UNIVERSITY OF UDINE
FACULTY OF AGRICULTURE

Doctoral Course in Food Science
Department of Food Science
Course XXIV

DOCTORAL DISSERTATION

Study and management of endogenous and exogenous colloidal systems of wines, finalized to the quality improvement.

Ph.D. Course Coordinator: *Prof. Alessandro Sensidoni*

Ph.D. Supervisor: *Prof. Emilio Celotti*

Ph.D. Student: *Valentina Cacciola*

ACADEMIC YEAR
2011-2012

A search always starts with Beginner's Luck
and ends with the Test of the Conqueror.

Paulo Coelho

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ABSTRACT

The endogenous and exogenous colloidal system remarkably conditions the stability characteristics and the sensory profile of a beverage.
Colloids are substances with a highly complex and heterogeneous chemical nature. They play a major role in the winemaking processes and in the characteristics and quality of wine. Furthermore, they are very important in the study and characterization of oenological adjuvants. Their presence in solution makes it hard to predict what kind of interactions they can have and how these interactions can affect the colloidal stability.
Since these molecules are naturally present in wine but can also be added as adjuvants, it becomes interesting to deepen the knowledge on their interactions in order to have more information to optimize the winemaking process and maintain a good quality of wine.
To this end, the research done for this thesis first focused on the improvement of the characterization of oenological adjuvants (tannins) using conventional and unconventional techniques (Dynamic Light Scattering (DLS) Streaming Current Detector (SCD) and Phenolic Oxidizable Materials- test (POM-test)), in order to obtain useful information that can help technicians in the wine industry choose the most suitable adjuvant to their needs, and that are necessary to avoid problems related to colloidal instability and precipitation.
The tannins were also tested in a technological trial in winery to assess whether the data obtained from laboratory tests could be considered valid. Moreover, with the same techniques, interactions between tannins and polysaccharides were investigated in order to understand the boundary between colloidal stability and intability related to their presence or use as exogenous adjuvants. The last part of this work considered the aroma component, which has a key role in the good quality of wines, evaluating the effects of macromolecules of the colloidal system on aroma volatility.
The results obtained confirmed that the unconventional techniques used are a valid tool for a better assessment of the adjuvant characteristics, but also for the study of the interactions that occur between colloids. The evaluation of the surface electrical charge and of the colloidal diameter of the particles combined with measures of turbidity helped in better understanding what the critical points linked to the use of these products as adjuvants are or what can be the issue when they interact with the ones naturally present in wines.
Colloids have proved to interact also with volatile compounds with positive or negative effects on their volatility depending on the type of colloid tested. In particular the presence of tannins generally reduced the volatility of these compounds, while the BSA often showed a salting out effect. The haze formed by

the simultaneous presence of tannin and BSA most of the times reduced the volatility of flavours. These tests confirmed that colloidal instability can negatively affect wine quality and for this reason it must be avoided by monitoring and controlling the colloidal system.

PREFACE

The management of the colloidal system of a wine is one of the most challenging issues in the wine industry, as the presence of colloidal substances makes it difficult to predict the interactions between the molecules and the result of these interactions, as opposed to what happens in "true" solutions.
The endogenous and exogenous substances in the colloidal system, interacting with each other, cause changes in the medium that can lead to instability.
The study of the colloidal system of wine and the molecules that constitute it is, therefore, essential to optimize the processes of vinification and the stabilizing and aging treatments, in order to ensure the colloidal stability, which, inevitably, affects the sensory quality of the product.

Part 1

Characterization of oenological tannins and technological trials in cellar

Part 2

Study on the interactions between tannins and polysaccharides.

1. Introduction

1.1 The colloidal system

Hydro-alcoholic solutions represent a particular environment for colloidal substances, in fact they often show a very complex colloidal system.
Colloids are substances with a highly complex and heterogeneous chemical nature. They play a major role in the winemaking processes and in the characteristics and quality of wine, furthermore, they are very important in the study and characterization of oenological adjuvants, including tannins.
But, in this case, it is correct to talk not so much of colloids, but more about colloidal behavior of some chemical species under certain conditions.
Colloids are particles that are part of disperse systems (Table 1), because their dimensions are intermediate between the larger molecules of "true" solutions and smaller particles in suspension. With respect to substances in solution, the colloids have larger dimensions, ranging from 2 to 100 nm, but may reach a size of 1000 nm and consist of approximately 10^3-10^9 atoms per particle (Zsigmondy, 1905; Ostwald, 1917 and Staudinger, 1947). Their small size explains the low rate of spontaneous sedimentation, which is directly proportional to the square of the radius of the particles (Stokes Law). The reduced forms make them difficult to be determined through the use of optical microscopes, so it is possible to detect them only through light diffusion, sedimentation, or osmosis.

	Particle size (nm; 10^{-6} mm)	Approximate number of atoms per particle	Particle properties
Ordinary solutions (or molecular dispersions)	<2	10^3	Pass through filters and ultrafilters, are not visible under a microscope or ultramicroscope, are dispersed in the solution and dialyze, do not settle
Colloidal solutions (or dispersions)	2–1000	10^3–10^9	Pass through filters but not ultrafilters, visible under an ultramicroscope but not a microscope, disperse in the solution with some difficulty and dialyze very slowly, settle very slowly
Standard suspensions	>1000	$>10^9$	Do not pass through filters, visible under a microscope, disperse in the solution with great difficulty, do not dialyze, settle very rapidly

Table 1 - Classification of dispersed systems.

These particles may occur in different states (Ribéreau-Gayon *et al.,* 1976):
SOL: fluid, elastic and slightly viscous, colloidal solution where the particles are free in relation to each other;
GEL: the particles are not mobile and are grouped in clusters which are hindered by Brownian movement;
JELLY: single mass obtained from amorphous substances that swell in a suitable liquid.
The transitions between the different states are shown in Figure 1:

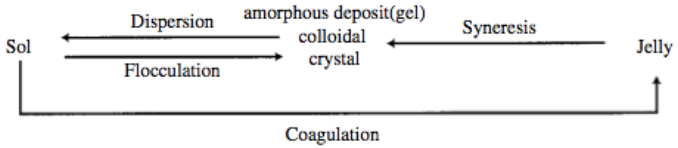


Figure 1 - Diagram of colloidal transformations.

A colloidal solution consists of small solid particles, maintained dispersed in a liquid by a set of forces that prevent their aggregation and flocculation. It includes two phases (liquid and solid), with a mutual boundary that constitutes the interface. Exchanges between the two phases take place at the interface. The total interface is one of the factors governing the physical and chemical properties of colloidal solutions.
Colloids can be macromolecules or aggragates of different molecules (micelles), this observation is based on a classification that divides them in macromolecular colloids and micellar colloids.

- **Macromolecular colloids** are composed of molecules linked by covalent bonds, whose electric charge is due to the dissociation of acidic or basic functional groups. These colloids, easily solvated, are very stable due both to surface repulsion and hydrophilicity, which provides a hydrated layer of the macromolecule. They consist of large molecules: in wine they are proteins, polysaccharides, and some phenolic fractions.
  "Protective colloids" of oenological interest are macromolecules characterized by the simultaneous presence of hydrophilic and hydrophobic groups able to stabilize the micellar colloids such as those made of phenolic compounds of wines or tannin-protein complexes.
- **Micellar colloids** are aggregates of molecules of the same or different chemical species linked not by covalent chemical bonds but by low-energy physical bonds, such as Van der Waals forces or hydrogen bonds or opposite charges or by all of these surface forces. In wine, most of the molecules that compose this kind of colloids are hydrophobic and are mostly represented by condensed phenolic compounds, colloidal

colouring substances, residues of ferric phosphate, ferric ferrocyanide and copper sulfide (Ribéreau-Gayon *et al.,* 2007).
The formation of these aggregates of molecules is ensured by the presence of hydrophobic heads outside and hydrophilic tails inside that guarantee protection (Figure 2).
The presence of electrical charges that cause continuous repulsions, guarantees the stability of the micellar colloids, their particles are also able to adsorb other substances ensuring the heterogeneity of the molecule. Induced the charge neutralization, the complex flocculates.

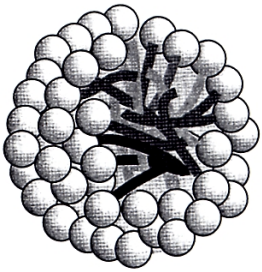


Figure 2 - Schematic representation of a spherical micelle. The hydrophilic groups are depicted as spheres, the hydrophobic hydrocarbon chains as stalks, these stalks are movable. (Atkins 1992).

The peculiarity of colloidal substances lies in their physical and technological properties (Ribéreau-Gayon *et al.,* 1976):

- they have a slower Brownian motion;
- dialyse through the membrane with difficulty;
- they are visible with the ultramicroscope;
- give amorphous precipitates;
- the boiling and freezing temperatures of the medium are not changed by their concentrations, at the freezing and boiling point, however, there are two distinct phases, one liquid and the other dispersed;
- they do not have defined compositions;
- for their precipitation they do not need either a more concentrated solution, nor a specific agent or to follow stoichiometric rules;
- scatter visible light (Tyndall effect)
- reactions that determine the turbidity do not coincide with the limit of solubility product.

Wine is a comlex system in which “true solution” and colloidal state coexist. The colloidal state of wine has solid particles larger than those in solution, different in composition and origin, maintained in a liquid dispersion by a set of forces that prevent aggregation and flocculation, promoting, in that way, the colloidal stability (Ribéreau-Gayon *et al.,* 2007).

1.1.1. Stability and flocculation of colloids

When a substance is in a colloidal dispersion, it is characterized by interactions with the medium that are not bonds or chemical interactions, but by the behavior of the surface governed by Brownian motion, Van der Waals forces and the electrical charge. Brownian motion is the chaotic and random motion of all the particles in suspension in a fluid, it is independent from convection or internal disturbances of the fluid, and so describable with the kinetic theory. According to it, the fluids in which we observe the phenomenon are made up of countless molecules, the particles in suspension, colliding constantly and in every direction. This kind of motion is closely related to thermal energy of the particles, and it provides stability to the colloidal state; morover, it is very limited with solutes (low diffusion).

Van der Waals forces are attractive, directly proportional to the diameter of the particles and inversely proportional to the distance between the particles. If the distance is less than the particle radius, Van der Waals forces of attraction are greater than the thermal motion and if the other forces of repulsion (surface electrical charge) are weak or neutralized, they take over, causing the swelling of the colloidal aggregate and its precipitation.

Colloids, in relation to the characteristics of the medium, in particular the pH, may have an electrical charge on their surface which can be positive or negative, the greater it is, the more the colloid will be stable, as charges of the same sign, exert a repulsion force opposing the cohesion of the particles. The surface electrical charge is determined in particular by the presence of nucleophilic and electrophilic groups. All wine colloids, except proteins, have a negative surface electrical charge.

By varying the pH of the colloids they can assume a more or less high electrical charge which vanishes at a certain pH value (isoelectric point), above which they assume a negative charge, while the sign is reversed at lower pH. If the charge is neutralized, the stabilized system becomes unstable, allowing firstly the aggregation up to a critical weight and thus the flocculation. For hydrophobic colloids it is sufficient the elimination of the charge, for the hydrophilic ones, dehydration is also essential for the removal of the solvation surrounding the particle.

The distribution of electrical charge on the surface of a colloidal particle is shown schematically in Figure 3.

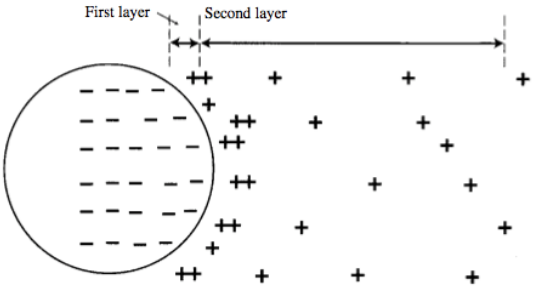


Figure 3 - Distribution of charges in a 'double layer' around a charged colloidal particle (Saucier, 1993).

