins. *Food Hydrocolloids*, *97*, 105176. doi: 10.1016/j.foodhyd.2019.105176

Okuda, T.; Fukui, M.; Takayanagi, T.; Yokotsuka, K. Characterization of major stable proteins in Chardonnay wine Food Sci. Technol. Res. **2006**, 12 (2) 131– 136

Paetzold M., Dulau L., Dubourdieu D. Fractionnement et caractérisation des glycoproteins dans les moûts de raisin blanc. Journal International des Sciences de la Vigne et du Vin (1990) 24(1): 13-28.

Paolo Ruzza, Claudia Honisch, Matteo Marangon, Andrea Curioni, Alan Bakalinsky, Simone Vincenzi, Pressure Liquid Chromatography-Mass Spectrometry (HPLC-MS). Journal of agricultural and food chemistry. 57. 4328-33. 10.1021/jf8034836.

Paranjpe, S.S., Ferruzzi, M., Morgan, M.T. (2012) Effect of a flash vacuum expansion process on grape juice yield and quality. LWT-Food Science and Technology, 48 (2), 147–155.

Parsons, M. E. M., McParland, D., Szklanna, P. B., Guang, M. H. Z., O'Connell, K., O'Connor, H. D., McGuigan, C., Ní Áinle, F., McCann, A., & Maguire, P. B. (2017). A Protocol for Improved Precision and Increased Confidence in Nanoparticle Tracking Analysis Concentration Measurements between 50 and 120 nm in Biological Fluids. Frontiers in Cardiovascular Medicine, 4. https://doi.org/10.3389/fcvm.2017.00068

Pascal, C., Poncet-Legrand, C., Cabane, B., & Vernhet, A. (2008). Aggregation of a Proline-Rich Protein Induced by Epigallocatechin Gallate and Condensed Tannins: Effect of Protein Glycosylation. Journal of Agricultural and Food Chemistry, 56(15). https://doi.org/10.1021/jf800790d

Pellerin P, Waters E, Brillouet JM. Characterization of two arabinogalactan-proteins from red wine. Carbohydrate Polymers. 1993; 22:187–192.

Petra Wigand, Stefan Tenzer, Hansjoerg Schild, and Heinz Decker. Analysis of Protein Composition of Red Wine in Comparison with Rosé and White Wines by Electrophoresis and High-Pressure Liquid Chromatography−Mass Spectrometry (HPLC-MS). *Journal of Agricultural and Food Chemistry* 2009 *57* (10), 4328-4333. DOI: 10.1021/jf8034836.

Pocock KF, Waters EJ. The effect of mechanical harvesting and transport of grapes, and juice oxidation, on the protein stability of wines. Australian Journal of Grape and Wine Research. 1998;4(3):136-139

Puig-Deu M, López-Tamames E, Buxaderas S, Torre-Boronat MC. Quality of base and sparkling wines as influenced by the type of fining agent added pre-fermentation. Food Chemistry. 1999; 66:35–42.

Puig-Deu M, López-Tamames E, Buxaderas S, Torre-Boronat MC. Quality of base and sparkling wines as influenced by the type of fining agent added pre-fermentation. Food Chemistry. 1999; 66:35–42.

R. Marchal, E.J. Waters, 7 - New directions in stabilization, clarification and fining of white wines,Editor(s): Andrew G. Reynolds, In Woodhead Publishing Series in Food Science, Technology and Nutrition, Managing Wine Quality,Woodhead Publishing, 2010, Pages 188-225, ISBN 9781845697983,

R. Ferrer-Gallego, N.F. Brás, I. García-Estévez, N. Mateus, J.C. Rivas-Gonzalo, V. de Freitas, M.T. Escribano-Bailón. Effect of flavonols on wine astringency and their interaction with human saliva.Food Chemistry, 209 (2016), pp. 358-364

Ribéreau-Gayon P, Glories Y, Maujean A, Dubordieu D. The microbiology of wine and vinifications. In Ribéreau-Gayon P, editor. Handbook of Enology. Vols. 1 and 2. Paris: Wiley; 2000.

