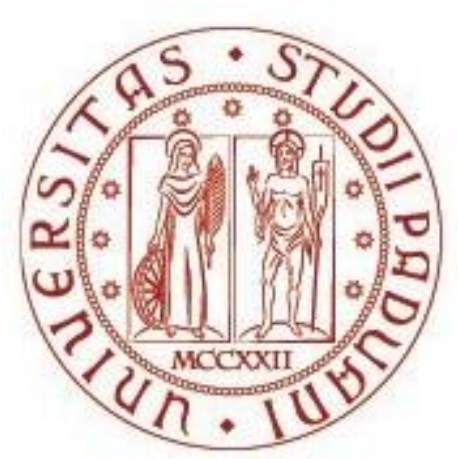

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Impact of the macromolecular composition on the colloidal state of red wines

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Index

Abstract

Red wine is a made of water, alcohols, organic acids, and other large and small molecules such as phenolic compounds, proteins and polysaccharides, all contributing to the wines' organoleptic properties that are in constant evolution during aging. This evolution implies that wines need to be stabilised against several potential instabilities before being bottled. Among these instabilities, red wine colloidal stability needs to be secured so that the wine does not form a precipitate during the period of storage after bottling to its consumption.

The appearance of turbidity in red wine can be triggered by different molecules such as proteins, polysaccharides, and phenolic substances, which interacting with each other over time, increase in size and become insoluble, thus causing haze in the bottled product. Indeed, wine proteins and polysaccharides interact differently with polyphenols, forming complexes of different sizes and properties. In this context, wine colloids can modify the physico-chemical properties of wines as their assembly can influence wine stability, colour, taste, and mouthfeel.

This thesis investigates, by means of colorimetric and chromatographic techniques, the impact of the macromolecular composition on the colloidal state of red wines monitored during aging at different temperatures and submitted to 4 different treatments able to modify the initial wine macromolecular content. The data obtained allow us to record different behaviours in the macromolecular composition of wines, caused by different effects such as: maceration, heat-treatment (thermos-vinification), deproteinization and storage at different temperatures.

In general, the samples that have undergone maceration in contact with the skins displayed a higher Colour Index and polyphenol content than the heat-treated samples, a fact attributable to the longer contact time with the skins allowing more pigments to be extracted. In addition, from the electrophoretic analysis it appears that the thermovinified samples contained free proteins, with an electrophoretic profile similar to those of white wines, while the samples produced with skin maceration showed only protein material in aggregated form.

When looking at the particle size of the colloids present in the wine, it generally appeared that larger colloids were present in samples treated with bentonite and Proctase, as the latter are treatments aimed at reducing the number of proteins. In fact, it is hypothesized that colloids made up of just tannins and polysaccharides are less compact than those containing proteins as well. Probably, the smaller size of colloids would make the wine less stable due to a lower risk of precipitation phenomena, a theory that, however, needs to be further tested.

Finally, the stability tests indicate that storage at cellar temperatures makes the wine more stable than storage at room temperature. This could have implications in the marketing phase of the product.

1. Introduction

Red wine is made of water, alcohol, organic acids, and other large and small molecules such as phenolic compounds, polysaccharides, nitrogen compounds, and volatiles (Ribéreau-Gayon et al., 2018). One of the key characteristics of red wine is that its composition and organoleptic properties are constantly evolving during aging. Among the characteristics that change over time, wine clarity is very important as it is essential for consumers that consider the presence of suspended particles a wine fault that influences the overall sensory evaluation of the wine. Winemakers recognise this potential problem and take measures to guarantee that the wine is clear at the moment of bottling, but it is also necessary that it remains clear during storage.

Despite the key importance of wine clarity and stability over time, the level of knowledge on the phenomena leading to the appearance of hazes in red wines is scarce. What is known is that, in addition to microbial contamination and tartaric precipitations, the cloudiness that can alter the limpid wine is due to colloidal phenomena (Ribéreau-Gayon et al., 2018). In particular, the formation of haze in red wines is mainly due to proteins, polysaccharides, and phenolic substances, which are the colloid-forming molecules that, over time, can become insoluble. In particular, the protein-tannin interaction appears to be the main cause of the formation of haze (Chursina et al., 2021).

Given the richness of phenolic compounds in red wines, many authors claim that the phenolic compounds are the unstable molecules in wine leading to haze formation as they tend to aggregate and precipitate over time (Nigena et al., 2019). However, wine contains other colloid-forming molecules, such as proteins and polysaccharide, so that the phenolics' physicochemical properties are modified by the presence of these compounds as they interact with each other to influence wine stability, taste, and mouthfeel. Indeed, wine colloidal particles are formed by molecular interactions involving proteins, polysaccharides, and condensed tannins. These macromolecules can come from grapes, yeast, bacteria, or originated, or be removed from the wine depending on the treatments used (e.g., fining treatments).

According to several studies, wine proteins and polysaccharides interact differently with phenolics, forming complexes of different sizes and properties (Marassi et al., 2021; Chursina et al., 2021).

Proteins and tannins are known to interact with each other to form insoluble complexes, and this property is exploited to remove the most reactive wine tannins via fining treatments with protein additions (e.g., animal or plant-based proteins) (Marangon et al., 2019). Conversely, polysaccharides interact with tannins, forming a soluble colloid that results in a decrease in the astringency perception in wine.

The study of wine colloids is technically challenging. One of the key parameters that researchers have been able to measure is the size of the wine colloidal particles, information that has been collected by several techniques, including dynamic light scattering (DLS), nanoparticles' tracking analysis (NTA), and Asymmetrical Flow field flow fractionation (AF4) (Bindon et al., 2016; Marangon et al., 2011; Marassi et al., 2021). Results from these studies indicate that the size of wine colloids is larger than the individual sizes of colloid-forming molecules, meaning that different molecules are necessarily present in the colloidal particles and that their dimension depends on the percentage of each of them within the complex. Additionally, their proportion is closely dependent on winemaking treatments like maceration, as each component has different kinetics of extraction, bringing differences in size and reactivity (Marassi et al., 2021).

1.1 Proteins

Wine contains a large variety of nitrogenous substances, but the most important are proteins. These molecules do not contribute to the nutritional value of the product given that their concentration is between 15 and 230 mg/L (Van Sluyter et al., 2015). Despite this, they play a role of considerable importance from an economic and technological point of view, as they influence the clarity and stability of wine.

By analysing different types of red wine, some authors have understood that the main cause that indicates variation in the nitrogen content in grapes are the climate, the soil, and the variety (Ferreira et al., 2002). The crucial moment for the extractability of proteins from the grape skins into the wine is the harvest. The main proteins found in the mature bunch are the pathogenesis-related (PR) proteins, which identify themselves in chitinases (PR3 family) and thaumatin-like proteins (TLP, PR5 family). These proteins are produced during the ripening of grapes and are involved in the plant's defence mechanisms against fungal attacks. They can hydrolyse chitin, which is a fundamental compound of the cell wall of the fungus. Furthermore, also invertase is considered one of the most abundant proteins in wine, representing a variable content from 9 to 14% of the total protein (Curioni et al., 2012).

The fermentation step causes the main difference in the protein content from the grape juice to the wine. During the fermentation proteins undergo processes of proteolysis and denaturation due to proteases, an occurrence also favoured by the change in wine pH. Therefore, wines normally contain less proteins than musts. PR proteins are generally soluble in acidic solutions, resistant to proteases, and have antifungal properties (Flamini et al., 2010), so they survive the vinification process and end up in the finished wines.

Wine proteins are characterized by compact globular structures and stabilized by a certain number of intramolecular disulphide bonds ranging from 6 to 8 (Curioni et al., 2012; Marangon et al., 2014). Reduction conditions induce the molecule's opening, which causes protein denaturation. This passage is crucial for protein aggregation and the formation of turbidity (Curioni et al., 2012).

The proteins that remain until the wine is ready are very resistant to proteolysis and low pH levels, which characterizes this product. Furthermore, the authors of this work discovered that about half of the proteins are bound to polyphenols from grapes, and during the vinification process, part of soluble proteins are precipitated thanks to the interaction with tannins (Ferreira et al., 2002). The work observed that the type of proteins produced is identical to those responsible for the haze. There are different isoforms of PR-proteins, and they vary according to the variety of the grapes (Flamini et al., 2010). The capacity of PR proteins to survive at low pH (3.0-3.8) and their resistance to proteolytic activity allow them to survive in the winemaking process, causing defects in wine.

There are also other types of proteins, like arabinogalactan proteins (AGPs), present in an amount of 4.3 and 5.2% of the total alcohol-precipitable colloids, whose protein content is less than 10%. The arabinogalactan proteins are released from the grape pectin, thanks to the endogenous activity of proteolytic enzymes. This type of protein reduces the filterability of wine (Ferreira et al., 2002).

According to a study by (Marangon et al., 2022), the level of ripeness of grapes can affect the number of proteins and polysaccharides present. Moreover, the extractability of these compounds increases during the ripening phase, due to the softening and cell wall. In addition, the variability of macromolecules in wine may be partially due to macromolecular interactions and phenolic compounds present in grape varieties. The result is the formation of soluble or insoluble compounds in different proportions.

According to the work analysed (Marangon et al., 2022), wines with different origins contain a very similar quantity of total tannins but differ in the number of reactive tannins with proteins. This fact could affect the formation of colloids. The electrophoretic profile analysed of different wine varieties appears to be very similar to each other. What differs is the electrophoretic mobility of the wine proteins, in fact, this is modulated by their interaction with the other components of the wine. It is hypothesized that it is due to the type of tannin present in the wine and the most obvious candidates in the formation of aggregates are the reactive tannins with proteins. The type of tannin is closely linked to the single variety.

1.2 Polyphenols

Polyphenols are the most important compounds regard the quality of red wine as they contribute to their organoleptic properties, color, astringency, flavour, bitterness, enzymatic and non-enzymatic

browning, and haze formation for the interaction with proteins, as well as aging potential and behaviour. The structure of polyphenols comprises a benzene ring with at least one hydroxyl group attached to it. These compounds can be divided into two main groups: flavonoids and non-flavonoids. The first is composed of flavan-3-ols, flavonols, and anthocyanins, while the second group contains hydroxycinnamates, hydroxybenzoate, and stilbenes (Watrelot et al., 2020).

1.2.1 Tannins

Tannins can interact with proteins and other molecules as polysaccharides and polyphenols. There are two types of tannins: Hydrolysable tannins and condensed tannins, where the first group is more common in fruits and leaves, and the second group is mostly found in stem, skin, flesh, and seeds of berries (Watrelot et al., 2020).

The name hydrolysable tannins is due to the fact that they are easily hydrolysed by acids, bases, hot water, and some enzymes. These are not natural grape tannins; they constitute the main commercial tannins used in the treatment of wine. They are divided into two main classes: gallotannins and ellagitannins (Figure 1.1). This denomination was decided because units of gallic acid or ellagic acid are released after a hydrolysis reaction. Ellagic acid can come from wooden barrels or from the addiction to oenological tannins. Gallic acid is present in all wines and can be also originated from the grape skin or the seeds (Ribereau-Gayon et al., 2018).

Condensed tannins are oligomers and polymers of flavan-3-ols, and the grapes of *Vitis vinifera* contain an amount of 3 to 110 mg/g berry weight. This type of tannin is found predominantly in red wine due to maceration time and skin contact and the concentration of tannins in red wine is between 50 mg/L to 4 g/L. The ethanol produced by yeast during alcoholic fermentation induces solubilization of lipids, which allows better extraction of tannins from seeds. The flavanols are composed of the carbonaceous skeleton of C6-C3-C6 with two aromatic rings A and B and one pyran ring. Catechins are the monomers of flavan-3-ols, and they can be distinguished based on the stereochemistry of the asymmetric carbons C2 and C3, the presence of galloyl groups, and the level of hydroxylation on the B-ring. It is called (+) catechin if there is a di-hydroxylation at C3' and C4' of the B-ring, whereas (-) catechin has a tri-hydroxylation on the B-ring (Watrelot et al., 2020).