On the surface of the particle there is a well-fitting first layer, dense of charge and therefore with a high potential (Stern layer) of opposite sign with respect to the charge inside the particle, after this layer there is a second layer (Gouy-Chapman) in which the counter ions are present diffusely. Only this second layer may be partially disrupted by factors such as thermal agitation (Brownian motion) or the motion of the particles or the medium in which the colloid is. Based on this principle there is an analytical technique used for the evaluation of the surface electrical charge. The limit potential on the border between the two layers is the potential ζ (zeta); it can clearly affect the colloidal behavior.
The most important colloidal phenomenon is the separation from the medium under certain conditions; these mechanisms depend on the surface behavior of colloids and thus on their surface electrical charges, but also on Van der Waals forces (high for hydrophobic colloids), the firsts with the effect of repulsion, the second ones of attraction between the particles. Also factors such as temperature, pH, salinity of the medium and the contribution of other charges, can influence the colloidal behavior, in any case it is necessary that forces of attraction prevail over those of repulsion. The theory that describes the kinetics of colloids precipitation (Ribéreau-Gayon *et al.,* 1976) provides, whatever is their type, hydrophilic micellar or hydrophobic macromolecular, the neutralizationtion of surface charge and the dehydration of the same, when hydrated (Figure 4). The dehydration of hydrophilic colloids may occur for heating or in the presence of substances acting as anti-solvation agents, i.e.. tannins, alcohol, but especially the action of electrolytes or salts is essential to foster the bond of the particles and the subsequent flocculation.

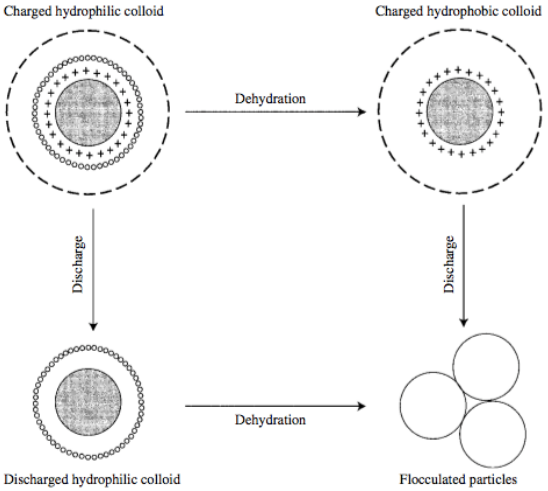


Figure 4 - Diagram of the flocculation of a hydrophilic colloid by elimination of the two stability factors: electrical charge and hydration (Ribéreau-Gayon *et al*., 1976).

Proteins, positively charged, would intervene on wine polyphenols forming tannin-protein complexes, tannins would adsorbe protein solvation, denaturating them by dehydration (Ribéreau-Gayon *et al.,* 1976) or simply would denaturate them due to adsorption (Kawamoto and Nakatsubo, 1997). The hydrophobic and electronegative colloid formed does not precipitate until the surface charge is neutralized due to intervention of the salts present in solution.
According to a recent theory on the behavior of colloidal tannins, hydration of hydrophilic colloids is not considered. When tannin molecules combine to form colloidal particles, the Van der Waals forces between tannins and proteins increase considerably, producing a non-specific adsorption phenomenon (Saucier, 1997). The mechanisms involved are as follows:

1. Tannins form colloidal particles by hydrophobic interactions.
2. The tannin particles are likely to be destabilized by proteins due to the Van der Waals attraction, forming aggregates that precipitate (fining mechanism in wine).
3. Cations, especially iron, promote agglomeration of tannins to form colloidal particles.
4. The formation of aggregates of tannin particles, or tannins and proteins, may be inhibited by the presence of polysaccharides (macromolecular colloids). This observation has been confirmed by several authors (Riou *et al*., 2002; de Freitas *et al*., 2003) (Figure 5).

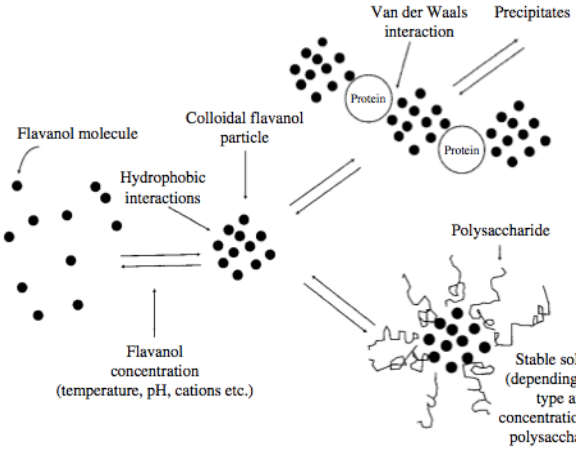


Figure 5 - Model of the colloidal properties of flavanols (tannins) (Saucier, 1997).

1.2 Tannins

By definition, tannins are substances that can originate stable combinations with proteins and other plant polymers such as polysaccharides (Ribéreau-Gayon *et al.,* 2007); they are a heterogeneous group of organic substances of phenolic nature, having a molecular weight between 500 and 4000 Daltons, widely present in the plant kingdom where they accumulate in large amounts in roots, leaves, barks, seeds, fruits, providing defense to plant tissues.
Tannins can be chemically classified into two distinct groups: hydrolysable and condensed (also known as catechin and proanthocyanidins). Since they are widely employed in musts or wines it becomes very important for the technician who uses them to have information on their chemical nature.

- Hydrolysable tannins (Figure 6): have a basic structure consisting of a carbohydrate fraction (usually glucose), whose hydroxyl groups are esterified by gallic, di-gallic or tri-gallic acid (Paronetto and Paronetto, 1986; Salagoity-Auguste *et al.* 1986). They are easily hydrolyzed by enzymes or in acidic or basic environment, releasing gallic acid (gallic tannins) or ellagic acid (ellagitannins).

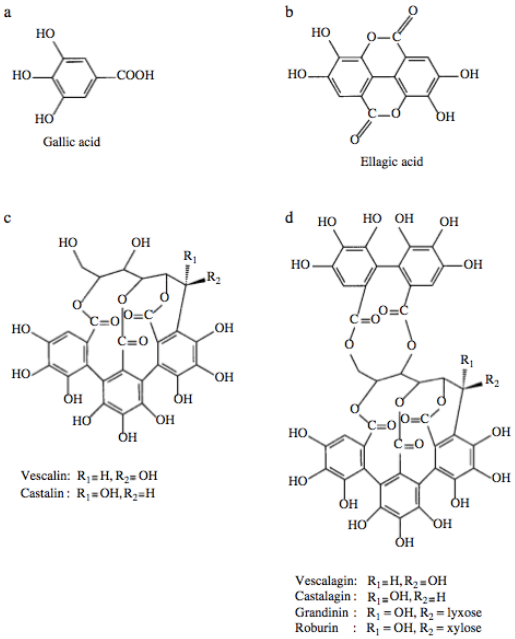


Figure 6 - Structure of phenolic acids (a and b) and ellagitannins (c and d) in extracts from the duramen of oak and chestnut wood (Vivas and Glories, 1996).

The values of molecular mass for ellagitannins are between 900 and 2.000 Daltons, moreover, they present darker colours and stronger smells of wood than gallic ones, especially when roasted or deriving from white oak (*Quercus alba*). They are usually less astringent than the gallic ones and are never found in wine, except for contributions arising from exogenous additions or deriving from wooden barrels. In oak wood there are eight different ellagitannins (Masson *et al.,* 1995): these are the monomers castalagin, vescalagin, grandinin, roburin E and roburin dimers A, B, C and D.

Gallic tannins, instead, are creamy white or yellow, they are not very fragrant, often bitter and astringent, particularly when the commercial formulated has a high acid content in gallic acid (Citron, 2007).

In general, there are no hydrolysable tannins in the polymer state, unlike the condensed tannins, and at most they can be composed of two or three basic units.

- Condensed tannins: This type of tannins origins anthocyanidins with an acid and hot treatment, hence the name of proanthocyanidins (Weinges and Nader, 1982), they are more or less complex polymers of flavan-3-ols or catechins. For example, in grape seeds, the condensed tannins liberate, during acid hydrolysis, cyanidin: they are then called procyanidins. In grape skin instead there is a mixture of procyanidins and prodelfinidins. When it is not precisely known the nature of the anthocyanidin formed, the name proanthocyanidin is used. (Vivas, 1997).

  The structural units, catechins, can not be considered as tannins, as their molecular mass is too small and their reactivity towards proteins is very low. Only starting from dimers their molecular mass is sufficient to allow the formation of stable bonds with proteins.

  There are many different bonds involved: the simpler forms consituted of dimers of types A and B (de Freitas, 1995) (Figure 7), and trimers C and D. Forms B and C are typical of grape. The more polymerized forms, represented by oligomeric procyanidins and polymers, are less known. Oligomeric procyanidins correspond to polymers formed by units of flavan-3-ols (from three to ten), linked by C4-C8 or C4-C6 bonds, while the polymeric ones are made up of more than ten flavan residues.

  The colouring of commercial products based on condensed tannins varies between brown and dark red depending on their botanical origin. The condensed tannins extracted from grape seeds are usually oligomers, unlike the grape skin ones, which have a higher degree of polymerization and are sometimes associated with other substances, such as polysaccharides. For the extraction of tannins, skins of white grapes are preferred in order not to enrich the final extract of anthocyanins, difficult to separate from tannins. This sort of problem does not arise for seeds, but, their degree of maturity may affect the degree of polymerization of

the extracted structures. The fact that skins are fermented or fresh has an influence on the degree of polymerization of the tannins present in the extract, in particular the latter ones present more polymerized tannins (Citron, 2007). Condensed tannins are mainly used in red wines already in the process of maceration and racking, because of their copolymerization with anthocyanins properties, producing soluble macromolecules, called combined anthocyanins, very stable over time. Wines added with this type of tannins maintain a higher colour intensity (Ribéreau-Gayon *et al.,* 2007).

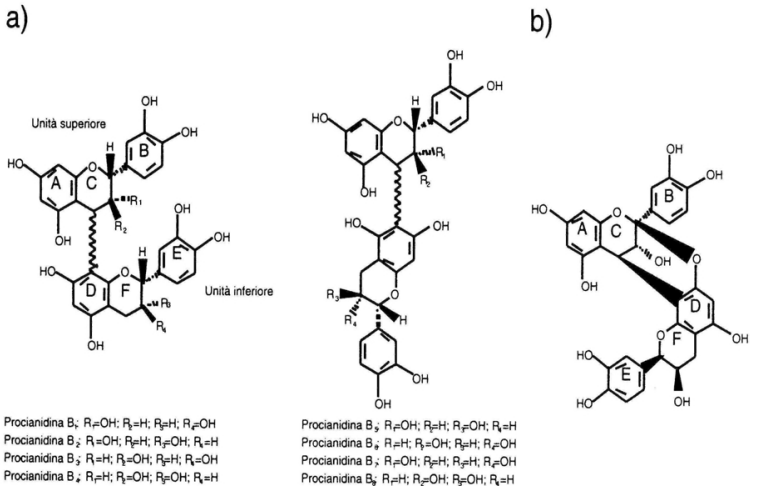


Figure 7 - Structure and of type-B dimeric procyanidins (de Freitas, 1995) and structure of the dimeric procyanidin A2 (Vivas and Glories, 1996).