Riou, V., Vernhet, A., Doco, T., & Moutounet, M. (2002). Aggregation of grape seed tannins in model wine—effect of wine polysaccharides. *Food Hydrocolloids*, *16*(1). https://doi.org/10.1016/S0268-005X(01)00034-0

S. Vidal, P. Courcoux, L. Francis, M. Kwiatkowski, R. Gawel, P. Williams, E. Wa- ters, V. Cheynier, Use of an experimental design approach for evaluation of key wine components on mouth-feel perception, Food Qual. Prefer. 15 (2004) 209–217 10.1016/S0950-3293(03)0 0 059-4 .

Sacchi, K.L., Bisson, L.F., Adams, D.O. (2005) A review of the effect of winemaking techniques on phenolic extraction in red wines. American Journal of Enology and Viticulture, 56 (3), 197–206.

Salagoity, M.-H.; Guyon, F.; Rene, L.; Gaillard, L.; Lagreze, C.; Domec, A.; Baudouin, M.; Medina, B. Quantification method and organoleptic impact of added carboxymethyl cellulose to dry white wine Anal. Methods 2011, 3, 380– 384 DOI: 10.1039/C0AY00549E

Salazar FN, López F, Chiffelle I, López R, Peña-Neira A. Evaluation of pathogenesis-related protein content and protein instability of seven white grape (Vitis vinifera L.) clones from Casablanca Valley, Chile. European Food Research and Technology. 2012; 234:509-515

Scollary, G. R., Pásti, G., Kállay, M., Blackman, J., & Clark, A. C. (2012). Astringency response of red wines: Potential role of molecular assembly. Trends in Food Science & Technology, 27(1). *https://doi.org/10.1016/j.tifs.2012.05.002*

Serrano-Megías M, Núñez-Delicado E, Pérez-López AJ, López-Nicolás JM. Study of the effect of ripening stages and climatic conditions on the physicochemical and sensorial parameters of two varieties of Vitis vinifera L. by principal component analysis: Influence on enzymatic browning. Journal of the Science of Food and Agriculture. 2006; 86:592-599

Silva, Gustavo Thalmer M. and da Silva, Karen M. and Silva, Cassio P. and Rodrigues, Ana Clara B. and Oake, Jessy and Gehlen, Marcelo H. and Bohne, Cornelia and Quina, Frank H. (2019) "Highly fluorescent hybrid pigments from anthocyanin- and red wine pyranoanthocyanin-analogs adsorbed on sepiolite clay", *"Photochem. Photobiol. Sci.",* 2019",18 (7) pages "1750-1760", The Royal Society of Chemistry",doi "10.1039/C9PP00141G", *http://dx.doi.org/10.1039/C9PP00141G*

Simonin S., Alexandre H., Nikolantonaki M., Coelho C., Tourdot-Maréchal R. Inoculation of Torulaspora delbrueckii as a bio-protection agent in winemaking. Food Research International (2018) 107: 451-461.

Smith, P. (2017). Factors affecting wine texture, taste, clarity, stability, and production efficiency. Australian Wine Research Institute. Project report 3.1.4

Sommer, S., Dickescheid, C., Harbertson, J. F., Fischer, U., & Cohen, S. D. (2016). Rationale for Haze Formation after Carboxymethyl Cellulose (CMC) Addition to Red Wine. Journal of Agricultural and Food Chemistry, 64(36). *https://doi.org/10.1021/acs.jafc.6b02479*

Tattersall DB, Pocock KF, Hayasaka Y, Adams K, Van Heeswijck R, Waters EJ, et al. Pathogenesis related proteins—Their accumulation in grapes during berry growth and their involvement in white wine heat instability. Current knowledge and future perspectives in relation to winemaking practices. Molecular Biology and Biotechnology of the Grapevine: Kluwer Academic. 2001:183-202

Tian B, Harrison R, Morton J, Jaspers M, Hodge S, Grose C, et al. Extraction of pathogenesis-related proteins and phenolics in Sauvignon Blanc as affected by grape harvesting and processing conditions. Molecules. 2017;22(7):1164

Tian B, Harrison R, Morton J, Jaspers M. Changes in pathogenesis-related proteins and phenolics in Vitis vinifera L. cv. 'Sauvignon Blanc' grape skin and pulp during ripening. Scientia Horticulturae. 2019; 243:78-83