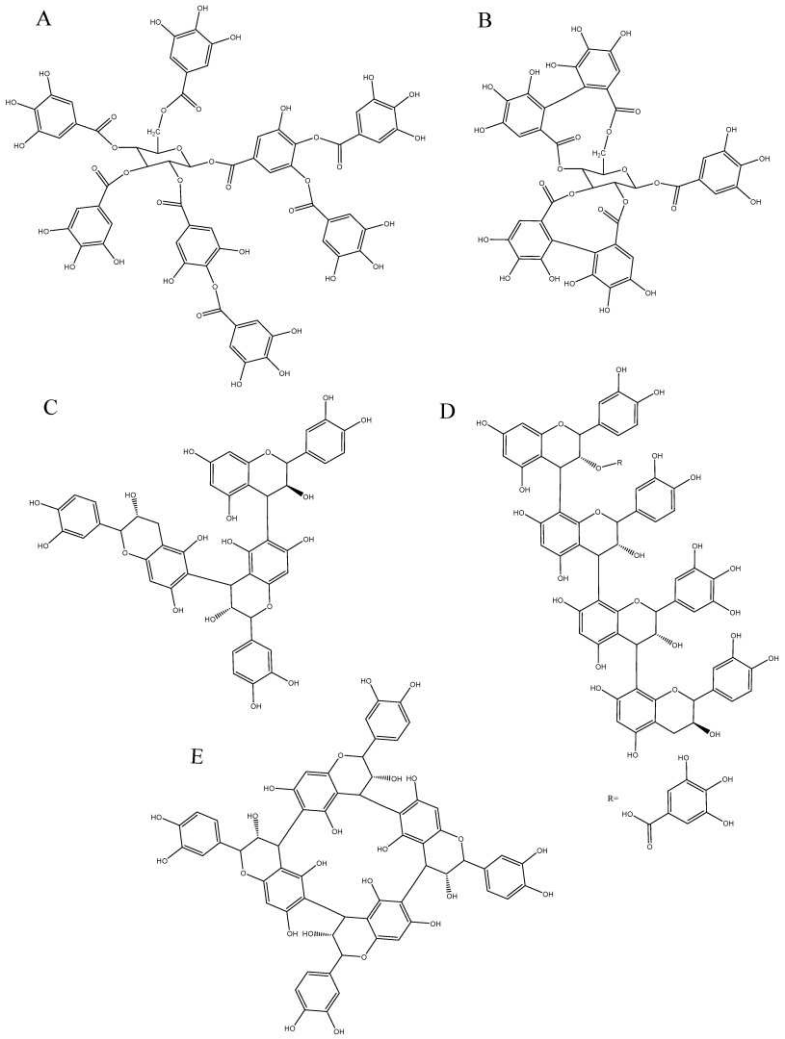


Figure 1.1. Chemical structure of hydrolysable tannins, gallotannins (A); and ellagitannins (B); and condensed tannins including trimer of catechin and epicatechin linked in C4-C6 (C); and crown procyanidin consisting in a tetramer of epicatechin linked in C4-C6 and C4-C8 (E) (Watrelot et al., 2020).

1.3 Polysaccharides

Polysaccharides come from the cell walls of the grapes, the yeast, and other microorganisms during the winemaking process (Rodrieguez et al., 2019). They are the main macromolecules of colloidal nature in wines, are characterized by high molecular weight, and are soluble in water.

These compounds do not directly influence organoleptic properties, but they are fundamental in the winemaking process. The polysaccharides take part in colloidal interactions helping the wine stabilization, in fact, they also reduce the formation of hazes and precipitates (Morendo et al., 2012). Polysaccharides are extracted during mechanical operations applied on the grapes, like pressing, pumping, and during some stages of winemaking, like maceration or aging on the red wine lees.

During the early stages there is an increase in macromolecules, but on the contrary, the next steps, such as filtration, occur a decrement in polysaccharides content.

The three major families of polysaccharides present in red wine are:

i) PRAG: polysaccharides rich in arabinose and galactose.

ii) RG-I and RG-II: polysaccharides rich in rhamnogalacturonans, which come from the pectin on the cell wall of grape berries.

iii) MP: mannoproteins, released by yeast during the fermentation and aging steps.

Other polysaccharides, such as glucans, can be found in wines only produced from grapes infected by Botrytis *cinerea*.

Rhamnogalacturonans-I represents a very small portion of the grape skin, and rhamnogalacturonans-II is formed during the maturation but is released during the winemaking process. They are important to reduce the astringency in wines because are reactive with tannins, forming large aggregates.

Arabinogalactan proteins (AGP) are in the cell walls and are extracted during winemaking. These polysaccharides come from the side chains of pectin and are connected themselves thanks to hydroxyproline-rich proteins. AGP and pectin together form polysaccharides rich in arabinose and glucose (PRAG). They contribute to the body and viscosity of wines.

Mannoproteins (MP) are proteoglycans, molecules that contain a high quantity of sugars (mannose up to 90%), which protect the protein part (about 10%). MP finds use in tartaric and protein stabilization, color stabilization, and the reduction of astringency (Rodrieguez et al., 2019).

1.4 Colloids and their properties

The colloidal system is a mixture of at least one dispersed phase and one dispersing phase. The main feature of this mixture is the negligible gravity effect compared to thermal agitation, the consequence of which is the absence of precipitation of colloidal particles. The mechanism involved in the colloidal phenomenon in wine consists of two stages: initially, occurs the formation of mainly colloidal particles (i.e., colloidal colouring substances), which remain in solution and leave the wine limpid. Later, due to various factors, they aggregate causing flocculation, responsible for turbidity (Ribereau-Gayon et al., 2018).

The practice of fining is an example of induced flocculation treatment, which consists in introducing a compound (typically a protein, but not always) into the wine that incorporates the particles present, creating flocculation. Thanks to these operations it is possible to stabilize the wine because the invisible and unstable colloidal particles are removed. At the same time, a clarifying effect is

obtained, because the proteins react with particles that are already in suspension, causing a decrease in wine turbidity (Ribereau-Gayon et al., 2018).

Colloidal particles are made of many types of substances characterized by different chemical families and their range is between 1 nm and 1 µm. Colloids can be grouped into two broad categories:

i) Macromolecular colloids: organic polymers such as proteins, polysaccharides, and tannins, affected only by covalent chemical bonds. This type of polymeric colloid is hydrophilic and therefore dissolves in water.

ii) Colloidal dispersions: aggregates of many different molecules, linked together by weak bonds such as Liftshitz-van der Waals, hydrogen, or hydrophobic interactions. The stability of colloidal dispersions is given by the presence of electric charges on the particles that cause repulsion between them.

These association colloids are present in wine: they can be formed naturally during storage (i.e., phenolic compounds condensed) or following some treatments (i.e., copper sulphide). The addition of electrolytes of opposite charge causes flocculation and therefore precipitation. The hydrophobic character of these colloids affects their instability (Marassi et al., 2021).

They have some properties in common (Moreno et al., 2012):

- Do not settle but they move with Brownian-type motions
- Do not diffuse or dialyze
- Do not modify fusion or ebullition point of solutions
- They create residues of amorphous precipitates, and it is not possible to recognize a defined structure
- The composition of a colloid is not perfectly defined
- They diffuse the light by Tyndall-effect
- They can flocculate
- Colloidal cloudiness is not regulated only by the law of mass action.

1.4.1 Colloidal interactions

The colloidal stability is strongly related to the physical-chemical interactions between the particles. According to many authors, the interaction potential of these colloids is the sum of the relative potential of Liftshitz-van der Waals, electrostatic, and polar hydrophobic or hydrophilic interactions. In aqueous solvents, Lifshitz-van der Waals between colloids are usually attractive and small: when they are in contact with each other the force is maximum and decreases with increasing distance.

The electrostatic interactions occur when colloids carry surface charges, thanks to the presence of ionized groups. This leads to the accumulation of counter-ions in the liquid and the formation of an electric double layer. The colloids come close to each other because there is an overlap of the double layer; they are repulsive forces if they have the same charge, while if the charge is opposite, they attract each other (Moreno et al., 2012).

Several authors state that electrostatic interaction decrease with increasing concentration of salt in solution. When the solution is saturated with salts, the electrostatic forces become negligible compared to Liftshitz-van der Waals: the particles tend to associate and then precipitate.

The colloidal stability was evaluated by Liftshitz-van der Waals and electrostatic interactions. When two particles with the same radius (100 nm) approach each other thanks to thermal agitation and they are very close, repulsive forces are weak and precipitation is easier. To reach this position, the particles must cross an energy barrier located at distance between 5 and 20 nm. when the concentration of salts is low, the energy barrier is elevated due to the presence of Liftshitz-van der Waals. In these conditions, the solution is stable because the thermal energy is not enough to cross the barrier. For higher salt concentrations, electrostatic interactions decrease and no longer compensate for Liftshitz-van der Waals: the energy barrier doesn't exist anymore, thus is created an irreversible aggregation (Ribereau-Gayon et al., 2018).

In water, the hydrophobic interaction is characterized by a strong attraction of hydrophobic surfaces and groups. The hydrogen bond is created when a hydrogen atom, covalently bonded to an electronegative atom, interacts non-covalently with a separate electronegative atom.

Many proteins and tannins can create bonds where are possible these types of interactions and their structure influence the force and the number of bonds (Watrelot et al., 2021). The kinetics interaction also depends on the concentration of colloids and their probability of colliding. In systems such as wine, macromolecules and colloids can interact with each other by participating in the stabilization of particles, by acting as "protective colloids", or on the contrary, they can induce flocculation (Curioni et al., 2012).

1.5 Reactivity of tannins with proteins

Colloidal aggregation in wines can develop during vinification or aging in the bottle. The colloidal instability is mainly related to the colouring matrix of wine; therefore, it could be given by the lower solubility of some particles, which tend to gradually aggregate during aging; all these processes follow the kinetics of chemical-physical interaction between the particles. The colloidal instabilities could also be caused by interaction between protein and polysaccharides (Moreno et al., 2012).

The limiting step for particle formation appears to be protein denaturation; once this happens, the aggregation phenomenon occurs easily. To induce the fast denaturation of proteins it is necessary to reach a temperature of about 50-60°C, but it is necessary to consider other limiting factors which are involved in the phenomenon of protein precipitation. Many authors have shown that purified proteins in model wine are unable to produce turbidity (Marangon et al., 2011). It has been shown that ions, polyphenols, and polysaccharides can modulate the formation of turbidity (Curioni et al., 2012).

Another factor affecting the stability of proteins is the presence of salts. For instance, the sulphate ion is fundamental for protein denaturation and therefore the turbidity in wines. Several studies have shown a greater susceptibility of chitinases to the formation of turbidity, in the presence of this ion (Curioni et al., 2012).

It is also necessary to consider the presence of tannins, which can have big effects on protein precipitation (Curioni et al., 2012). The two main bonds that can occur between tannins and proteins are hydrophobic interactions and hydrogen bonds. It is possible to state that the greater the degree of hydrophobicity, the greater the degree of tannin reactivity. In the presence of reduced amounts of proteins, the polyphenols are arranged on the surface decreasing their hydrophilic character. This causes an agglomeration between proteins and eventually they precipitate (Ribereau-Gayon et al., 2018).

Under normal conditions, the hydrophobic regions are hidden within the protein. Thanks to heat treatment, the protein structure opens and exposes the hydrophobic portions. The tannins, already present on the external surface of the molecule, can bind to the new binding sites causing cross-linking and complex precipitation (Curioni et al., 2012).

Interactions between tannins and macromolecules are influenced by the structural variability of tannins. Depending on the constitutive units, the types of linkages connecting these units, the mean degree of polymerization or size of tannins, and the conformation of longer chain tannins, it is possible to obtain different behaviours. These characteristics depend on the grape variety, and they could be influenced by the growing season.

The pyran ring of flavan-3-ols is involved in the binding potential and aggregation of tannins to proteins. According to this study, the presence of the galloyl group on the (+) catechin and (-) catechin induces an aggregate haze when the molecules are exposed to poly-L-proline. This does not happen in absence of the galloyl group bound to (+) catechin and (-) catechin (Watrelot et al., 2020).