According to the Codex Œnologique International de l'O.I.V., "... they are extracted from gall nuts, from wood particularly rich in tannin: chestnut, oak or grape seed ... Tannin is a mixture of gallic and ellagic acid glycosides, or catechol ... " Among the hydrolysable tannins, the gallic tannins are usually derived from galla nuts due to hypertrophy of plant tissues generated by an insect bite, from tara (*Caesalpinia spinosa*) or dried fruits of myrobalan (*Therminalia chebula*). Chestnut (*Castanea sativa*) and oak (*Quercus spp.*) woods are the main sources of ellagitannins (Salagoity-Auguste *et al.,* 1986). Condensed tannins are generally obtained by extraction from grape seeds, from marc, from bark of mimosa (*Acacia mearusii*) or exotic woods such as quebracho (*Quebracho spp.*) (Figure 8).

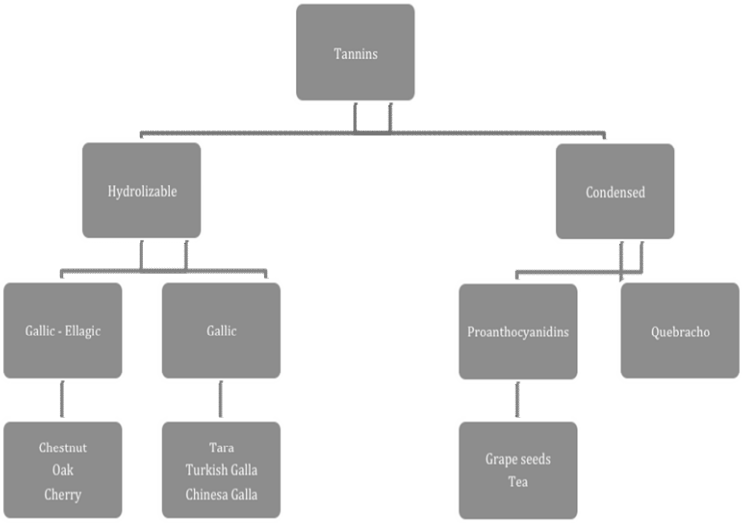


Figure 8 – Classification of the main types of oenological tannins.

Many research works show the composition of commercial tannin extracts deriving from different plant sources (Salagoity-Auguste *et al.*, 1986; Vivas, 1997) (Table 2).

Origin	**Extraction**				
Species	**Vegetal part**	**Method**	**Solvent**	**Preparation**	**Nature**
	galla	S/L	EtOH/EtOH	liof.	G
Cherry plum	fruits	S/L	EtOH/H_2O	liof.	G/E
Tara	fruits	S/L	EtOH/H_2O	liof.	G
	galla	S/L	EtOH/EtOH	liof.	G
Oak	wood	S/L	H_2O	liof.	E
Oak	wood	S/L	H_2O	liof.	E
Chestnut	wood	S/L	H_2O	liof.	E
Acacia	galla	S/L	EtOEt	evap.	G
Grapevine	seeds	S/L	H_2O/SO_2	evap.	P

Table 2 – Origin and method of extraction of oenological tannins (Vivas, 1997)
S/L = solid/liquid; G= gallotannins; E= ellagitannins; P= procyanidins; liof.= liofilization; evap.= evaporation; EtOH= ethanol; EtOEt= diethyl ether.

Several factors affect the final content of the commercial preparation: botanical origin but also the part of the plant from which the extraction is carried out, the extraction solvent, velocity and temperature of extraction (time-consuming extraction lowers the final content of tannin in the formulation); furthermore, the method of drying the extract: freeze-drying or evaporation.
Obtaining the commercial powders is based on a solid-liquid extraction, made on chips of wood material, with solvents such as water or steam, diethyl ether or ethanol, alone or in mixture (Vivas, 1997, Chauvet *et al.,* 1992). After the extraction the solvent is removed by evaporation or lyophilization (Vivas, 1997).
The solvent mostly used in the production of tannin extracts is ethanol mixed with water, the different percentage ethanol/water affects the degree of polymerization of tannins extracted. If the extract is derived from toasted wood fragments (it is the case of derivatives of oak), also other molecules developed by thermolysis of polysaccharides and lignin with a strong sensory input appear. The type of solvent used during extraction, the temperature at which it is made, and the duration of the process affect the solubility but also the polyphenolic amount of the extracts.
Becomes important, then, the quantity of active substance, that is the sheer quantity of tannin contained in the formulation. The Codex Oenologique International de l'O.I.V. regulates that presence, by providing a minimum content of tannin, equal to 65%.
The final composition of the preparation also influences the taste: bitterness may be due to coumarins and di-gallic acid, sweetest notes to polysaccharides, of which the positive contribution in terms of softness and structure in wines is also known. The extracts derived from chestnut wood are generally characterized by greater bitterness. Another qualitative aspect of the extract is the degree of polymerization of tannins because it affects their reactivity, as their degree of oxidation: already partially oxidized tannins absorb smaller amounts of oxygen and show a lower propensity to react with proteins due to the decreased electronegativity of the molecule (Citron, 2007).
Since there are commercially available preparations both of hydrolysable tannins or procyanidins but also, in certain formulations, several classes of tannins differently mixed depending on the formulation itself, it becomes important for the winemaker to be aware of the actual formulation of the product that intends to buy. In this regard, there are different methods for the immediate characterization of commercial oenological tannins. Among them, the most used is certainly the tracking of the spectrum of absorption by spectrophotometry UV / visible.
The characteristics of oenological tannins, therefore, depend on:
• the chemical nature (gallic tannins, ellagic and proanthocyanidins);
• the degree of oxidation;
• the degree of polymerization (condensed tannins);
• the extraction technique and the type of solvent;
• the botanical origin.

1.2.1 Oenological properties of tannin

Tannins have properties that explain their wide use in industrial processes, in food industries, in pharmacology and even in certain heavy industries, especially in the leather industry, in which their use makes leathers rotproof and resistant. To this group of substances are attributed important properties that make them suitable for use in the wine world both for red winemaking and for the elaboration of white wines with different technological purposes.
In fact, as well as the ability to precipitate the proteins of the medium, thay also have (Citron, 2007):

1. Antioxidant and antioxidasic action;
2. Capacity of chelating metals;
3. Anti-radical action;
4. Capacity of capturing sulfur compounds, responsible for undesirable odours;
5. Anti-protein action;
6. Capacity of stabilizing wine colour;
7. Aromatic role;
8. Capacity of increasing the wine structure;
9. Bacteriostatic effects.

1. Antioxidant (limitation of redox potential rising and reduction of SO_2 consumption in the bottled wine) and antioxidasic action (inhibition of polyphenol oxidase and laccase) (Figure 9); tannins all consume oxygen directly, thus preserving anthocyanidins and flavans from oxidation (Vivas and Glories, 1996), the ellagitannins, in particular, are very effective;

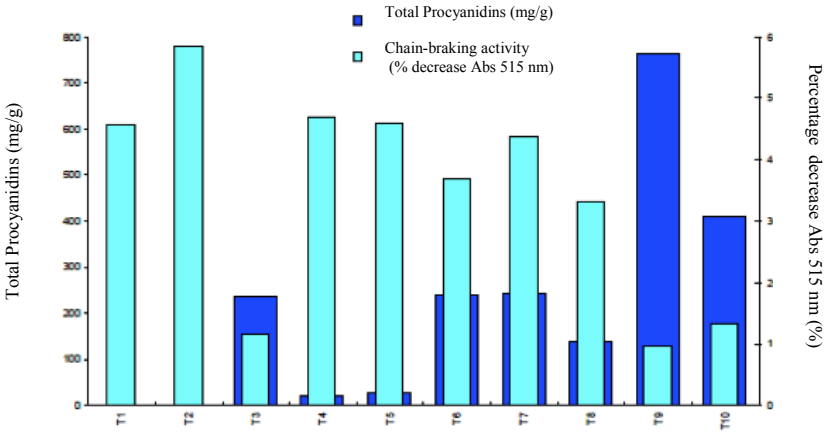


Figure 9 – Antioxidant activity of some commercial preparations of tannins in model wine solution (1g/L) (Celotti *et al.,* 1999).

The hydrolysable tannins raise the redox potential avoiding excessive reduction to which all the wines tend even when they are oxidized. The action is more pronounced in the case of ellagitannins. The condensed tannins (procyanidins) lower the redox potential (Figure 10).

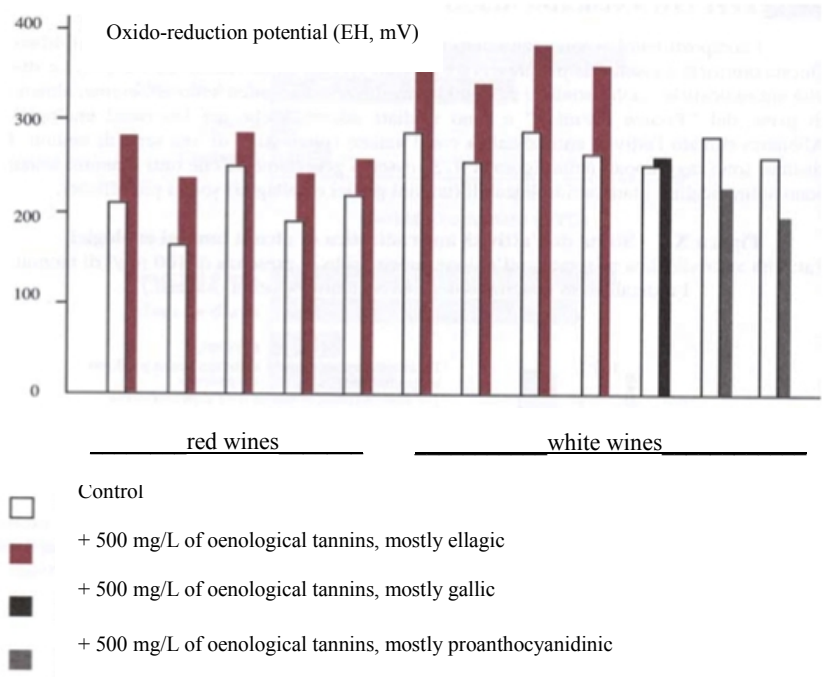


Figure 10 – Influence on wines oxido-reduction potential of oenological tannins containing ellagitannins, gallotannins and proanthocyanidins (Vivas *et al.,* 2003).

The whole class of polyphenolic compounds shows, essentially, anti-oxidant activity. In particular the group of hydrolysable tannins shows greater propensity to capture oxygen than the condensed ones, due to a higher number of -OH groups present in the aromatic rings. This action, therefore, appears to be synergistic with the activity of sulfur dioxide, showing, however, with respect to the latter, a faster reaction on oxygen. Depending on the speed of absorption of oxygen there is the following order: ellagitannins > gallic tannins > condensed tannins.
The antioxidant shield thus exercised has positive effects in the case of white wines, prolonging the shelf-life and protecting the aromatic profile, in red wines by preventing violent oxidations of anthocyanins, that would lead to loss of colouring matter.
It is also known the ability of ellagitannins to produce, by oxidizing themselves to quinones, hydrogen peroxide (H_2O_2), which can oxidize ethanol to acetaldehyde,

favoring the formation of anthocyanin-tannin complexes, of considerable importance for the stabilization of red colour during the maturation of red wines (Vivas and Glories, 1996).
Antioxidasic action takes place on enzymatic complexes that catalyze the transfer of oxygen on polyphenols (polyphenol oxidase).
The most dangerous of these enzymes pool is undoubtedly the laccase, present in large quantities in musts that have been attacked by fungal *Botritys cinerea*. In this case gall tannins are the most active in inhibiting the enzyme. Added directly to grapes during the pressing in a dose varying from 5 to 15 g/100 kg, according to the extent of the parasite attack, they act both by removing oxygen available to the enzyme and by replacing the ideal substrate for the enzyme attack, avoiding the above issues. It should also be remembered that even the extracts obtained from chestnut are recommended in the case of unhealthy harvests (Citron, 2007).