Tian B, Harrison R, Morton J, Jaspers M. Influence of skin contact and different extractants on extraction of proteins and phenolic substances in Sauvignon Blanc grape skin. Australian Journal of Grape and Wine Research. 2020;26(2):180-186

Tian, B., & Harrison, R. (2021). Pathogenesis-Related Proteins in Wine and White Wine Protein Stabilization. *Chemistry And Biochemistry Of Winemaking, Wine Stabilization And Aging*. doi: 10.5772/intechopen.92445

Valentina Marassi, Matteo Marangon, Andrea Zattoni, Simone Vincenzi, Andrea Versari, Pierluigi Reschiglian, Barbara Roda, Andrea Curioni, Characterization of red wine native colloids by asymmetrical flow field-flow fractionation with online multidetection, Food Hydrocolloids, Volume 110, 2021,106204,ISSN 0268-005X, https://doi.org/10.1016/j.foodhyd.2020.106204. (https://www.sciencedirect.com/science/article/pii/S0268005X20302551)

van Sluyter, S. C., Marangon, M., Stranks, S. D., Neilson, K. A., Hayasaka, Y., Haynes, P. A., Ian Menz, R., & Waters, E. J. (2009). Two-Step purification of pathogenesis-related proteins from grape juice and crystallization of thaumatin-like proteins. Journal of Agricultural and Food Chemistry, 57(23), 11376–11380. *https://doi.org/10.1021/jf902365r*

Van Sluyter, S. C., McRae, J. M., Falconer, R. J., Smith, P. A., Bacic, A., Waters, E. J., & Marangon, M. (2015). Wine protein haze: Mechanisms of formation and advances in prevention. *Journal of Agricultural and Food Chemistry, 63*(16), 4020–4030. https:// doi.org/10.1021/acs.jafc.5b00047.

Vassilopoulou, E.; Zuidmeer, L.; Akkerdaas, J.; Tassios, I.; Rigby, N. R.; Mills, E. N.; van Ree, R.; Saxoni-Papageorgiou, P.; Papadopoulos, N. G. Severe immediate allergic reactions to grapes: part of a lipid transfer protein-associated clinical syndrome Int. Arch. Allergy Immunol. **2007**, 143 (2) 92– 102

Vidal, S., Francis, L., Williams, P., et al. (2004) The mouth-feel properties of polysaccharides and anthocyanins in a wine like medium. Food Chemistry, 85 (4), 519–525.

Vincenzi, S., Marangon, M., Tolin, S., & Curioni, A. (2010). Protein evolution during the early stages of white winemaking and its relations with wine stability. Australian Journal Of Grape And Wine Research, 17(1), 20-27. doi: 10.1111/j.1755-0238.2010. 00113.x

Waters EJ, Wallace W, Tate ME Williams PJ. Isolation and partial characterization of a natural haze protective factor from wine. Journal of Agricultural and Food Chemistry. 1993; 41:724–730.

Waters, Elizabeth & ALEXANDER, G. & Muhlack, Richard & POCOCK, K.F. & Colby, C.B. & O'Neill, Brian & HØJ, P.B. & Jones, Patrik. (2005). Preventing protein haze in bottled wine. Australian Journal of Grape and Wine Research. 11. 215 - 225. 10.1111/j.1755-0238. 2005.tb00289.x.

Watrelot, A. A., Schulz, D. L., & Kennedy, J. A. (2017). Wine polysaccharides influence tannin-protein interactions. Food Hydrocolloids, 63, 571–579. https://doi.org/ 10.1016/j.foodhyd.2016.10.010.

Zimman, A. and Waterhouse, A.L. (2004) Incorporation of malvidin-3-glucoside into high molecular weight polyphenols during fermentation and wine aging. American Journal of Enology and Viticulture, 55 (2), 139–146.

Zimman, A., Joslin, W.S., Lyon, M.L., et al. (2002) Maceration variables affecting phenolic composition in commercial- scale Cabernet Sauvignon winemaking trials. American Journal of Enology and Viticulture, 53 (2), 93–98.

Zoecklein, B. (1987) Protein fining agents Part III. Practical Winery Vineyard November/December: 42-43.