The authors speculated that the galloylated monomers could interact with two protein units bilaterally, and consequently cause aggregation. Non-galloylated monomers did not cause an insoluble aggregate for two possible reasons: the interaction did not cause the insolubilization of complexes or the non-

galloylated monomers were more soluble than galloylated monomers and then interacted less with proteins.

It has been shown that the larger the size of tannins, the greater the interaction with proteins and then their precipitation. This is due to the increase in the number of functional groups capable of interacting with proteins (Watrelot et al., 2020).

The bonds between procyanidins, which derive from condensed tannins, and the polysaccharides of grape skin, constitute another type of association mechanism. Acid polysaccharides, such as arabinogalactans, react strongly and in the presence of proteins, they favour the formation of complexes with tannins (Ribereau-Gayon et al., 2018).

1.6 Colloidal stability in red wine

Wine clarity is an essential requirement that the consumer asks for. The presence of particles impacts the presentation and influences the sensory evaluation of wines. Thanks to various technological processes, such as filtration or centrifugation, it is possible to obtain the desired clarity. It is necessary to underline that it is not enough to maintain its clarity until bottling, but that it also needs to be maintained during storage. The interventions that are carried out in the cellar to obtain stability and clarity are different. The filtration clarifies but does not stabilize, while the fining operation stabilizes but does not clarify. The mechanisms of clouding of white and red wines are based on the properties of colloids. These are linked to the conditions of enlargement of the particles, which cause their flocculation and sedimentation (Ribereau-Gayon et al., 2018).

In the past years, studies concerning colloidal stability were purely focused on white wines as clarity is a requirement for this type of wine. Despite this, recently several studies have also focused their attention on red wines, as they are characterized by the problem of colloidal instability too. Therefore, it is necessary to investigate the dynamics of colloid formation and how they can influence the stability of red wine.

2. Aim

The aim of this thesis is to try to elucidate, by means of colorimetric and chromatographic techniques, the impact of the macromolecular composition on the colloidal state of red wines monitored during aging at different temperatures and submitted to different treatments able to modify the initial wine macromolecular content.

3.1 Materials

All reagents used were purchased from Sigma-Aldrich (Milan, Italy), Merck KGaA (Darmstadt, Germany), and Biorad Laboratories S.r.l. (Segrate-MI, Italy) unless otherwise stated. The grapes used for producing the experimental red wines were 100% Raboso Piave, sourced from the Conegliano area (Veneto, Italy) in September 2021.

3.2 Experimental winemaking

About 30 kg of handpicked and healthy Raboso Piave grapes were manually destemmed, an operation that yielded 24kg of grape berries. These were divided into 2 lots of 12 kg each and then further divided into two parts each. From this point on, two separate experiments were conducted by applying different treatments (Figure 2).

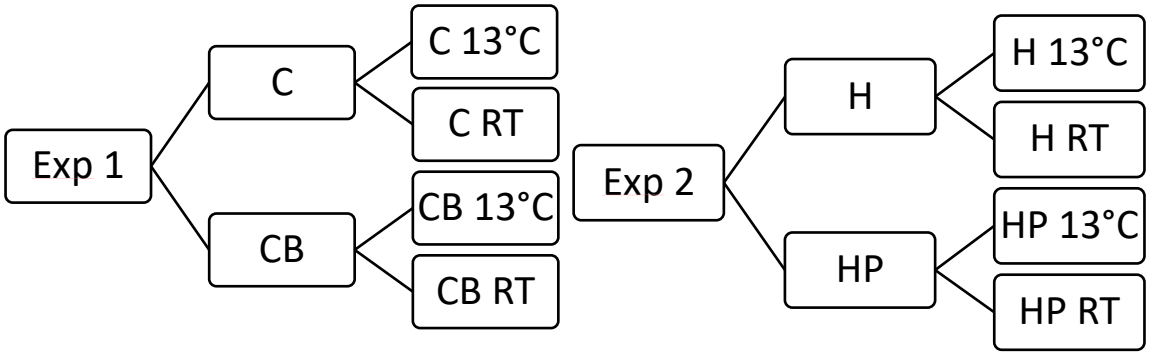


Figure 2.1. Experimental design (C: Control sample with 300 mL of Milliq-water; CB: sample treated with 10 mg/L bentonite; H: sample heat-treated; HP: sample heat-treated and with 50 mg/L of Proctase; 13 °C: storage at 13 °C; RT: storage at room temperature).

The first experiment aims to understand the trend of colloids, by comparing the wine treated with bentonite (CB) and the untreated control wine (C). The second experiment aims to understand the trend of colloids in heat-treated wine with addiction of the enzyme Proctase (HP), compared to the one just heat-treated wine (H).

3.2.1 Experiment 1: effect of bentonite fining on the colloids' formation

Raboso grapes were placed in two large plastic bags and pressed manually. Once the must was obtained, it was placed in two food-grade plastic beakers of 5L volume each. Subsequently, was added 2.5 g of yeast (conc. 500 mg/L) dissolved in water at 37 °C. Meanwhile, a stock solution of

bentonite (Pluxbenton, Enartis, Trecate-NO, Italy) was prepared by adding 30 mg of clay in 300 mL of Milli-Q water with constant stirring for 48 hours. Bentonite was then added at a concentration of 3 g/L in one container (CB), while in the control sample the equivalent amount of sole water was added. Immediately, 300 mg of DAP, a nitrogenous fermentation activator (Minvit, IOC, Epernay Cedex, France), was added to both samples C and CB.

3.2.2 Experiment 2: effect of heat and protease on the colloids' formation

Starting with a quantity of 12 kg of destemmed grapes, this was divided into 6 equal parts of 2 kg, and the berries were inserted in plastic zip-lock bags. Three of the six bags were added a dose of Proctase enzyme (a mix of Aspergillopepsin I and II, Meiji, Japan) equal to 50 mg/L diluted in 2 mL of Milli-Q water, while in the other three added just 2 mL of Milli-Q water to have the same dilution. All 6 samples were shaken gently and then placed inside a water bath set at a temperature of 70 °C for 10 minutes. The treatment has the purpose of favouring the heating of the berries, thus inducing the denaturation of proteins required for the Proctase to degrade them (Marangon et al., 2012).
Once the heat treatment was completed, the contents of each bag were transferred into food-grade plastic beakers, where a manual pressing was performed using the bottom of a smaller beaker. Subsequently, bags containing pressed grapes were placed again in a water bath set at 70 °C for 15 minutes. The attainment of the internal temperature of the samples was monitored thanks to a food thermometer. This process aims to activate the enzyme so that it can act on denatured proteins, thus degrading them. Once the procedure was completed, the resulting juice was filtered using a rather large mesh strainer, to allow the juice to flow out, but also to retain the skins and the seeds. The filtrate of both samples, named H (heated) and HP (heated + Proctase), was transferred into two carboys of a volume equal to 5L.
The H and HP musts were kept at room temperature for about 1 hour before being added with 1 g/L of yeast diluted in lukewarm water. The demijohns were then closed with parafilm. After 48h, 300 mg/L of DAP fermentation activator (Minvit, IOC, Epernay Cedex, France) were added to each container. This is intended to provide the yeast with the nitrogen necessary to carry out the fermentation.

Fermentations were carried out at room temperature, and it was completed for both experiments after 7 days. At the end of fermentation, 100 mg/L of potassium metabisulfite was added to each sample to avoid oxidation and microbial spoilage. Wines were let to settle in plastic demijohns for 2 months with no ullage. Subsequently, an additional dose of potassium metabisulfite (60 mg/L) was added, and the racked wines were bottled in 250 mL glass bottles closed with a tin screw cap. The samples

were kept at cellar temperature (13 °C) for about two months before the first round of analysis. Subsequently, it was decided to keep part of the samples at a temperature of 25 °C and another part at a temperature of 13 °C, to verify the difference in the behaviour of the macromolecules on the stability of the wine induced by the temperature.

3.3 Analytical methods

The concentration of organic acids (citric, tartaric, malic, succinic, lactic), glucose, fructose, glycerol, and ethanol were determined using the method proposed by Marangon (Marangon et al., 2011). To determine, the organic acids it was used a size exclusion chromatography with the Column Hi-Plex H Flow Serial No 000658609-134 was purchased from Agilent, and the running buffer was Sulfuric Acid 5 mM in water. To analyse the pH was used HI98100 Checker Plus purchased from Hanna instruments. Before doing each analysis, the wine was filtered using Whatman quality standard filter paper, Grade 1.

3.4 Protein analysis and sample preparation

Protein quantification and characterization in red wines was performed using a combination of a colorimetric method, SDS page electrophoresis, and HPLC and the sample preparation is the same for each method. The quantification of red wine proteins is always complicated, due to their strong interaction with tannins, which could interfere with the results. For this reason, various sample preparation methods have been applied, which include precipitation of proteins with TCA/acetone, KDS, and ethanol.

The TCA/acetone method followed the procedure described by Marangon (Marangon et al., 2022). The KDS method of protein precipitation followed the protocol explained by Vincenzi (Vincenzi et al., 2005). As regards the method using ethanol, the protocol described was followed by Kassara (Kassara et al., 2022).

3.4.1 Protein Assay

For the colorimetric methods, the pellet obtained with TCA/acetone was dissolved in 250 µL of Milli-Q water. For analysis, a volume of 20 µL was inserted and randomly added to the cells of a 96-well plate and then added with 200 µL Bradford solution for protein determination (A6932,0500, PanReac AppliChemBradford, 1976). The pellet obtained with the KDS and ethanol methods underwent the same treatment, but the results obtained with the spectrophotometer were more promising than using

the pellet from TCA/acetone method. The colour developed after the reaction was analysed by a Spectrometer Synergy HTX (Biotech) at 595 nm.

3.4.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoresis analysis SDS page followed the method described by Laemmli (Laemmli, 1970). The pellets obtained with TCA/acetone, KDS, and ethanol methods were dissolved in a 40 µL 4X Laemmli Sample Buffer (Biorad) and loaded onto a pre-cast gel (Mini-PROTEAN TGX Stain-Free Gels, Biorad).

The results obtained by TCA/acetone proved inconclusive. For this reason, it was then considered appropriate to change the procedure, using the KDS protocol. For the reading of the results, there were problems using QC Colloidal Coomassie Stain (Bio-Rad), so it was decided to proceed with the Silver Staining for high sensitivity protein detection (Blum et al., 1987). However, the best results were obtained by using the method of precipitation with ethanol and reading the results by Silver Staining. The intensities of the bands were analysed using a scanner connected to the computer.

3.4.3 HPLC Protein Analyses by Size Exclusion Chromatography

The protein fraction was analysed by exclusion chromatography using the HPLC instrument 1260 Infinity II Prime LC 800 bar (Agilent Technologies). The Buffer used was Ammonium Acetate 0.3 M and the column was PL aquagel-OH 40 8 µm Flow (Agilent Technologies). The pellet was dissolved in 1 mL of running buffer, then it was mixed with the vortex and centrifuged (14000 g, 5 minutes). In each HPLC-vial was added 900 µL of wine sample (centrifuged), and the injection volume was 100 µL.

3.4.4 Protein quantification by Nessler reagent

Wines at T1 were extensively dialysed against water (3.5 kDa MWCO). The equivalent of 50 mL of wine was divided in two falcon tubes, frozen and freeze dried. The freeze-dried material was weighed before being mineralised. The resulting solution containing nitrogen in water was titrated using the Nessler reagent, and the amount of proteins in the samples was calculated by multiplying the nitrogen concentrations x 6.25. The analysis was done using 6 samples per wine.

The method involves the transfer of the previously prepared sample in the digestion flask. The freeze-dried wine was added with 4 mL of concentrated sulfuric acid and heated at 440 °C. Once the temperature was reached, the sample was maintained hot for 4 minutes. Then 10 mL of 50% hydrogen peroxide were added, and the mixture was let to boil for over 1 minute until the excess of hydrogen

peroxide had evaporated but avoiding drying the sample. The sample was left to cool and diluted with milliQ water up to 100 mL.