2. Chelation of metals: the tannins have, thanks to their phenolic nucleus, hydroxyl substituents that can chelate the cations of heavy metals (Fe^{3+}, Cu^{2+}), which are important catalysts of oxidation (Vivas *et al.,* 2003) (Figure 11), thus limiting at same time the risk of ferric casse (Salagoity-Auguste *et al.,* 1986);

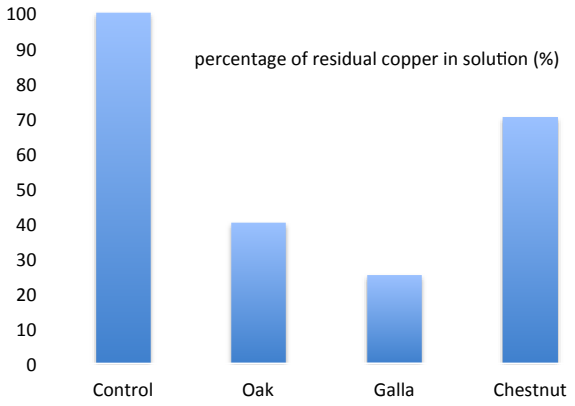


Figure 11 – Capacity of copper chelation of some oenological tannins (Vivas *et al.,* 2003).

3. Anti-radical action (radical scavengers) (Figure 12);
In general the activity of free radicals is known to be harmful even at the level of human cells, triggering phenomena of cytotoxicity and tissue degeneration. All phenolic compounds are known as effective free radical scavengers (Uchida *et al.,* 1987; Ariga and Hamano, 1990), stopping the triggered reactions (radical scavengers), in particular, various studies have identified how tannins block the superoxide ion ($.O^{2}$-) (Vivas, 1997; Vivas *et al.,* 1997). Hence the importance in medical and pharmaceutical field that is attributed to tannins: numerous studies

have demonstrated their "healthy" implications as vascular protectors (Masquelier, 1988).
In wines, also antiradical activity exerted by tannins appears to be far superior to that of sulfur dioxide and ascorbic acid (Vivas *et al.,* 1997).

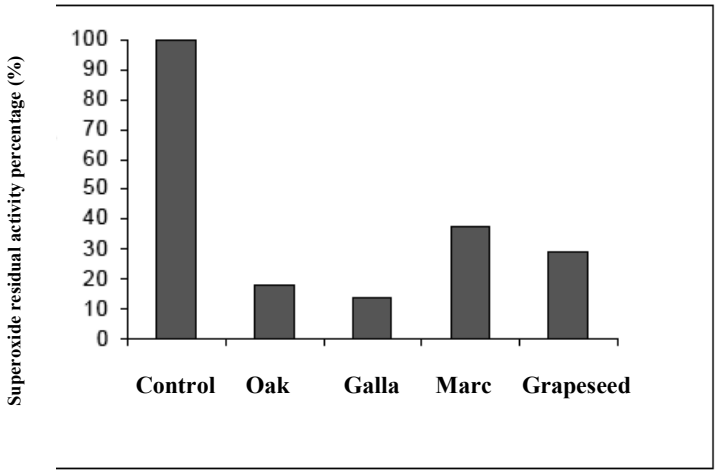


Figure 12 – Oenological tannins. Superoxide ion inactivation in model wine solution. Comparison with a control added with tannin (Vivas *et al.,* 2003).

4. Capture of sulfur compounds: tannins promote the inactivation of the thiol component and of the sulfur compounds responsible for unwanted smells (Vivas, 1997; Ribéreau-Gayon *et al.,*2007); the ellagic ones are the most effective (Figure 13).

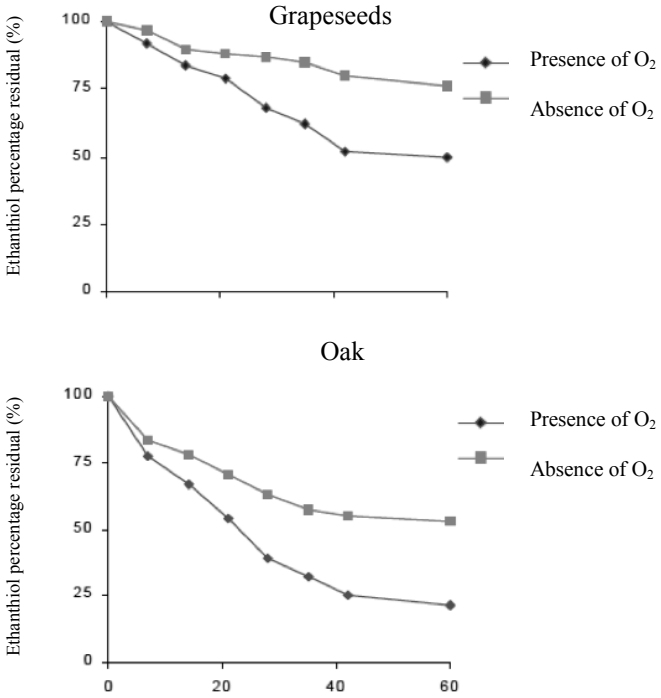


Figure 13 - Sulphur compounds inactivation by tannins of different origins (Vivas, 1997).

It must be specified that this type of intervention does not reduce the content of thiols in the short term, but replaces treatments with pure oxygen that give immediate results, but with the collateral risk of oxidation of aromatic compounds or colour (Citron, 2007).

5. Anti-protein action: the ability of tannins to form complexes with protein molecules which then precipitate (Figure 14). There are several mechanisms that underlie the tannin-protein complexes: hydrogen bonds, electrostatic and hydrophobic interactions (de Freitas, 1995; Guinard *et al.,* 1986, Oh *et al.,* 1980); Ellagitannins are less effective than proanthocianydins in reducting soluble proteins.

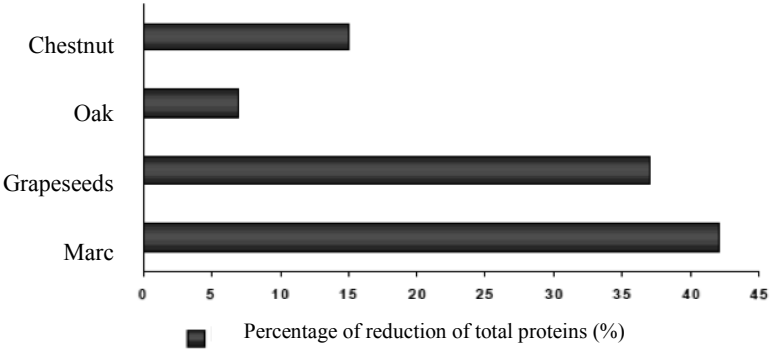


Figure 14 – Activity of different commercial preparations of tannins on proteins (Vivas, 1997).

The formulations made of condensed tannins, preferably deriving from grape seeds, which have increased reactivity due to their lower degree of polymerization, show greater reactivity to proteins than the extracts of other botanical origins. It is therefore preferable to use the first ones as a remedy for overfined wines (Vivas, 1997).

6. Stabilization of colour: the addition of tannins, especially during the winemaking process, helps in stabilizing the colour in red wines. The process of colour stabilization is quite complex and has slow kinetics that probably continue throughout the life of a wine, even in the bottle. It leads to the formation of stable molecular associations between tannins and anthocyanins with the participation of ethanal, hence they become not susceptible to further oxidations or precipitations.
Proanthocyanidins intervene directly and preferably on these complexes, by binding with anthocyanidins. It should be noted how ellagic tannins offer a marked synergistic action by stimulating, once oxidized, the formation of ethanal (Guerra, 1997; Saucier, 1997)

7. Aromatic role: in addition to the bitterness (clearer in chestnut and gall tannin) and astringent (more rapidly perceived with oak tannins) sensations, they can determine typical sensory notes of refinement in wood and improve the characteristics of weakly structured wines (Vivas, 1997);

8. Increase in the wine structure (increase of final extract and improvement of the body of the beverage).
In wines, the interest is to have tannin preparations whose nature is identical to that of the grape, so that they can participate in the global wine tannin structure.
In this case, it is possible to:

• change the balance of tannins/anthocyanins, ensuring an improvement in the stabilization of the colouring matter;
• provide structure to wines allowing their successful evolution during aging, limiting the risk of "leaning" and dryness;
• change the ratio skin tannins/seed tannins, working on products based on white grape marc or fresh grape seeds, acting in this way, on the profile of wines and their response to oxygen consumption;
• conduct an effective removal of excess proteins, because it appears that only condensed tannins are active in protein flocculation (Vivas *et al.,* 1997).

9. Bacteriostatic effects: tannins tend to inhibit significantly the growth of bacteria, lactic acid ones are the most sensitive. For acetic acid bacteria sensitivity depends on the type of tannin, they are not very sensitive to gallic and ellagic tannins and moderately sensitive to condensed ones.

1.3. Polysaccharides

1.3.1 Gum Arabic

Gum Arabic is a natural vegetal resin produced in different regions including areas from east to west in the southern Saharan belt in Africa; currently the largest producer is Senegal. This resin is the dried exudate (gum) obtained from two different species of Acacia through special tappings made on the trunk of the Acacia Senegal tree (for alimentary sector, pharmaceutical and beverage industry) and Acacia Seyal (for oenological and biotechnological applications).
Gum arabic is a neutral or weakly acid hydrocolloid composed of a branched polysaccharide (Arabinogalactan II) associated with a glycoprotein (Figure 15). This natural substance has an average molecular weight between 300,000 and 800,000 Daltons. It is made for 95% of its dry weight of polysaccharides and for 1-2% of proteins, depending on the tree species provenance.
It consists of three fractions that differ in their molecular weight and protein content:

- fraction 1: about 90-99% of the total; arabinogalactans (AG) with numerous ramifications and little amount of nitrogen;
- fraction 2: approximately 1-10% of the total; arabinogalatcan-protein complexes (AGP) (50% protein);
- fraction 3: about 1% of total; glycoproteins (GP) very rich in protein component (25% of total), representing the skeleton of the structure of gum arabic.

The arabinogalactans are the ramifications of the protein backbone. The polysaccharide fraction is composed of linear chains of galactose linked by β-1,3 bonds. These chains are branched in position 1,6 with chains of rhamnose and galactose. Rhamnose, glucuronic acid and methyl-glucuronic acid are found in the terminal position of the chains.

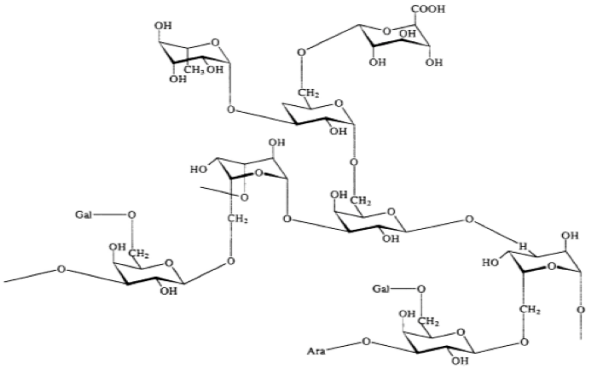


Figure 15 - Gum arabic structure.