The preparation of the Nessler's reagent was carried out in a cuvette. It was added 0.1 mL of NAOH 6N to the diluted sample and all made up to a volume of 2.5 mL with water. Once this was done, it was added 0.1 Nessler Reagent. The absorbance on the spectrophotometer was read at 425 nm.

The preparation of the standards was done by constructing a straight line starting from a concentration of 0.1 to 10 mg/L of nitrogen.

The composition of the Standard sample included 0.1 mL of NaOH 6N, 200 µL of Standard, 2200uL of water and 100 µL of Nessler reagent.

3.4 Polyphenol determination

For the analysis of the total polyphenols, the protocol described by Guillermo was followed (Guillermo et al., 2017). That is a Folin-Ciocalteu colorimetric method, and the results, expressed in mg/L of gallic acid equivalent (GAE), require the preparation of a calibration curve. It used Folin & Ciocalteu's phenol reagent F9252-500 mL (Sigma-Aldrich). The results were analysed using the spectrophotometer Synergy HTX multi-mode reader (Biotek) at 725 nm.

3.6 Polysaccharide determination

The total polysaccharide content was measured by a method based on the one proposed by (De Iseppi et al., 2021) and the results were expressed in mg/L of glucose. It used a size exclusion Chromatography with the Column PL aquagel-OH 50 8µm (Agilent) and the running buffer was Ammonium Formate 50 mM. The pellet was dissolved in 1 mL of buffer, then it was mixed with the vortex and centrifuged for 5 minutes. In each HPLC-vial was added 900 µL of wine sample, with an injection volume of 10 µL.

3.7 Colour index analysis

The protocol applied for the colour analysis provided a dilution of the sample with model wine (12% Ethanol, 2.5 g/L tartaric acid, pH 3.5) in the proportions 1:10; everything was done in triplicate. Then, 200 µL of diluted wine were randomly inserted into a 96-well plate and the absorbance was analysed at three different lengths (420 nm, 520 nm, 620 nm) using the spectrophotometer Synergy HTX multi-mode reader purchased from Biotech (Guillermo et al., 2017).

3.8 Stability tests

The wine colloidal stability was analysed using a turbidimeter (Range 0 – 1000 NTU; HI 93703 Portable Microprocessor Turbidity Meter, Hanna Instruments) in three different situations: room temperature, cold test (wine at 4 °C for 72 hours, room temperature for 1 hour), and heat test (wine at 80°C for 2 hours, room temperature for 2 hours, Waters et al., 1992). Everything was done in triplicate. The wine turbidity was then measured after wines re-equilibrated at room temperature.

3.9 NTA analysis

This type of analysis involves the use of the NanoSight tool. The protocol foresees a dilution of the samples with model wine with the proportions 1:2. Then the sample was inserted into the instrument, the NanoSight NS 300 from Malvern Panalytical Ltd (United Kingdom), applying the correct focus and the camera level 12.

3.10 Statistical analysis

All data were processed, statistically analysed, and visualised using Minitab software (Microsoft). One-way analysis of variance (ANOVA) followed by a post hoc Tukey test was used to determine statistical significance using an alpha value of 0.05.

Following small scale winemaking, the 4 types of wines produced (C and CB for Experiment 1; H and HB for experiment 2) were submitted to a series of physicochemical analyses aimed at elucidating the impact of the treatments on several parameters. The analyses were conducted shortly after the end of the alcoholic fermentation (T0), and again after 3 (T1) and 6 (T2) months of aging. From the 3rd to the 6th month of aging, an additional variable was added to the experiment, namely temperature for wine storage. Therefore, the four types of wine were stored at 13 °C and at room temperature (RT) (see Figure 2.1).

4.1 Fermentation kinetics and physicochemical parameters

The sugar content of the wines was recorded during fermentation to assess whether the treatments induced modification in the fermentation rates (Figure 4.1).

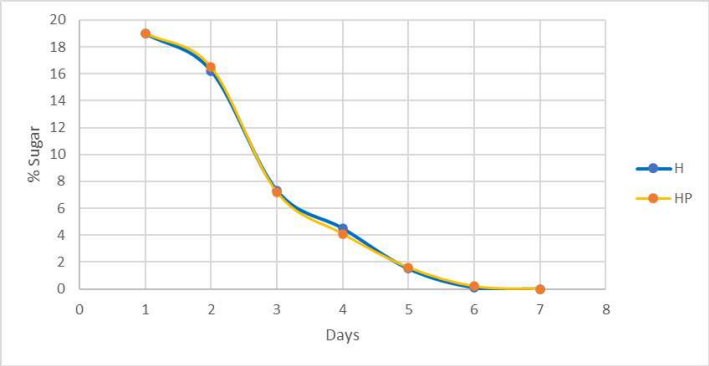


Figure 4.1. Fermentation kinetics of wines from experiment 2.

Figure 4.1 shows that no modification in the fermentation rate was attributable to the treatments, indicating that the heat treatment of the musts did not impact the ability of the yeasts to ferment the sugars to dryness. For practical reasons, the kinetic of sugar consumption during fermentation of C and CB was not recorded, but the quantification of residual sugars on the produced wines indicated that the fermentation was completed without problems (see Figure 4.2).

To further understand the general composition of the red wines produced in the laboratory, these were submitted to a series of analyses to determine their residual content in sugars (glucose and fructose, Figure 4.2), ethanol content (Figure 4.3), and organic acids (Figure 4.4).

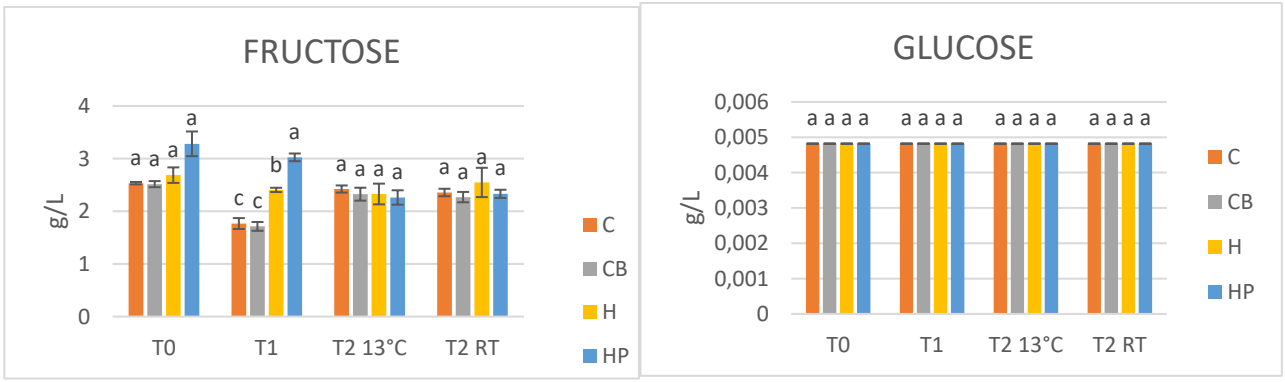


Figure 4.2. Concentration (expressed in g/L) of glucose and fructose during the experiment. Within each timepoint, different letters represent statistically significant differences between treatments (post-hoc Tukey test, $\alpha = 0.05$).

Results show that in all wines the glucose content was basically 0, while the fructose content varies from 2 to 3 g/L independently of the treatment considered. This is in agreement with the findings of Figure 4.1 and indicates that none of the treatments interfered with the ability of the yeasts to ferment the sugars. The sum of glucose and fructose is typically used to define the level of dryness of a wine. In this case, with about 2 or 3 g/L of residual sugar the wines could be defined as dry.

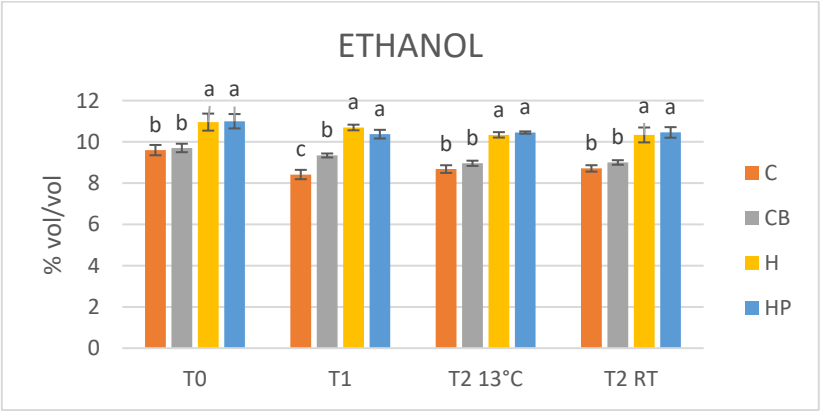
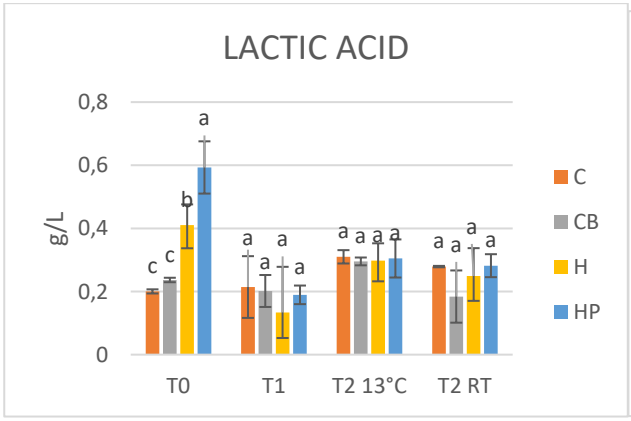
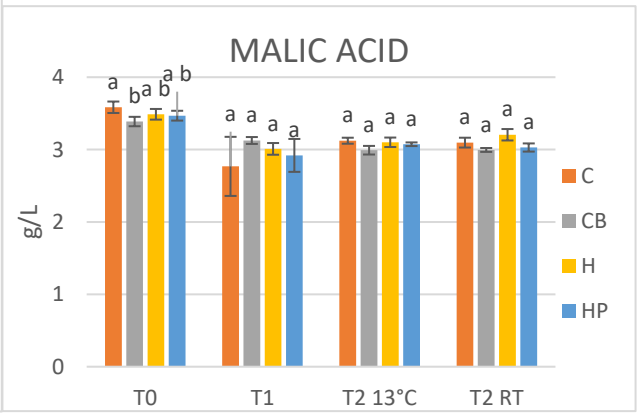


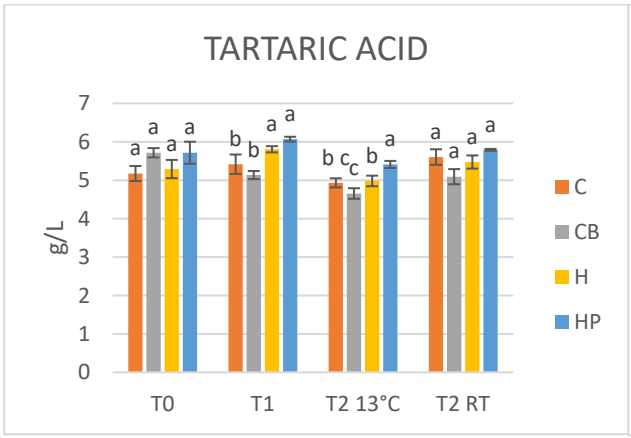
Figure 4.3. Concentration (expressed in % vol/vol) of ethanol in the wines throughout the experiment. Within each timepoint, different letters represent statistically significant differences between treatments (post-hoc Tukey test, $\alpha = 0.05$).