After complete acid hydrolysis, the products obtained are in the following respective percentages:
- 35 to 45% galactose;
- 25 to 45% arabinose;
- 4 to 13% rhamnose;
- 6 to 15% glucuronic acid
- 1 to 2% protein

Commercial gum arabic is a totally odourless white powder, readily soluble in cold water until a 40% concentration. Given the presence of uronic acids, partially salified, the solution becomes acidic in nature and gives a slightly viscous aspect.
It has the capacity of stabilizing colloidal systems and in this property its high solubility and its low viscosity play a key role.
Based on these characteristics, gum arabic is able to form thin films on the surface of particles in suspension in wine, making them hydrophilic and creating repulsive forces. Therefore, it acts as a protective colloid, preventing swelling and flocculation of particles. To perform this function fully, the concentration of gum arabic in solution should be sufficient to cover the interface between the particles in suspension and the solvent. These properties persist without alteration over time and of course they change depending on the quality of the gum and the characteristics of the small protein backbone.
The main applications of gum arabic are based on its properties as an emulsifying agent, stabilizer, flavour fixer, gelling agent, thickener, binder and film-forming.
The functions of gum arabic on the wines can be summarized in:

- Improving softness: Thanks to the high content of non-fermentable polysaccharides, gum arabic has a slightly sweet taste, which greatly improves the balance of wines, especially those with particularly acute acidic or tannic notes.
  The complex molecular structure of gum allows its positioning on the receptors of taste buds, temporarily isolating and thereby delaying and reducing astringent and bitter perceptions (Taira *et al.,* 1997, Troszynska *et al.,* 2010). It also prevents the combination of tannins with salivary proteins.
- Increasing aromatic intensity and fragrance: Substances making up the bouquet of wine, in order to be perceptible to the senses, must be present in the volatile form. These substances tend to spontaneously separate from the liquid solution, dispersinng in gas phase, depending on the balance between the two phases. Polysaccharides, the group to which gum arabic belongs, can increase the volatility of aromas in wine (Vidal *et al.,* 2004, Mitropoulou *et al.,* 2011). Therefore, the addition of exogenous carbohydrate colloids is the only practical and legal tool to better highlight pleasant sensory characteristics of wine, action more interesting as more high is the potential quality of the product.

- Stabilizing the colour of red wines: In young red wines, the fraction of the colouring matter in the colloidal state may be eliminated by using bentonite, but this practice would produce an absorption of anthocyanins and therefore a loss of colour intensity. It would be better, then, to stabilize the natural colouring of young red wines with the addition of gum arabic (Giacomini, 1980). Gum arabic also slows down the polymerization and subsequent precipitation of any colouring matter in the bottle.
- Increasing the chemical and physical stability: The hydrophobic colloids of wines are often made of amorphous aggregates: forming ferric and copper casse, colloidal colouring matter, different kind of precipitates. These hydrophobic colloids are derived from a first physico-chemical or chemical transformation, which resulted in the passage from molecules in solution to dispersed colloidal micelles. Although the phenomena related to their formation are still poorly known, it is certainly known that some compounds are able to prevent the flocculation of these micelles and the resulting haze of the wine. One of the most active substances in this sense, as a "protective colloid" is gum arabic, which prevents turbidity and all kinds of nature colloidal deposits (ferric and copper casse, insolubilization of colouring matter, etc..). Gum arabic has also a greater effect than meta-tartaric acid in its preventing the growth of crystals of bitartrate because it is absorbed on their faces. It also acts as protector against protein precipitation.

The dose used in wines should not exceed 30 g/hL, but there is no regulatory limit of use. The addition is generally made after the filtration, at bottling, as it may be cause of difficult filterability of wines.

1.3.2 Mannoproteins

Mannoproteins are a family of macromolecules in which each structural element confers different properties and activities.
There are two industrial methods for the extraction, and lead to obtain preparations with different characteristics:

- Thermal Method: (Peat *et al.,* 1961) consists of putting both active dry yeast, previously washed with distilled water, and yeast cell walls and hulls, derived from autolysis, in an autoclave at 125 °C for 90 minutes. After autoclave treatment, yeasts and the hulls are placed in suspension in a citrate buffer at pH 7. This operation is repeated twice, then they are subjected to centrifugation. The supernatant, which in the laboratory can be precipitated with ethanol and then dried, can be dried by lyophilization or heat treatment in a pilot scale.
- Enzymatic Method (Moine-Ledoux, 1996) consists of an extraction of mannoproteins from yeast cell wall by digestion with an industrial β-

glucanase preparation and proteases. Its use is authorized in wine industry for the hydrolysis of the glucan deriving from *Botrytis cinerea* and to facilitate the filtration of wine. Mannoproteins extracted enzymatically are richer in proteins and their polysaccharide fraction contains only mannose. Analyzed by molecular exclusion HPLC, mannoproteins extracted by enzymatic digestion have an additional peak corresponding to an average molecular weight between 30 and 50 kDa. At the end of enzyme treatment, the hydrolyzate is separated by centrifugation, purified, and lyophilized.

The two main constituents of the yeast cell wall are glucans (polysaccharides) and mannoproteins (glycoproteins) (25-50% of the walls) (Figure 16).

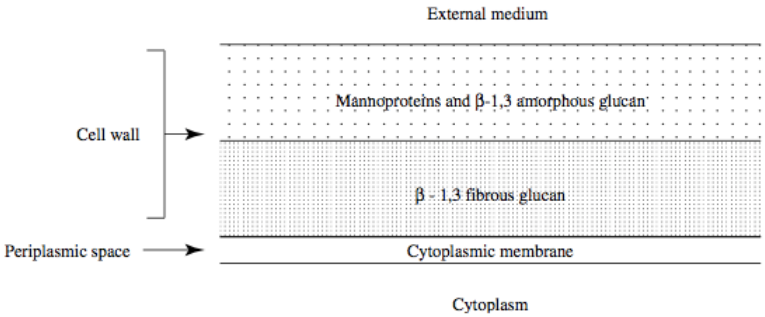


Figure 16 - Cellular organization of the cell wall of *S. cerevisiae.*

The mechanism of yeast autolysis involves a liberation of mannoproteins, after the wall glucans hydrolysis. These consist of a peptide fraction and a polysaccharide fraction mainly represented by mannose. Their molecular weight ranges from 20 to over 450 kDa, and they can have varying degrees of glycosylation.

The amount of polysaccharides released depends on the strain of the yeast, on the fermentation and storage conditions, on the temperature, on the agitation of the medium and on the retention time on biomass.

In the case of fermentation of dry white wines, polysaccharides are released especially during aging on the lees; the phenomenon is slow because the storage temperature is low (12 to 16 °C). The enrichment of red wines in the colloids of yeast, instead, occurs primarily during the post-fermentation maceration at a temperature of 30-35 °C, but it is limited because most of the yeasts are separated from the wine when racking.

It has been demonstrated that mannoproteins are the class of polysaccharides able to interact more effectively with other molecules in wine, with the result of significant improvements in the organoleptic quality. The most important role of yeast mannoproteins is their stabilizing effect on protein precipitation in white wine and tartrate crystallization (in both red and white wines)**.** Lubbers *et al.,* in

1993, indicated that mannoproteins have a positive effect on tartrate stability. It seems as though the mechanism of activity is based on competitive inhibition, which limits crystal formation (Moutonnet *et al.,* 1999). The findings above are confirmed by the fact that if white wine is left on the lees for a considerable period of time (some months), it becomes relatively tartrate stable and does not therefore require cold stabilisation. In fact, the most "protective" mannoproteins are extracted from yeast cell walls during aging on the lees.
Mannoproteins have positive influence on tartaric (Lubberes *et al.,* 1993, Waters *et al.*, 1994), protein (Ledoux *et al.,* 1992, Moine-Ledoux and Dubourdieu, 2002) and phenolic stability (Saucier *et al.,* 1996), but they also show other positive properties, such as:
• positive interaction with aroma, as the protein fraction of mannoproteins binds the aromatic compounds, stabilizing them; the practical result is that it decreases the volatility of some compounds with a slight decrease of olfactory intensity, however, there is a much more prolonged retention of aromas over time (Lubbers *et al.,*1994a);
• adsorption and removal of malodorous thiols (Lavigne and Dubourdieu, 1996);
• influence on the "fullness" of a wine (Vidal *et al.,* 2004) ;
• positive influence on malolactic fermentation: Guilloux-Benantier *et al.* (1995), as well as Rosi *et al.* (1999) highlighted the positive influence of mannoproteins on the onset and development of malolactic fermentation. It was apparent that in a wine fermented primarily with a yeast strain that produced naturally higher levels of polysaccharides, the onset and completion of malolactic fermentation occurred more quickly than the malolactic fermentation of a wine fermented with a yeast strain that produced inherently lower concentrations of polysaccharides. Apparently the association of tannins with mannoproteins seems able to decrease the antiseptic effect of the latter on the malolactic bacteria.

1.3.3 Carboxymethylcellulose (CMC)

Carboxymethylcellulose (CMC) is a polysaccharide that is obtained by etherification of the primary alcohol functions of the glucopyranose units linked by hetheroxide β- 1–4 bonds (Figure 17).

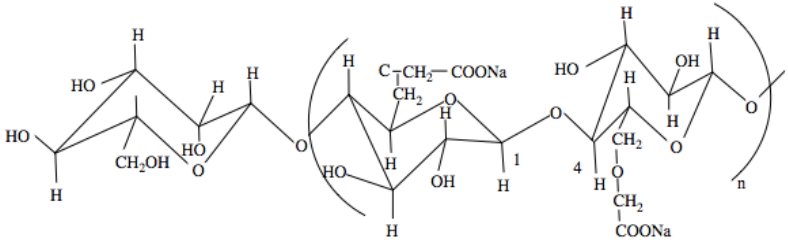


Figure 17 - Structure of a carboxymethylcellulose (CMC) chain.

It is characterized partly by the degree of etherification of its alcohol functions, known as the degree of substitution (DS), and partly by its degree of polymerization (DP), i.e. the average number of glucopyranose units per polymer molecule.

The CMC viscosity depends on its DP (increases with molecular weight, which may vary from 17,000 to 1,500,000 Daltons) and on the cation present, divalent cations (i.e. calcium, magnesium, iron, etc.) reduce viscosity.

Its structure makes it a perfect "protective colloid" and Lubbers *et al*. in1993 found out that the more cation anchor sites it has, and the more effective it is as a protective colloid (Lubbers).

CMCs are reputed to promote solubilization of proteins and stabilize solutions containing them (Federson and Thorp, 1993), that could be useful in preventing protein casse.

Carboxymethylcellulose, identified with the initials "E466", has long been used as a food additive

in the preparation of cream-based products, fish, meat and fruit, desserts, ice creams, creams, or as a coating for tablets in the pharmaceutical industry.

Its properties along with the fact that it is easy to use, relatively inexpensive, and do not require special investments allowed, in 2009, the O.I.V. to authorize the use of carboxymethylcellulose in white wines and in sparkling wines (Resolution OENO 2/2008) in a maximum dose of 10 g/hL, though recommending that care should be taken as to potential allergenic risks due to this substance (Bosso *et al.,* 2010).

Many research works demonstrated its effectiveness in reducing the formation and the growth rate of potassium bitartrate crystals (Gerbaux, 1996, Crachereau *et al*., 2001, Bosso *et al.,* 2010, Gerbaud *et al.,* 2010) and its stability even at high temperatures (Ribéreau-Gayon *et al*., 1977).

Further research is required to assess the effectiveness in different types of wine, especially tannic red wines, which have a particularly complex colloidal structure.

1.3.4 Grape Polysaccharides

Polysaccharides constitute one of the main groups of macromolecules occurring in wine.
A fraction of them is directly derived from grapes, they can be acid or neutral and they are essentially pectins and polysaccharides such as arabinanes, galactanes and arabinogalactanes or rhamnogalatturonans with a molecular weight ranging from 40 to 250 kDa. These substances are hardly removed from the medium, because they behave as stable colloids in the solution. They are located in the outer part of the peel and in cell membrane of the pulp.
Pectins represent the acid polysaccharides, they are chains formed almost exclusively of galacturonic acid (α-D-galacturopyranoside acid) units partially esterified by methanol. The degree of esterification of grape pectins is high (70–80%) and they have α-(1,4)-type oside bonds. These homogalacturonanes are not present in musts and wines because of the action of endogenous or exogenous pectolytic enzymes.
Acid pectic substances in grape are made of long chains of galacturonic acid (homogalacturonan) units (Figure 18), interrupted by rhamnogalacturonan structures, where rhamnose units (Rha) alternate with galacturonic acid units (GalA).

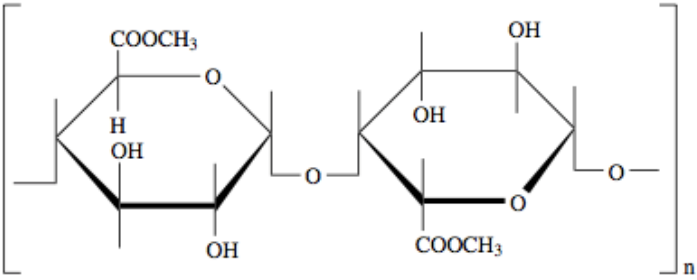


Figure 18 - Basic structure of α-homogalacturane. Chain of partially methylated galacturonic acid units, linked by α-(1,4)-type oside bonds.

Besides galacturonic acid, neutral polysaccharides consist of neutral oses, such as arabinose, rhamnose, galactose and small quantities of xylose, mannose and glucose.
Their role in winemaking is still little known, it has been demonstrated by some authors, that they play an important role in the stabilization of anthocyanins and mitigating astringency of tannins (Vidal *et al.,* 2004) but they impart viscosity to

wine, sometimes necessitating the use of pectolitic enzymes to clarify wine and make filtration easier.
Both rhamnogalacturonans (RG-I and RG-II) act as tartrate crystallization inhibitors in wine (Vernhet *et al.,* 1999). The natural inhibition of tartrate crystallization at low temperatures is more marked in red than white wines. This difference is due to the effect of polyphenols, which are also crystallization inhibitors, as well as the presence of higher quantities of RG-I and RG-II in red wines.

1.4 Tannins interactions

1.4.1 Interactions with proteins

The interactions between tannins and proteins have been extensively studied (Hagerman, 1989; Haslam *et al.,* 1988, Baxter *et al.,* 1997, Hagerman *et al.,* 1998), depending on their important role in the formation of turbidity, in the perception of astringency, and their nutritional and anti-nutritional effects resulting from inhibition of various enzymes and the reduction of the digestion of protein in the diet. Other effects include reduced absorption of β-casein on the gas-liquid interface in presence of epigallocatechin gallate with potential consequences on the properties of the foam (Saucier and Glories, 2000).
The interaction between flavonols and protein is linked to London-Van der Waals interactions and hydrogen bridges (Oh *et al.,* 1980, Luck *et al.,* 1994, Murray *et al.,* 1994, Charlton *et al.,* 1996).
These hydrophobic effects occur between tannins and the non-polar regions of the proteins. Certain authors (Oh *et al*., 1980) even consider that this is the predominant interaction mode, due to the hydrophobic nature of the tannins. The reactions seem to be the origin of the complexation reinforced by hydrogen bonds, for example, between the carbonyl group of the secondary amine function of the proline and the phenol -OH. These surface phenomena depend not only on the number of phenol groups on the periphery of the molecule (Haslam *et al.,* 1988) but also on the relative proportions of each of the two families.
At low protein concentrations, polyphenols bond to the surface of the protein at one or more sites, forming a monolayer that is less hydrophilic than the protein alone. This is followed by aggregation and precipitation. When the protein concentration is high, an identical phenomenon occurs, with the superposed formation of cross-bonds between various protein molecules. This explains the non-stoichiometry of the tannin-protein reaction observed by many authors (Ribéreau-Gayon *et al.,* 1977).
The link between flavonols and flavones with certain proteins such as serum albumin, which is involved in their transport in plasma, has been extensively documented (Boulton *et al.,* 1998, Dangles *et al.,* 2005). The flavonols have also been shown to be absorbed by Polyvinylpolypyrrolidone (PVPP) (Laborde *et al.,* 2006) but did not show any interaction with proteins.
A study carried out using NMR spectroscopy (NMR - Nuclear Magnetic Resonance) showed a clumping of the phenolic ring with the proline residues in the sequences of proline and the stabilization of the complex through hydrogen bridges between the acceptor sites of hydrogen ions of the adjacent peptide bridges and the hydrogen atom of the hydroxyl group of the phenolic compound (Murray *et al.,* 1994).
More recently, an isothermal calorimetric titration experiment (Isothermal Titration Calorimetry - ITC) has shown that the interaction of flavonols with poly-L-proline

involves both entropic phenomena (associated with hydrophobic effects and conformational changes) and enthalpy (attributed to hydrogen bridges) (Poncet-Legrand *et al.,* 2007a). The latter situation is common in the case of flavanol monomers, while in polymers in the case of the former. The interaction does not necessarily lead to precipitation.

The absorption of flavonols on PVPP involves Van der Waals forces associated with hydrophobic effects and hydrogen bridges (Laborde *et al.,* 2006).

The protein foam due to interaction between flavanols and proteins or peptides of flavanols is well documented in beer (Outtrup, 1989) but it also occurs in wine.

The protein precipitation also occurs as a result of aging treatments, which consist of adding exogenous proteins in order to precipitate tannins to stabilize the wine and reduce its astringency.

The protein most commonly used for aging of red wines are gelatin, albumin and casein. Proteins extracted from lupine or wheat have been tested as alternatives to gelatin (Maury *et al.,* 2003). All, as well as saliva proteins (Sarni-Manchado and Cheynier, 2002) selectively precipitate high molecular weight flavanols (Ricardo da Silva *et al.,* 1991; Maury *et al.,* 2001; Sarni-Manchado *et al.,* 1999), which also show high astringency (Vidal *et al.,* 2003).

However it should be noticed that the decrease in astringency observed after aging can be partly due to the union of flavanols forming soluble complexes.

The interactions and the formation of insoluble complexes with proteins increase with the number of phenolic rings, especially the ortho-diphenolic ones and then with polimerization and galloylation (Haslam *et al.,* 1988, McManus *et al.,* 1985). The intensity of the signal obtained by mass spectrometry corresponds to soluble flavanol-peptide complex increased by the presence of monomers and dimers and with galloylation (Sarni-Manchado and Cheynier, 2002).

And in addition, the interaction of flavanols momomers with proteins follows the same order as their partition coefficient between octanol and water, which leds to an increase in hydrophobicity of the phenolic compounds (Poncet-Legrand *et al.,* 2007a). The phenolic oxidation generated by particular polymeric species, leads to an increase of protein interactions as evidence of a high enzymes inhibition (Guyot *et al.,* 1996).

Within gelatin (Maury *et al.,* 2001) or gluten (Maury *et al.,* 2003), the lowest molecular weight proteins are much more selective than the ones with a higher weight. The mechanism of interaction depends on the concentration of protein. At low concentrations, it occurs in three stages depending on the increase of the polyphenol/protein ratio, as described above: saturation of the sites of interaction, formation of metastable colloids, and aggregation leading to the formation of turbidity. At elevated protein concentrations, there is the direct formation of bridges, resulting in lower thresholds of absorption and turbidity.

The interactions of flavonols with proteins (Dangles *et al.,* 2005) as well as their absorption of PVPP is much more efficient with aglycones, because the residue on the sugar glycosides weakens the driving force (Laborde *et al.,* 2006). Other

parameters, such as the characteristics of the solvent, the presence of other solutes and temperature, influence the associations polyphenol/protein and the properties resulting from the formation of the related complexes. Thus the affinity between tannins and PRPs is less at high temperatures (Charlton *et al.,* 2002). The presence of polysaccharides prevents coprecipitation of tannins and proteins (Luck *et al.,* 1994, Cheynier *et al.,* 2006).

The complexation between flavonoids and proteins shows some specificity. However, the lowest molecular weight of flavonoids (eg. flavonols, non galloyl flavanols monomers) show moderate affinity towards proteins and do not form aggregates. Similarly, although all proteins interact with tannins, proline-rich structure, as already seen in proteins that are very commonly used as fining agents (gelatin, casein) or in proline-rich salivary proteins (PRP) involved in perception of astringency, are particularly prone to interact with tannins (Hagerman and Butler, 1989; Charlton *et al*., 1996). The importance of proline is probably due to its incapacity to form helixes, leaving the protein open and accessible to tannins.

Tannin-protein interactions depend on the characteristics of the tannins: size, structure, charge, etc. These interactions increase with the degree of polymerization of the procyanidins (Ricardo da Silva *et al*., 1991) and also according to their galloylation rate (Charlton *et al*., 1996). Tannin-protein interactions also vary according to the composition of the tannins: condensed tannins formed from procyanidins linked by ethyl cross-bonds, tannins combined with anthocyanins or tannin–polysaccharide complexes. At pH 3.5, these molecules have different charges depending on their origin. Furthermore, it has been observed that this surface charge density is affected by the pH of the solution. The higher the pH, the more charged the flavanols, with a variation on the order of 100% between pH 3 and 4. This phenomenon is obviously useful in fining.

Turbidity (Siebert *et al*., 1996), as well as the type and quantity of tannin-protein precipitates, depend on the relative concentrations of the various components (Calderon *et al*., 1968).

Ionic strength and pH affect the solubility of proteins. The precipitation of the complexes tannin-protein is higher at the isoelectric point of the protein because the electrostatic repulsion is minimum (Calderon *et al.,* 1968, Perez-Maldonado *et al.,* 1995, Charlton *et al.,* 2002; Kawamoto and Nakatsubo, 1997). The effect of ionic strength and ethanol content in epigallocatechin gallate interaction with salivary PRP has already been studied (Pascal *et al.,* 2006). The increase in ionic strength with sodium chloride or tartrate ions results in an increase in aggregates stability, which means that the aggregation is not driven by repulsive electrostatic forces. At a concentration of 12% alcohol, the protein is not completely dissolved and the aggregation with epigallocatechin gallate requires much higher concentrations of tartrate ions, confirming the role of hydrophobic interactions. It has been proved that high alcohol contents influence this kind of interactions by reducing the affinity between tannins and proteins (Calderon *et al*., 1968)

The presence of Na^+, K^+, Ca^{2+}, Mg^{2+} and especially Fe^{3+} cations is indispensable

for flocculation and the precipitation of tannins and proteins.
Polysaccharides may also have an 'activating' effect. The presence of pectins, arabinogalactans and polygalacturonic acids increases the intensity of turbidity and is favorable to fining, while neutral polysaccharides have no effect.
A low temperature (15 °C) enhances precipitation and clarification, due to the decrease in Brownian movement that facilitates flocculation of the colloids

1.4.2 Interactions with polysaccharides

Polysaccharides are one of the major groups of macromolecules in wine. Among them are pectic substances and neutral polysaccharides, which come from grapes. Others are of fungal origin, such as glucans, with a molecular weight of 1000 kDa produced by Botrytis cinerea when it infects the grapes (Escot *et al.,* 2002).
There is also one important group of polysaccharides, produced or liberated by yeasts during alcoholic fermentation (Llaubères, 1988) or during the lysis of yeast (Feuillat *et al.,* 1989).
The effect of macromolecules called "protective colloids" on the stability of the wine is known since 1933 (Ribéreau-Gayon). However in the past, these colloids were removed by filtration or fining treatments (Feuillat, 1987) using not suitable membranes.
Most of the polysaccharides present in wine, including mannoproteins from yeast and originated from the constituents of the plant cell wall (Arabinogalattan proteins (AGP) and Rhamnogalatturonan II (RGII)), have shown the ability to interact with flavanols (Riou *et al.,* 2002).
The composition and structural characteristics of mannoproteins present in wine are summarized in Table 3.