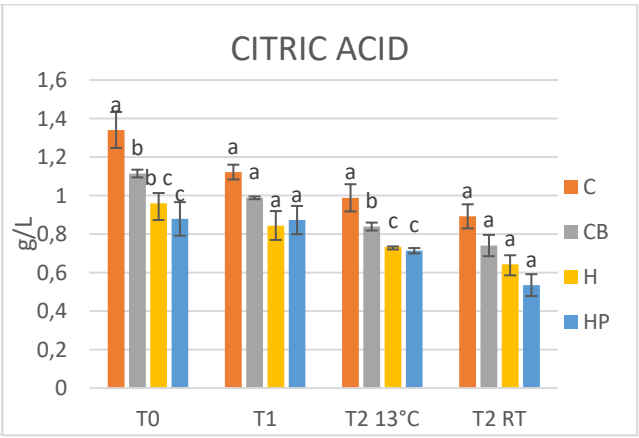
The samples have an alcohol content ranging from 8.42% to 11%, with differences visible between wines from experiment 1 (C and CB) and experiment 2 (H and HP). It seems that the heating resulted in higher ethanol content and that the treatment used to deproteinize the wines (Bentonite or Proctase)

did not affect this parameter. It is likely that the heat treatment applied in samples of experiment 2 resulted in juices richer in sugars, probably due to a higher sugar extraction from the grape skins, hence in wines with more alcohol. However, this occurrence could also result from the adsorption of ethanol by the grape skins in the C-CB samples, hence their lower alcohol content. The Anova test was performed on the wine samples to verify if there were statistically significant differences. According to the Tukey test, it was found that samples C and CB significantly differed from samples H and HP, in fact in no case do they share the same letter. Additionally, the ethanol content in both experiments (C vs CB and H vs HP) did not vary significantly, thus indicating that the treatments did not interfere with this parameter.









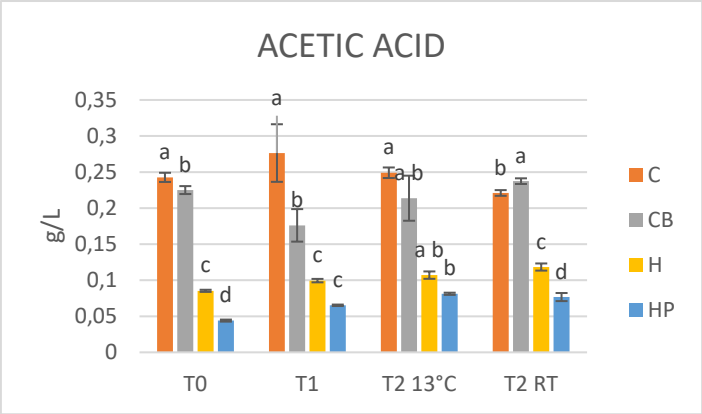


Figure 4.4. Concentration (expressed in g/L) of the five major wine organic acids throughout the experiment. Within each timepoint, different letters represent statistically significant differences between treatments (post-hoc Tukey test, $\alpha = 0.05$).

When looking at the 5 major organic acids a few interesting differences emerged. Citric acid content is maximum in the control at T0 and is in lower quantities in wines deproteinated (CB) or heat treated (H and HB). The general trend is for a notable decrease for all samples from time T0 to time T2 in citric acid content, a decrease that seems faster when wines are stored at RT. The wines from experiment 2 have a lower content in citric acid than wines from experiment 1.

Tartaric and malic acid, the two main wine organic acids, were quite stable and did not seem greatly affected by the treatments selected. The value of tartaric acid has almost remained unchanged, around 5-5.8 g/L. Malic acid, at time T0, was about 3.5 g/L for all samples, while at time T2 there was a slight decrease up to 3-3.2 g/L. Malolactic fermentation was stopped in these wines, and results confirmed that this worked as malic acid content were around 3 g/L and did not change over time. Equally, lactic acid content showed some initial variability between treatments, but in later timepoints, this variability disappeared, and all wines showed very low amounts of this acid (around 0.3 g/L).

Acetic acid values were very low and in line with quality red wines. Heating the juice resulted in wines with a lower acetic acid content, a fact probably due to the elimination of microbial competition for the commercial yeast used when heat treating the juice. Indeed, it is known that when yeast needs to compete with other microorganisms, they are more likely to secrete compounds related to stressful fermentation conditions such as acetic acid (Rantsiou et al., 2012).

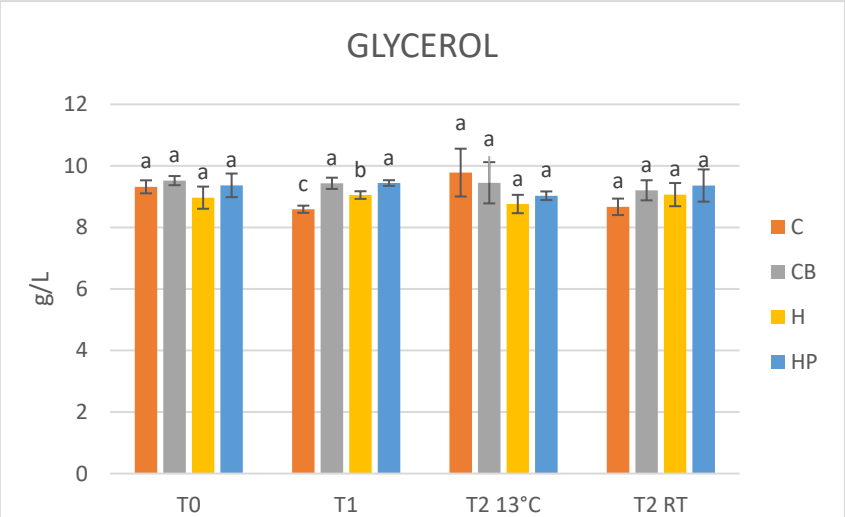


Figure 4.5. Concentration (expressed in g/L) of glycerol throughout the experiment. Within each timepoint, different letters represent statistically significant differences between treatments (post-hoc Tukey test, $\alpha = 0.05$).

The glycerol value is within the norm in line with the study by (Yanniotis et al. 2007), it turns out to be 8.5-9 g/L from the end of fermentation (T0) to T2, therefore after six months of aging.
The measured organic acid parameters are quite high, and in line with reports by other authors (Zohar et al., 2004) (Giacosa et al., 2021). Indeed, Raboso Piave grapes are well known for their high acidity (De Rosso et al., 2019).

4.2 Color and polyphenols

A series of analyses were performed to check whether the treatments had an impact on wine colour (Figure 4.6) and the extraction of total phenolic compounds (Figure 4.7).

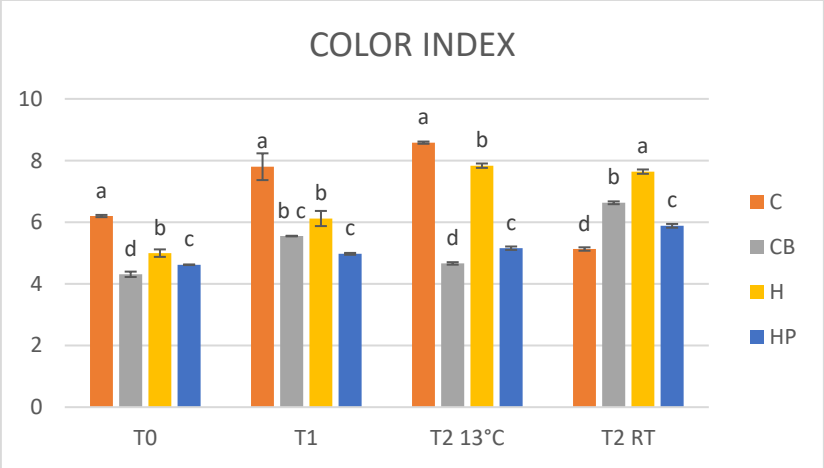


Figure 4.6. Color index values of wines throughout the experiment. Within each timepoint, different letters represent statistically significant differences between treatments (post-hoc Tukey test, $\alpha = 0.05$).

From the data shown in Figure 4.6, it is possible to highlight many differences. At time T0 sample C has a higher colour index than CB, the value for the first is 6.2 and for the second is 4.3. This could be caused by the treatment with bentonite removing some pigments from the wines (Dordoni et al., 2015). As red wine colloids are formed by associations of polysaccharides with proteins and phenolic compounds (including anthocyanins), the bentonite treatment likely removed some of the anthocyanins and polyphenols responsible for colour. At T0, the heating treatment resulted in wines with a lower colour index than C, and with values similar to CB. Additionally, the HP sample also showed a lower colour index than the H control. The colour index increased at T1, and again at T2 13°C, but the ranking between the 4 treatments remained the same, meaning that the impact that the initial treatment had on the colour was maintained over 6 months. Interestingly, comparing the graphs at time T2, the samples stored at 13 °C were more colourful than the samples stored at room temperature. Especially in sample C, at T2 13 °C the colour index was 8.58 and at room temperature is 5.13, which showed a strong colour decrease if stored at room temperature. It is possible to hypothesize that the higher temperature in samples stored at room temperature have destabilized some of the coloured wine colloids, with a consequent loss of colour most likely through precipitation of coloured matter. This resulted in sample C having the worst colour. Conversely, at a storage

temperature of 13 °C, this did not happen, so the colour did not precipitate either. The opposite happens for CB samples, at 13 °C the value is equal to 4.6, while at room temperature it is equal to 6.63. In general, the treated samples, therefore CB and HP, have a lower colour index than the samples C and H.

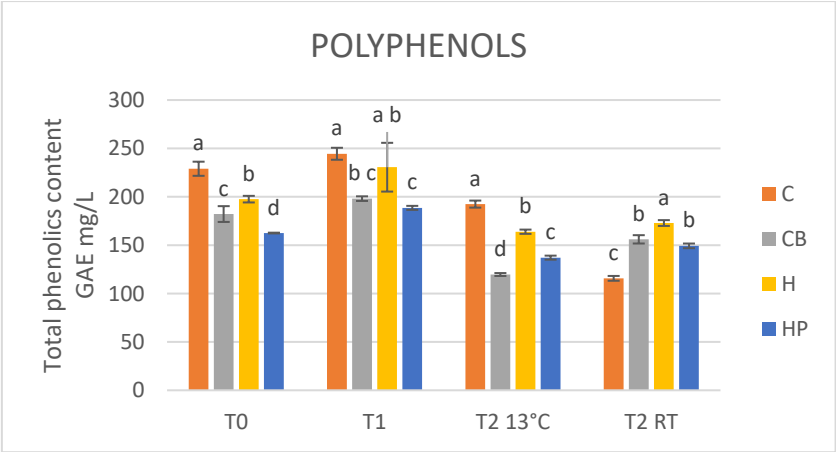


Figure 4.7. Concentration (expressed in mg/L of Gallic acid equivalent) of polyphenols throughout the experiment. Within each timepoint, different letters represent statistically significant differences between treatments (post-hoc Tukey test, $\alpha = 0.05$).

When looking at the total polyphenols' content (Figure 4.7), results are in line with those of the colour index (Figure 4.6), so the same commentary described in Figure 4.6 applies here too. The higher valuer for the wines from experiment 1 is attributable to the maceration on skins that took place only for this series.

In general, the fact that red wine colloids are unstable and can over time precipitate thus bringing down some colour, especially when stored at high temperatures, is a finding in agreement with the literature. This can be explained by the study by Bindon (Bindon et al., 2016) which hypothesizes the presence of a mechanism of loss of tannins extracted from grapes. Tannins bind easily to proteins creating aggregates and causing unwanted sedimentation or haze. The authors of the study state that about 50% of natural tannins from grapes can complex soluble proteins and cause precipitation.

Heat-treated samples H and HP at T2 RT, appear to be the most colourful and therefore also the most stable, probably due to the lower extraction of unstable compounds from grape skins during the fermentation/maceration step.

Interestingly, Figures 4.6 and 4.7 show that sample C RT had accelerated aging due to the high T, while C 13 °C was more coloured, and has more polyphenols. However, if the latter goes into the

trade, there is a risk of losing the polyphenols and the colour, of making sediment and therefore the wine will lose quality. Also in this case, CB 13°C has a lower value (119.6 GAE mg/L) of polyphenols than CB RT (156.0 GAE mg/L). At T2 the values of the samples H 13°C and H RT do not differ much, the samples stored at room temperature have a slightly higher value than those stored in cellar conditions. This also happens for HP samples.

4.3 Protein content

The initial aim of this work was to apply a natural enzyme (Proctase) extracted from the fungus *Aspergillus Niger* (Marangon et al., 2012) to remove proteins from the wines before fermentation, to be able to study the role of proteins on the formation and stability of wine colloids. Proctase appears to be a specific enzyme for proteins; however, it requires heat treatment at 75 °C for 5 min to cause the unfolding of wine proteins, a step that facilitates the proteolysis of these proteins by Proctase (Marangon et al., 2012). Based on this information, it was decided to add the enzyme immediately after the crushing operation and perform a heat treatment similar to a thermovinification protocol.
The quantification of proteins in red wines is challenging due to the phenolic compounds interfering with the measurement (Marangon et al., 2022). In this case, several approaches were attempted to obtain meaningful results, and figure 4.8 shows the findings of the 2 most successful methods used.

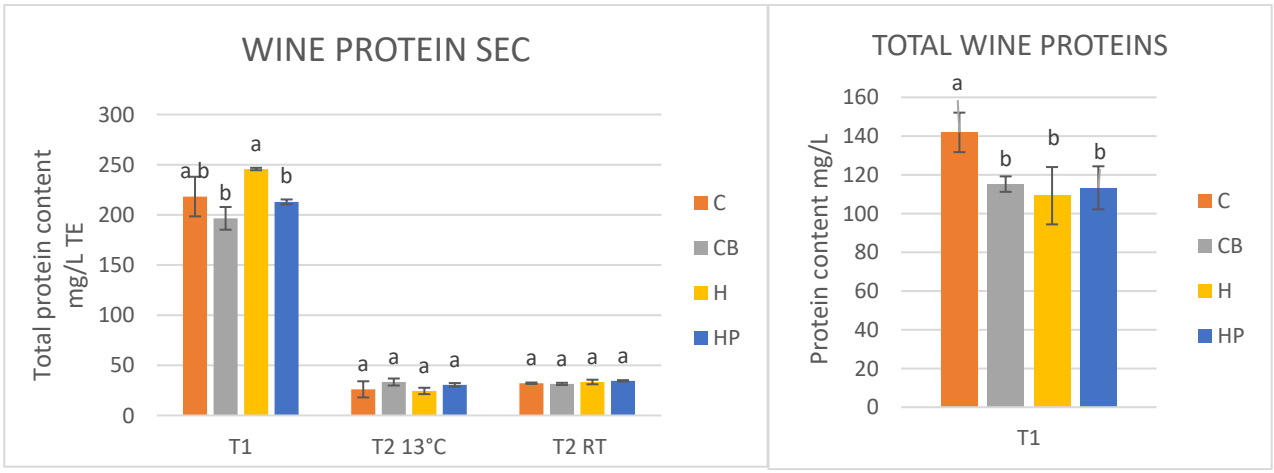


Figure 4.8. Left: Concentration (expressed in mg/L of Thaumatin Equivalent) of total wine proteins throughout the experiment. Right: Concentration (expressed in mg/L) of total wine proteins at T1 measured after mineralization and titration with Nessler reagent. Within each timepoint, different letters represent statistically significant differences between treatments (post-hoc Tukey test, $\alpha = 0.05$).

However, when looking at the protein content of the finished wines (Figure 4.8) it is clear that the attempt to deproteinize the wines did not work, except for the small but significant difference between C and CB at T1 (see figure 4.8, left). There are some potential explanations for the lack of protein removal observed in samples HP and CB: it is very likely that the proteins, once the wine is made, are already covalently attached to phenolic compounds, and this prevents them from being attacked by the enzyme (as in HP) or bound by bentonite (as in CB) and subsequently removed.

Indeed, Figure 4.8 shows that, at T1, the treatments aimed at removing proteins (CB and HP) only resulted in a limited decrease in total proteins. However, a great and generalised decrease in proteins from time T1 to time T2 emerged, with values recorded in the second case in the range between 24.4 mg/L and 34.5 mg/L, in line previous studies (Ferreira et al., 2002; Marangon et al., 2022). This decrease in proteins during storage could be attributed to two phenomena: on one side, proteins part of the red wine colloids (Marassi et al., 2021) could precipitate over time; on the other side, the evolution of the wine colloids over time, with a likely increase in size, could make these proteins less accessible to the analytical method used for their quantification. In either case, it seems clear that the treatments did not have a significant impact on the protein content, at least with the quantification method used.

From the analyses, it can be understood that at the protein level there are not many differences between the samples. The quantities are very similar to each other, contrary to what we expected from the initial hypotheses. The proteins did not decrease as it was hoped, neither with the treatment with Bentonite nor with the Proctase and this could be because the proteins are bound with the polyphenols. In fact, gel electrophoresis shows that the polyphenols are attached to large components and do not allow their migration, especially in samples produced with maceration (C and CB) (Figure 4.9 and 4.10).

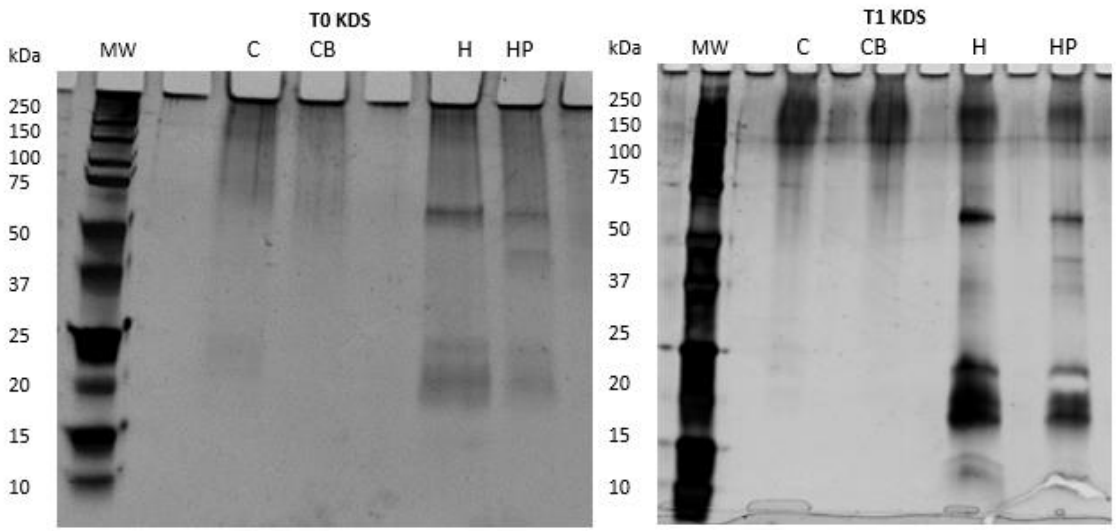


Fig. 4.9. Electrophoresis gel (T0) and (T1) with KDS method and Silver Staining.

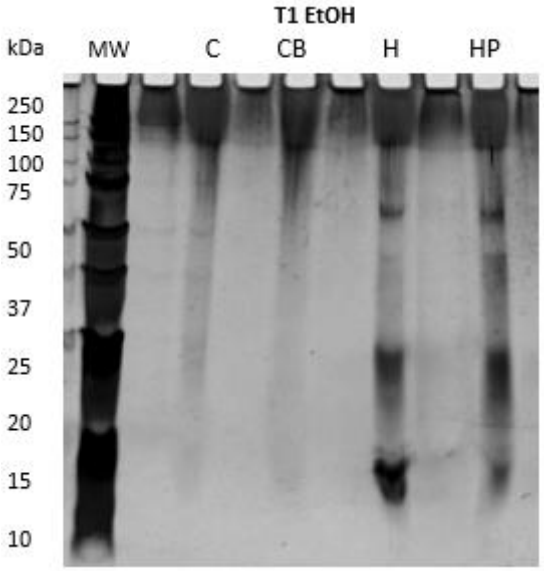


Fig. 4.10. Electrophoresis gel (T1) with Ethanol method and Silver Staining.

The gels were produced with two different sample preparation methods. In Figure 4.9, a combination of KDS protein precipitation and silver staining was used for samples at T0 and T1, while ethanol precipitation replaced KDS in Figure 4.10 (only T1).

Results differed from the protein quantification data shown in Figure 4.8. Indeed, when looking at Figure 4.9 some considerations can be made. At T0, the intensity of the bands of CB and HP is lower when compared to those at C and H, and this lower intensity is maintained at T1 (Figure 4.10), suggesting that the treatments with bentonite and Proctase indeed resulted in a decrease in protein

content. This would support the hypothesis made in the commentary of Figure 4.8, that are that the protein quantification method used was inadequate. The situation is somewhat similar, but less evident, when looking at Figure 4.10.

Another interesting observation is that samples vinified with conventional maceration on skins (C and CB) contained much less proteins than those thermovinified (H and HP), with the latter not only showing the high MW protein aggregates visible in and CB at around 250 kDa MW, but also free protein bands visible at MW consistent with those of wine proteins, so at around 60 kDa (likely invertase), and between 20 and 30 kDa (likely thaumatin-like proteins and chitinases) (Van Sluyter et al., 2015). It therefore seems that thermovinification results in a very different macromolecules profile than conventional maceration, and the implications of this on wine quality and stability have been investigated in the following sections.

4.4 Polysaccharides content

An important class of molecules participating in the formation of wine colloids are polysaccharides (Marassi et al., 2021). Their quantity can be quantified by HPLC-SEC, as well as their molecular weight distribution (Figure 4.11).

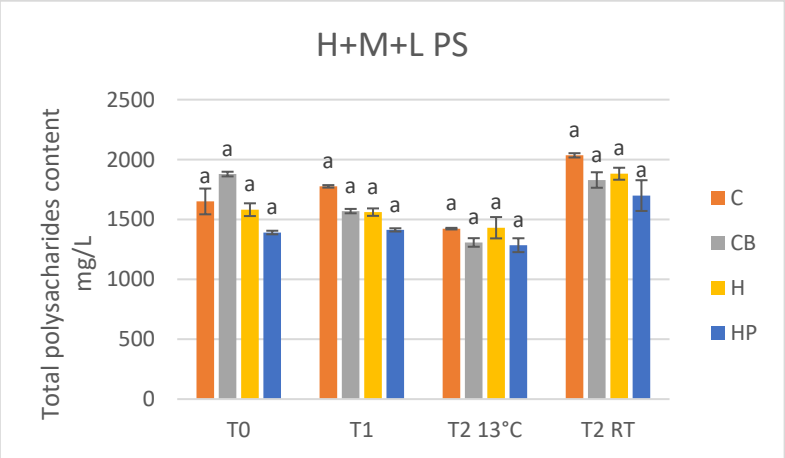


Figure 4.11. Concentration (expressed in mg/L of pectin and dextran) of the sum of low, medium, and high wine polysaccharides throughout the experiment. Within each timepoint, different letters represent statistically significant differences between treatments (post-hoc Tukey test, $\alpha = 0.05$).

Figure 4.11 shows values tendentially higher than those recently reported for Italian red wines (Marangon et al., 2022). However, the experimental nature of the wines could have resulted in higher extraction of these compounds, hence the higher values reported. Nevertheless, the treatments did not

seem to affect the concentration of these molecules, even if some trend was visible for HP which was the sample with the lowest values throughout.

The analysis allows to gather information on the concentration of polysaccharides of low (Figure 4.12), medium (Figure 4.13), and high (Figure 4.14) molecular weight polysaccharides.

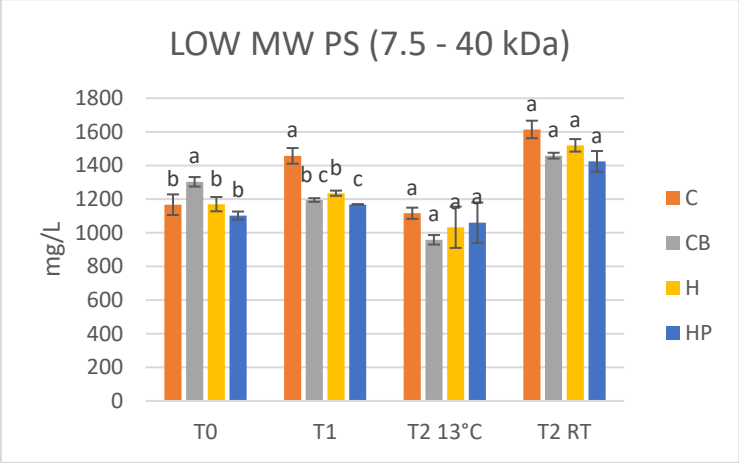


Figure 4.12. Concentration (expressed in mg/L of pectin and dextran) of the low molecular weight wine polysaccharides (7.5 - 40 kDa) throughout the experiment. Within each timepoint, different letters represent statistically significant differences between treatments (post-hoc Tukey test, $\alpha = 0.05$).