	MP0 (Vernhet)	MP0a (Vidal)	MP0b (Vidal)	MP0c (Vidal)
Concn in red wine (g L^{-1})	0.10-0.15			
Molecular weight (kDa)	30-400	337	62	51
Negative charge at pH 3.2 (C g^{-1})	7.3			
Protein (% dry matter)	6.2	1.4	1.6	3.5
Uronic acid	nd[a]	–	–	–
Rhamnose (molar ratio)	–	–	–	–
Arabinose (molar ratio)	0.9	4.1	5.2	–
Mannose (molar ratio)	96.9	92.4	88.8	97.1
Galactose (molar ratio)		2.53	2.9	1.2
Glucose (molar ratio)		–	2.6	1.9

Table 3 - Composition and characteristics of wine mannoproteins (Vernhet *et al.,* 1996, Vidal *et al.,* 2003).

In addition, gum arabic (a mixture of arabinogalactans arabinogalactan-proteins) can be added as protective colloid to limit or prevent the aggregation, flocculation and precipitation of tannins and tannin-protein complexes (Pellerin and Cabanis, 1998). However, at high doses, can lead to instability and foam formation (Saucier *et al.,* 1996, Siebert *et al.,* 1996).

Understanding the influence of mannoproteins on the stability of phenolic compounds needs a better knowledge of their composition as well as of the release and action mechanisms. For this reason many observations were performed in laboratory and some under real conditions in the cellar (Escot *et al.,* 2002).

Studies using laser light scattering technique showed that the interaction of some mannoproteins and arabinogalactan-proteins with procyanidins prevents the aggregation of the AGP and leads to the formation of particles of small size, stable over time. Adding other polysaccharides such as RGII monomers there was no effect, while RGII dimers increased the aggregation leading to precipitation (Riou *et al.,* 2002). The effectiveness of mannoproteins as stabilizing agents decreases with the increase of their molecular weight, suggesting that the mechanism involved is steric stabilization; at high concentrations of polysaccharides (high active surface), the low molecular weight of mannoproteins provides ideal conditions for the steric stabilization (Poncet-Legrand *et al.,* 2007b) (Figure 19).

Polysaccharides have also been shown to reduce the precipitation of tannin-protein complexes (Cheynier *et al.,* 2006, Luck *et al.,* 1994). This has been attributed to the formation of ternary complexes protein-polysaccharide-soluble flavonoid, often mediated by hydrogen bridges and hydrophobic effects (McManus *et al.,* 1985).

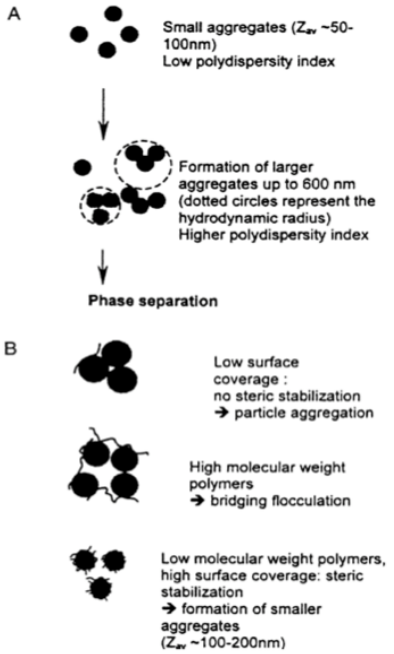


Figure 19 - Outline of the combination of polyphenolic molecules (A) and steric stabilization or flocculation of the bridges of tannin molecules by polymerization (B). (Poncet-Legrand *et al.,* 2007b).

Among polysaccharides of wine, mannoproteins play an important role in stabilizing the protein turbidity (Waters *et al.,* 1994; Dupin *et al.,* 2000). Ledoux *et al.,* in 1992, indicated that some mannoproteins had a noticeable influence on the protein stability of white and rosé wines. The presence, as well as the addition of such mannoproteins, would obviously necessitate less bentonite for the stabilisation of white and rosé wines. Therefore smaller volumes of aromatic molecules would subsequently bind and perhaps be removed from the wine by the bentonite.

Clarification with the gelatin of a wine phenolic extract in wine-like solution shows a high percentage of precipitation compared to effect of the same treatment as it is in wine. The addition of polysaccharides at the usual concentration present in wines, reduced the precipitation to the level measured in the wine as it is, confirming the stabilizing effects of polysaccharides (Cheynier *et al.,* 2006).

Knowledge of size and structure of polysaccharides and polyphenols is essential. The presence of hydrophobic cavities such as those encountered in the cyclodextrin molecule promotes interaction with phenolic molecules of appropriate form or mobility (Smith *et al.,* 1994).

The polysaccharide effects also depend on the composition of the medium. Therefore mannoproteins mostly influence the procyanidins aggregation at higher concentrations of alcohol. At high ionic strength, all mannoprotein fractions, including the high molecular weight ones, are effectively stabilized by polyphenols.
Polysaccharides represent a large class of compounds responsible for the expression of some sensory characteristics in wines that are becoming increasingly important, such as softness, roundness, fullness, balance, etc. They have a huge influence on the aromatic quality of wines. Different studies have shown that the polysaccharides can fix aromatic compounds, forming interactions that involve a significant increase in aromatic persistence, which is opposed by a decrease in the intensity, especially at low temperature (Lubbers *et al.,* 1994b).
Some authors have proposed an index to measure the structure of the wine in the mouth, showing that polysaccharides, and particularly mannoproteins, have a major impact on a sensory level than glycerine (Bertuccioli *et al.,* 1999). This effect is certainly, or at least in part, due to the decrease in astringency of tannins. As already said in Paragraph 1.1.1, French researchers have developed a theoretical model that would be able to explain the interactions between polysaccharides and tannins, which form the basis of the increased softness of wines (Saucier *et al.,* 1996).
As part of these studies, it was highlighted the ability of certain complex polysaccharidic molecules, originated from yeast, to stabilize the colouring matter in red wines (Saucier and Glories, 2000).
Wines stored for long periods in contact with the lees of fermentation show, at the end of fining process, a significant improvement in protein and tartrate stability. Several authors have studied these phenomena, showing that some macromolecules belonging to the large family of mannoproteins, released by the yeast during the period of aging, are responsible for this stabilization (Dubourdieu and Moine, 1995, Ledoux *et al.,* 1992).
It has been demonstrated by these authors that the improvement of protein stability of wines stored on lees is primarily attributable to MP32, a particular mannoprotein, corresponding to a specific fragment of invertase, with a molecular weight of 31.8 kDa, hydrolyzed by vacuolar proteases during cell lysis.
From a practical point of view, the improvement of protein stability during fining of white wines in barrels is strongly influenced by different parameters:

- duration of storage;
- quantity of lees present;
- age of the barrel;
- frequency of resuspension of yeast.

The improvement of tartaric stability was attributed to another fragment of mannoprotein, MP40, which has a lasting effect over time. For a mechanism that is not fully elucidated, it has the capacity to inhibit both the formation of

crystallization germs and their growth, thus avoiding the tartrate precipitation. In this way, the concentration of tartrates in wines remains costant.
Another interesting aspect is the absorption of indesirable volatile thiols by certain classes of polysaccharides. It was shown that, due to formation of disulfide bonds between mannoproteins and -SH groups, some molecules such as methanthiol and etanthiol are inhibited, resulting in loss of their negative effects.
The inhibitory effect of carbohydrates in relation to tannin-protein aggregation has been proposed as a likely result of the ability of the polysaccharide to form a ternary complex protein-polyphenol-carbohydrate, increasing its solubility in aqueous medium, or molecular associations between carbohydrates and polyphenols in solution, competing to bind the protein (Figure 20) (Mateus *et al.,* 2004).

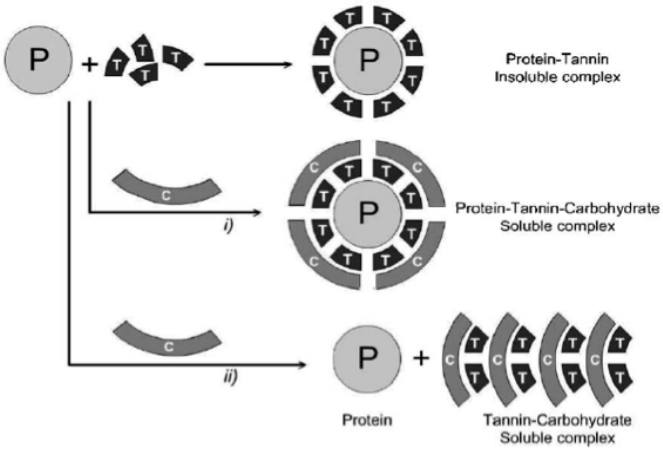


Figure 20 - Possible mechanisms (i and ii) involved in the inhibition of the aggregation of tannins and polyphenols mediated by polysaccharides. P: protein, T: tannin; C: carbohydrate. (Mateus *et al.,* 2004).

Unfortunately, some polysaccharides may be undesirable, that is the case of glucans produced by *Botrytis cinerea*, which slow the flow of filtration and confer instability to the wine (Dubourdieu *et al.,* 1981).

1.5 Non conventional analytical techniques

Highlighted the complexity of the colloidal system in a hydro-alcoholic solution, it is necessary and desirable to increase the knowledge about the endogenous and exogenous colloidal substances of wine.
In this research work it has been chosen to take advantage of some non-conventional techniques of analysis for the wine industry, such as: the dimensional measurement of the hydrocolloidal radius (particle size) using the technique of the dynamic diffusion of light (DLS) and the surface electric charge (SEC) by measuring the streaming potential through a streaming current detector (SCD). Even if these techniques are not used in enology they are already well known and used in other fields and in other food sectors

1.5.1 Dynamic Light Scattering - DLS

In recent years, the technique of dynamic light scattering (DLS), also called quasi-elastic light scattering (QELS) or photon correlation spectroscopy (PCS), has proven to be an invaluable analytical tool for characterizing the size distribution of particles suspended in a solvent (usually water). The useful size range for the DLS technique is quite large, from below 0.005 micron to several microns. The power of the technology is mostly apparent when applied to particular particles with diameters of 300 nm submicron size range, where most competing measurement techniques lose their effectiveness or fail altogether. Consequently, DLS-based sizing instruments have been used extensively to characterize a wide range of particulate systems, including synthetic polymers (e.g. latexes, PVCs, etc.), oil-in-water and water-in-oil emulsions, vesicles, micelles, biological macromolecules, pigments, dyes, silicas, metallic sols, ceramics and numerous other colloidal suspensions and dispersions.
A simplified schematic diagram of the DLS module is shown in Figure 21. Light from a laser is focused into a glass tube containing a diluted suspension of particles. The temperature of this scattering cell is held constant. Each of the particles illuminated by the incident laser beam scatters light in all directions.
The intensity of light scattered by a single, isolated particle depends on its molecular weight and overall size and shape, and also on the difference in refractive indices of the particle and the surrounding solvent. The incident light wave can be thought of as consisting of a very rapidly oscillating electric field, of amplitude E_o (frequency approx. 1015 Hz).

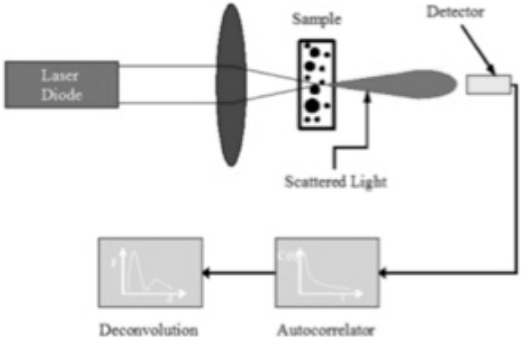


Figure 21 - Simplified block diagram -- NICOMP DLS Instrument (Nicomp ZLS user manual).