The graph of low molecular weight polysaccharides shows differences between the various samples. Usually, they are mannoproteins with low MW (7.5 - 40 kDa) coming from the autolysis of the yeast and then released spontaneously in wines aged on yeasts (Goncalves et al., 2002). First, it is possible to note that at time T0 the quantity of low MW PS is lower than at times T1 and T2. Above all, the quantity of low MW PS is lower in the samples stored at cellar temperature, compared to that stored at room temperature. Moreover, a difference can be noticed by comparing the control samples C and H with the respective treated samples CB and HP, at the time of T2 RT they show a higher quantity of low MW PS. This does not happen in T2 13 °C samples, where only the CB sample has a lower quantity than the others.

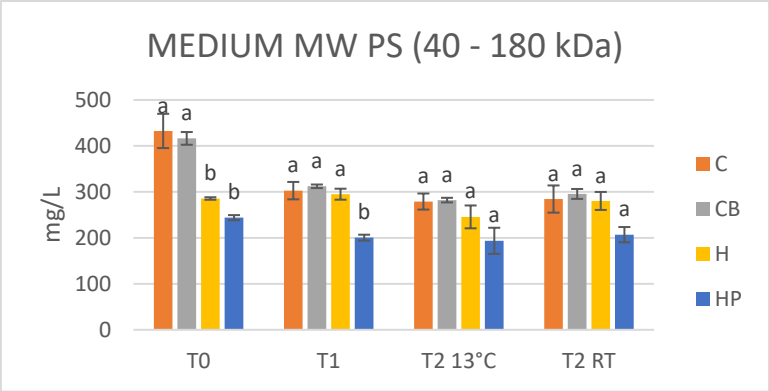


Figure 4.13. Concentration (expressed in mg/L of pectin and dextran) of the medium molecular weight wine polysaccharides 40 - 180 kDa) throughout the experiment. Within each timepoint, different letters represent statistically significant differences between treatments (post-hoc Tukey test, $\alpha = 0.05$).

As regards the polysaccharides with medium molecular weight, certain stability in terms of quantity can be noted. Samples C, CB, and H are very similar in time T1, T2 13 °C, and T2 RT. On the other hand, the HP sample differs, which has a much lower quantity than the others. The proteolytic activity combined with the heat may affect the solubility and/or integrity of this class of polysaccharides.
In addition, sample H already has the same quantity of Medium MW PS at the start (T0), while all the others undergo a reduction in number.

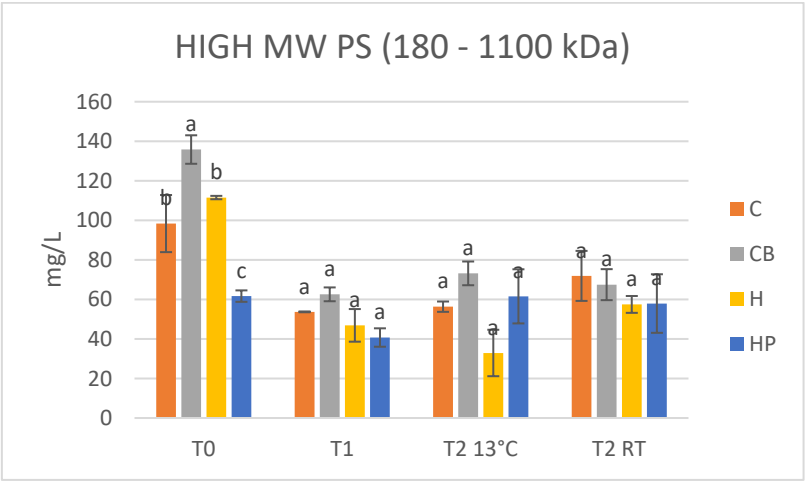


Figure 4.14. Concentration (expressed in mg/L of pectin and dextran) of the high molecular weight wine polysaccharides (180 - 1100 kDa) throughout the experiment. Within each timepoint, different letters represent statistically significant differences between treatments (post-hoc Tukey test, $\alpha = 0.05$).

The high molecular weight polysaccharides are numerically lower in all samples than low and medium MW PS. Usually, the high MW PS are mannoproteins with high MW (180 - 1100 kDa) with 30% of the protein part (Goncalves et al., 2002). The content of high MW polysaccharides at time T0 is generally high, especially in the CB sample. Samples C and H have a more modest content, while the sample HP has a reduced number of high MW PS, and it seems that the number stays more or less stable also in the other tests.

At time T1, C and CB have a lower number than T0, but not compared to H and HP.

At time T2 13°C, C has a lower content of high MW PS than C at T2 RT, and this does not happen for CB, where the quantity remains approximately the same. There is a big difference between H T2 13°C with H T2 RT, in the first case high PS is 32.9 mg/L and in the second case, they are 57.45 mg/L, almost double.

In general, thermally treated samples have a lower high MW PS content than C and CB. There is a clear decrease from the end of fermentation (T0) to T2.

4.5 Nanoparticle Tracking Analysis (NTA)

Nanoparticle Tracking Analysis was used to gather information on the dimensions of the colloids of the different wines (Figure 4.15).

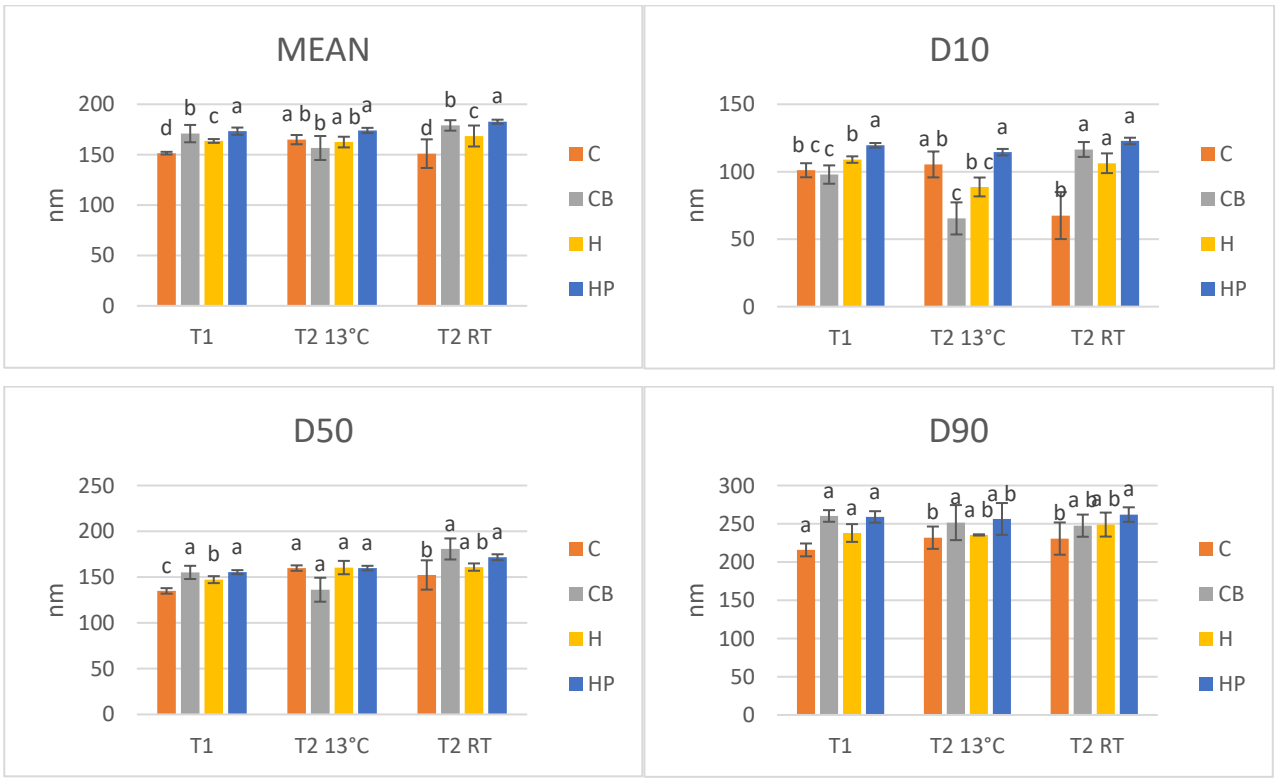


Figure 4.15. Dimension (expressed in nm) of the wine particles observed by Nanoparticle Tracking Analysis throughout the experiment. Mean average size; D10: 10% of particles below this size; D50; 50% of particles below this size; D90: 90% of particles below this size (Bindon et al., 2016). Within each timepoint, different letters represent statistically significant differences between treatments (post-hoc Tukey test, $\alpha = 0.05$).

By looking at the average size of colloids it appears that values are quite similar between treatments and during storage, with a tendency to increase in size with time. A small treatment effect is noticeable when looking at the mean diameter of colloids, with samples CB and HP having bigger colloids than C and H at T1, and at T2 but only for HP. This information, together with those from the electrophoresis results (see Figure 4.9), could indicate that in samples where proteins have been partially removed early during winemaking, the colloids formed are slightly bigger. This hypothesis would be supported by previous results by Marassi and colleagues, that indicate that the presence of proteins resulted in colloids more compact (Marassi et al., 2021).

As for the temperature effect, it seems that wines stored at room temperature contained colloids larger in size than those kept at 13 °C; with also here a small effect of deproteinization (CB and HP) that led to colloids larger in size. This situation could be due to a precipitation of some colloids, as discussed in the phenolic and proteins sections, resulting in the remaining in a solution of the larger colloids, hence a higher average.

Considering graph D10 at T1, it seems that wines thermovinified (H and HP) had larger colloids than those macerated (C and CB). Also, samples stored at cellar temperature are very different from those stored at room temperature. The D10 in sample C T2 13 °C is higher than C T2 RT, this indicates that in the former there are much larger particles than in the sample stored in room T, which has colloids of reduced size. The opposite occurs instead for CB T2 RT presents colloids with a larger size than CB T2 13°C. As for the H T2 RT and HP T2 RT, the HP sample has a larger colloid size than H.

In graph D50 it is observed that also in this case the CB T2 13 °C samples have a smaller colloid size than CB T2 RT. The other samples have approximately the same values and the same commentary applied to the mean values are valid here too.

When looking at the D90 at T2, H RT and HP RT have a larger colloid size, while at cellar temperature, the size is smaller. However, we see that in both treated samples, i.e., CB T2 and HP T2, the size of the colloids is greater when compared with the respective untreated control, i.e., C T2 and H T2.

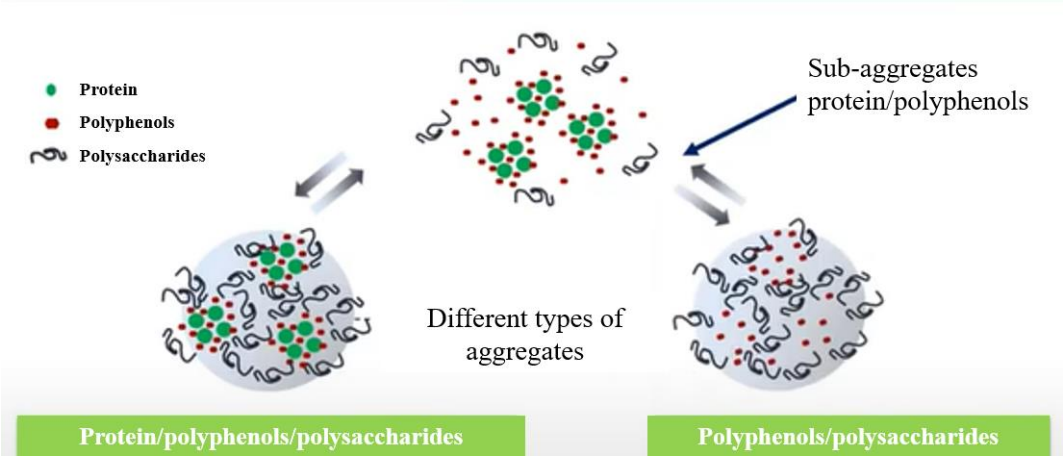


Figure 4.16. Hypothetical mechanism for colloidal aggregation in red wine (Marassi et al., 2021)

In general, there seem to be a couple of potential explanations to understand the changes in dimensions shown in Figure 4.15. On one side, the increased average dimensions of colloids over time could be due to growth phenomena, with colloid-forming molecules aggregating with each other to form larger colloids. Another possible explanation, as shows figure 4.16 could be that the colloids containing more proteins (C and H), that are those more compact and smaller as demonstrated in a previous study (Marassi et al., 2021), could have precipitated over time, thus leaving fewer but larger colloids in the wine with the result of an increased average dimension.

4.6 Wine stability

To gain information on the stability of wine colloids, and on the effect of the treatment on wine stability, classic heat and cold stability tests were applied to the wines during the storage period.
Initially, the turbidity of the wines resulting from the storage conditions was only measured by a nephelometer (Figure 4.17).

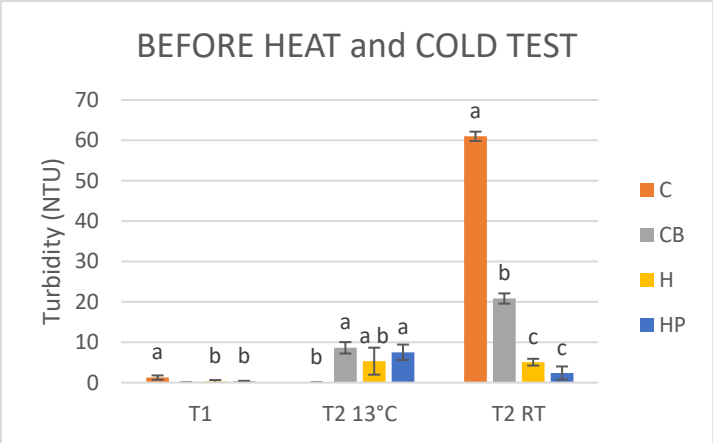


Figure 4.17. Wine turbidity (expressed as NTU) without application of heat and cold tests. Within each timepoint, different letters represent statistically significant differences between treatments (post-hoc Tukey test, $\alpha = 0.05$).

Results indicate that at T1 all wines were limpid, with NTUs values ranging from 0 and 1.2. The storage temperature showed a great effect on the wine turbidity developed naturally, with samples maintained at cellar temperature (13 °C) showing low turbidity values (range 0 - 8.6 NTUs), while those stored at room temperature showed a marked instability, especially for the wines vinified without the use of heat (C and CB). Interestingly, H and HP, despite not being fully limpid, remain quite clear and showed values similar to those of wines kept at 13°C. From these data, it seems that heat contributed to producing wines that, at least in the short term, could be more stable without the need for fining, while wines made conventionally were quite unstable, even if bentonite fining significantly decreased this instability as visible in CB at T2 (RT).

Therefore, those observations can lead to the hypothesis of different behaviours of the colloids depending on the temperature in which the wine is stored.

When wines were also submitted to the heat stability test (Figure 4.19), and other differences emerged.

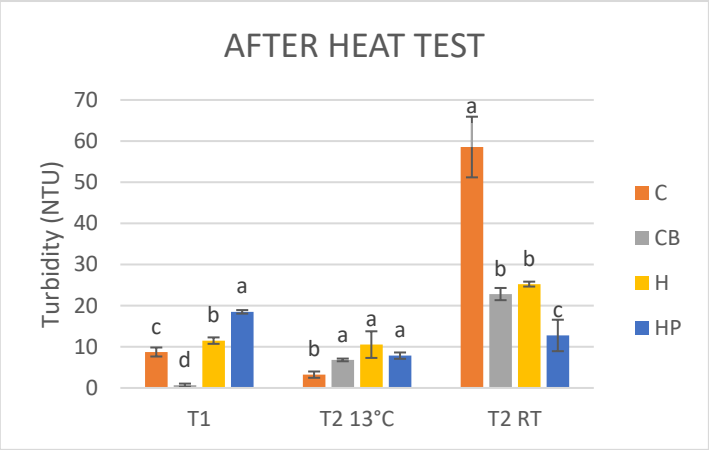


Figure 4.18. Wine turbidity (expressed as NTU) after heat test. Within each timepoint, different letters represent statistically significant differences between treatments (post-hoc Tukey test, α = 0.05).

From these data, some differences were already present at T1. In particular, the sample treated with bentonite (CB) was the most stable of all, while the heat-treated samples (H and HP) were slightly more unstable than the control C. Confirming what was said about data of Figure 4.17, also in this case the T2 13 °C samples resulted much more stable than the RT samples. At time T2 13 °C it seems that CB and HP have an almost equal value, H slightly lower. At time T2 RT, the HP sample has a much lower value than CB. There is a big difference between C T2 13°C and C T2 RT, where the first is stable, instead the second is unstable. This is probably due to the high temperature of storage. The same wines were also submitted for cold stability test (Figure 4.19).

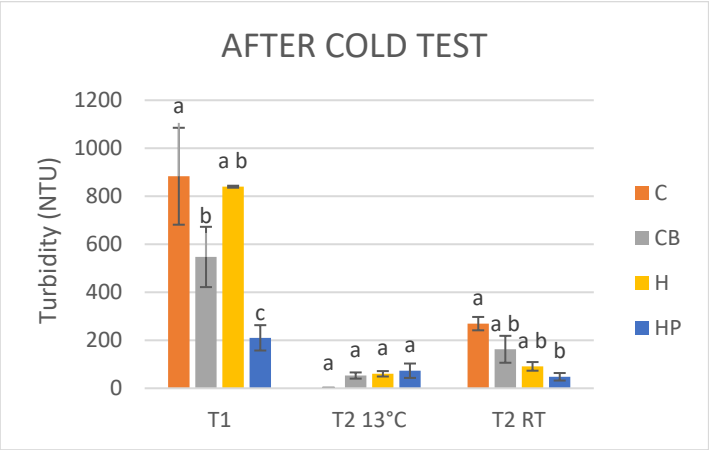


Figure 4.19. Wine turbidity (expressed as NTU) after the cold test. Within each timepoint, different letters represent statistically significant differences between treatments (post-hoc Tukey test, α = 0.05).

The "after cold test" graph showed high instabilities for wines at time T1. We note that samples C and H are much more unstable than CB and HP. Moreover, it seems that HP is the sample with the lowest value compared to all the others at T1. As regards to the comparison between T2 13 °C and T2 RT, also in this case at cellar temperature the samples are more stable. The thermally treated samples (H and HP) show lower values than C and CB, stored at room temperature. This event appears in all three tests, so it could be interesting during the wine marketing period. Overall, the higher stability of wines at T2 when compared to T1 might be attributable to the natural cold stabilization of wines that happens over time, so more material was available to precipitate at T1 than at T2.

The wine bottles are sold on the shelves at a temperature of about 20-25 °C. Our heat-treated samples are more stable at these temperatures than samples that have not undergone the initial heat treatment.

5 Conclusions and future perspectives

The impact of the macromolecular composition on the colloidal state of red wines was monitored during aging at different temperatures and submitted to different treatments able to modify the initial wine macromolecular content. This resulted in the production of 4 experimental wines that were analysed in terms of wine quality and stability, thus providing information on the effect of the vinification method (maceration vs. thermovinification), on the effect of deproteinization (with bentonite and Proctase) and of the storage temperature (13 °C vs room temperature). The experiment allowed to collect several important information that were used to improve the understanding of the wine colloidal state and on some of the factors that can affect it.

The data collected on the wines chemical composition showed that none of the treatments (bentonite fining, heating, and heating + Proctase) caused major modification in terms of wine composition. Indeed, fermentation kinetics were unaffected, and all wines produced were dry, and had similar organic acids profiles regardless of the treatment undergone, with values in line with those reported in the literature. As for acetic acid, it seems that the heating of the juice has caused a reduced content of this parameter, probably due to the elimination of the microbial competition by the heating. Furthermore, it appears that the ethanol values are higher in the heat-treated samples, probably due to the heating causing higher extraction of sugar, or to the absorption of ethanol by the grape skins in the macerated wines.

Maceration vs. thermovinification also greatly affected the phenolics compounds of the resulting wines. Indeed, the Color Index and polyphenols show considerable differences between the various samples. In both analyses, it appears that macerated wines (C and CB) had more phenolics and colour than thermovinified wines (H and HP), a result that was expected. Additionally, protein removal by bentonite (CB) resulted in less phenolics in the wine, a fact probably caused by the treatment with bentonite which is known to remove some wine pigments (e.g., anthocyanins and pigmented polymers) that contribute to wine colour. Furthermore, the polysaccharide content appears slightly higher for the samples that have undergone maceration in contact with the skins. This could be caused by a natural extraction of polysaccharides by the grape skins that, at higher temperature, could have released more of these materials from the cell walls. From the gel electrophoresis it appears that the maceration in contact with skins and the thermovinification have produced a clearly different protein profile between the samples. This analysis reveals protein bands typical of wines only in thermovinified samples, while wines that have undergone maceration in contact with the skins have only one aggregate protein band at the top. This is an observation that warrants further investigation, but it is likely that the higher presence of phenolics in macerated samples resulted in

the formation of tight protein-phenolics aggregates that could not be separated by the reducing conditions of the analysis (e.g., use of heat, reducing agent, SDS) to migrate in the gel. Conversely, thermovinified wines contained less total phenolics, so that the above-mentioned aggregates could not be formed, or could only partially be formed, thus producing wines with free proteins that could migrate in the gel. Considering the analysis using the Nanosight graph D10 at T1, it seems that thermovinified wines (H and HP) have slightly larger colloids than macerated ones (C and CB), a fact that must be due to the differences in macromolecular content and profiles that the treatments adopted caused in the wines.

The data collected from the stability tests indicate that the heat-treated samples are generally more stable than those produced with conventional maceration. Indeed, it appears that thermovinified samples stored at room temperature have a high stability like the samples stored in the cellar, while non-thermovinified samples stored at room temperature appear to be rather unstable.

Regarding the effects on deproteinization, the proteins did not decrease as it was hoped. This occurrence is probably to the fact that proteins are already bound with the polyphenols from the early vinification stages, making the deproteinization treatments (bentonite and Proctase) less or not effective in interacting with their target (wine proteins). Despite this, it is possible to notice several effects caused by the attempted protein reduction in wine. The deproteinizing effect combined with thermovinification may have caused a reduction in the extraction of polysaccharides, since in the HP samples, their content is always lower. Some considerations can also be made regarding the size of the particles. In general, there is an increase in the colloidal size due to the interactions of the particles over time, but the samples treated with bentonite or with the enzyme are smaller than the others. This is in line with a previous hypothesis (Marassi et al., 2021) that stated that the presence of proteins creates more compact aggregates with polysaccharides and polyphenols, thus affecting the size of the formed colloids and, consequently, their stability.

The different storage temperatures have highlighted different phenomena. As regards the Color index, it is noted that the value recorded at 13 °C is clearly higher than that at room temperature. This could be caused by the high temperatures, which have destabilized some colloids that consequently precipitated, also taking with them pigments responsible for wine color. The same goes for polyphenols. In general, samples stored at room temperature have a higher content in Low MW PS than those stored at 13 °C. The cold and heat stability tests report interesting results, with an evident instability in samples stored at room temperature compared to those stored in the cellar. A cellar storage temperature allows to obtain wines much more stable than storage at room temperature. This phenomenon could be taken into consideration when marketing wine.

In general, the here-presented experiments allowed to gather additional information on the interactions of macromolecules in red wines, and on their impact on several wine quality parameters. However, more studies are needed in order to test the hypothesis made during this study, also using experimental conditions that could effectively reduce the protein content of the wines, using other grape varieties, and other fining agents.

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