The arrival of this alternating field in the vicinity of a particle causes all of the electrons which are free to be influenced ("polarizable" electrons) to oscillate at the same frequency. These oscillating electrons, in turn, give rise to a new oscillating electric field which radiates in all directions, the scattered light wave. The quantity of interest in a scattering measurement is the intensity of the scattered wave, I_s, rather than its amplitude, Es. The intensity is given simply by the square of the amplitude: $I_s = (E_s)^2$. The dependence of the scattered light intensity I_s on the molecular weight (MW) or volume (V) of the particle is particularly simple when the particle diameter is much smaller than the laser wavelength λ, the so-called Rayleigh region. In this case, all of the polarizable electrons within a particle oscillate together *in phase*, because at any given time they all experience the same incident electric field.
Hence, the scattered wave amplitude E_s is simply proportional to the number of polarizable electrons, times the incident wave amplitude, E_o. The former quantity is essentially proportional to the overall molecular weight of the particle, MW, or its volume, V (for a given particle density).
The constants of proportionality that connect these various physical quantities depends on the indices of refraction of the particle (n_p) and solvent (n_n). That is, how well a given particle scatters light depends not only on MW, or V, but also on the polarizability of the particle (related to n_p) relative to that of the solvent (related to n_s). For the very small particles in the Rayleigh region, we arrive at the expressions for the scattered intensity I_s:

$$I_s = f(n_p,n_s)\,(MW)^2\,I_o \qquad (1a)$$

or

$$I_s = g(n_p,n_s)\,V^2\,I_o \qquad (1b)$$

where I_o is the incident laser intensity, and $f(n_p,n_s)$ and $g(n_p,n_s)$ are functions of the indices of refraction of the particle and solvent, which are fixed for a given system composition. For these small particles in the Rayleigh region (i.e. diameters < approx. 0.1 micron, or 100 nm), there is negligible angular dependence in the scattered intensity.
The simple expressions above must be modified when the characteristic particle dimension (i.e. the diameter, in the case of spheres) is no longer negligible compared to the wavelength of the incident light beam. In this so-called Mie scattering region, Equations la and 1b must be altered to take account of intra-particle interference. With a larger particle, the oscillating electrons no longer oscillate together in phase; the individual scattered waves originating from different regions of the particle interfere at the distant point of detection (Figure 22). The resulting total scattered intensity I_s is therefore diminished relative to the values given by Equations la and 1b, which assume that all of the effective scattering mass is packed into a very small particle size. The expressions in Equations la and 1b can be repaired to include the effects of interference by multiplying them by a Mie "form" factor; this quantity has a limiting value of 1.0 (i.e. no effect) in the Rayleigh region, but falls below unity in a non-monotonic way as the particle size grows.

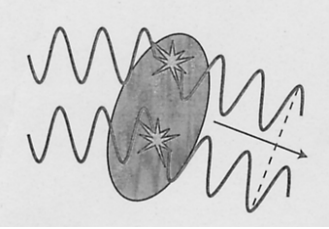


Figure 22 - When light is scattered from different regions of the same molecule, the emerging waves interfere changing the distribution of intensity of the Rayleigh diffusion. That change can be due to the shape of the macromolecule (Atkins, 1992).

Using Equation la or lb, one can, in principle, determine either the molecular weight or the volume of the particles from a measurement of the scattered intensity I_s, using known calibration standards, together with empirical determinations of $f(n_p,n_s)$ and $g(n_p,n_s)$. This forms the basis for the technique of "classical" light scattering. The newer DLS method, however, departs radically from this traditional approach to light scattering. The quantity of interest is no longer the magnitude, *per se*, of the scattered light intensity. Rather, DLS concerns itself with the time behavior of the fluctuations in the scattered intensity.
To understand why the scattered intensity fluctuates in time, it should be appreciated that it is the result of the coherent addition of many individual scattered waves, each of which originates from a different particle located in the

illuminated/detected volume. This is the physical phenomenon known as "interference". Each individual scattered wave arriving at the detector bears a phase relationship with respect to the incident laser wave, which depends on the precise position of the suspended particle from which it originates. All of these waves mix together, or interfere, at a distant slit on the face of a photomultiplier detector (PMT), which measures the resulting net scattering intensity at a particular scattering angle (90°).

The suspended particles are not stationary; rather, they move about, or diffuse, in random-walk fashion by the process known as Brownian motion (caused by collisions of neighboring solvent molecules). As a consequence, the phases of each of the scattered waves arriving at the PMT detector fluctuate randomly in time, due to the random fluctuations in the positions of the particles that scatter the waves. Because these waves interfere together at the detector, the net intensity fluctuates randomly in time. It is important to appreciate that only relatively small movements in particle position are needed to effect significant changes in phase and, therefore, to create meaningful fluctuations in the final net intensity. This is because the laser wavelength is relatively small.

The connection between the diffusion of particles and the resulting fluctuations in scattered intensity is perhaps more easily understood by considering a simplified situation in which there are only tw*o* particles in suspension, shown in Figure 23.

The net intensity at the detector (located far from the scattering cell, with a pinhole aperture) is a result of the superposition of only two scattered waves. In Figure 24 we have defined the two optical path lengths, $L_1 = l_{1a} + l_{1b}$ and $L_2 = l_{2a} + l_{2b}$. (More precisely, the optical path length is the distance corrected by the index of refraction, but for simplicity it is assumed an index of 1.0 and L_1 and L_2 are shown to be simple distances in Figure 24.) When the positions of the two particles are such that the difference in optical path lengths, $\Delta L = L_1 - L_2$ becomes equal to an integral multiple of the laser wavelength λ, then the two scattered waves will arrive in phase at the detector. This is called total "constructive" interference and produces the largest possible intensity at the detector.

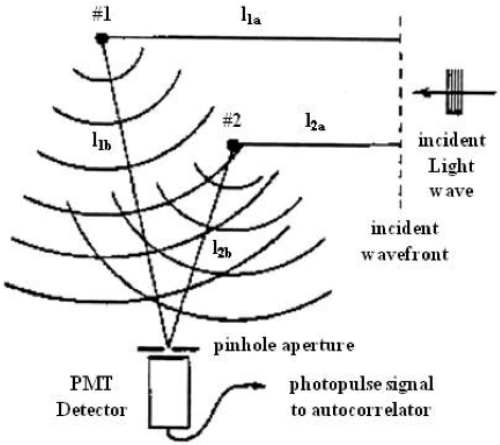


Figure 23 - Simplified scattering model: two diffusing particles (Nicomp ZLS user manual).

At the other extreme, it is possible for the two particles to find themselves at positions such that ΔL equals an odd number of half wavelengths, $\lambda/2$. In this case the two scattered waves arrive at the detector totally out of phase with each other. This is total "destructive" interference, resulting in zero net intensity. Over time, diffusion of the particles will cause the net intensity at the detector to randomly fluctuate, like a typical "noise" signal, between these two extreme values. A representative total intensity signal is shown in Figure 24. The intensity varies between the maximum value and the minimum value (zero) when the optical path length difference changes (i.e. increases or decreases) by $\lambda/2$.

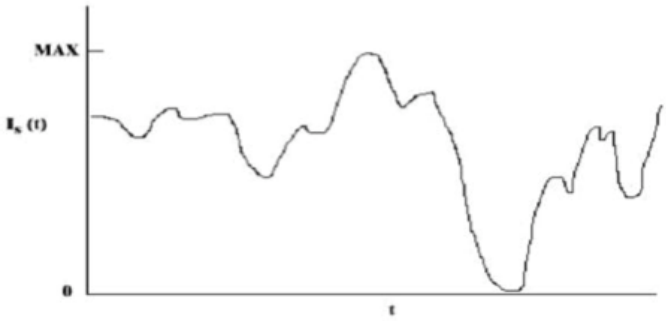


Figure 24 - Typical intensity vs time for two diffusing particles (Nicomp ZLS user manual).

The key physical concept that underlies the DLS particle sizing measurement is the fact that the time scale of the fluctuations shown in Figure 24 depends on the size of the particles. For simplicity it is assumed that the particles are uniform in size, with a single, well-defined diffusion coefficient. Small particles will repel each

other in solution relatively rapidly, resulting in a rapidly fluctuating intensity signal; by contrast, larger ones will diffuse more slowly, resulting in a more slowly varying intensity.
At this point it is simply assumed that the temperature of the particle suspension is held constant. We shall see that the temperature plays an important role on the particle size measure, in determining the diffusivity and, hence, the time scale of the resulting intensity fluctuations. In any real situation of interest, of course, there are many more than two particles in suspension which contribute to the scattered intensity signal. However, the principle of interference remains the same. The resulting signal will be observed to fluctuate average level, which is proportional to the number of particles illuminated/detected volume and their individual scattering power (Equations 1a and 1b). The time scale of the fluctuations depends on the particle diffusivity, and hence on the particle size. This is illustrated in Figures 25 a, b and c for "small", "medium" and "large" size particles (using the same time scale on all three horizontal axes). Again, it must be remembered that the fluctuations in the net scattered intensity are not caused by the addition or subtraction of particles in the illuminated/detected volume. Rather, they are the result of the variations in position of an essentially fixed number of particles within the scattering volume.

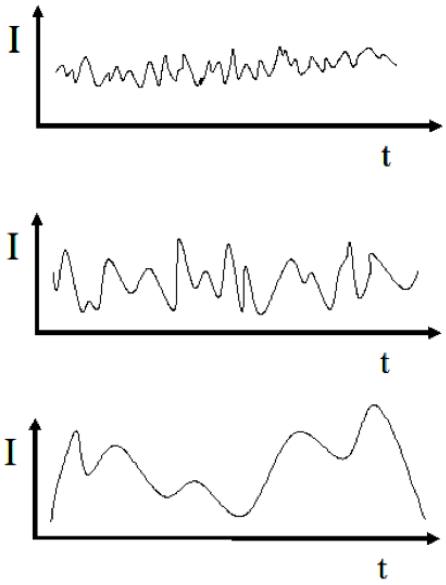


Figure 25 a,b,c - Representative intensity vs time for "small"(a), "medium"(b) and "large"(c) size particles (Nicomp ZLS user manual).

The goal of the DLS technique is to determine the diffusion coefficient D of the

particles (assumed uniform here) from the "raw" data (i.e. the fluctuating light scattering signal). From D we can easily calculate the particle radius R. using the well-known Stokes-Einstein relation,

$$D = kT/6\pi\eta R \quad (2)$$

where k is Boltzmann's constant (1.38 x 10^{-16} erg K^{-1}), T the temperature (°K = °C + 273) and η the shear viscosity of the solvent. Thus, we see that the rate at which the particles jitter about in the suspension, as measured by D, is inversely related to the particle radius R.

From Equation 2 we see that, in general, the diffusion coefficient D of particles of a given size increases with increasing temperature T. This is due primarily to the T-dependence of the solvent viscosity η. Clearly, the less viscous the solvent, the more rapid will be the random-walk diffusion of the particles and the faster the resulting intensity fluctuations. Hence, changes in T are completely indistinguishable from changes in particle radius R. as they affect D. For this reason, the sample temperature must be constant (and accurately known) in order to obtain a meaningful measurement of D and, hence, of R using Equation 2.

Obtaining quantitative information from these kinds of scattering signals is another matter altogether. What helps is the mathematical operation known as autocorrelation.

The essential point about the autocorrelation function is that it serves as a useful probe of the characteristic lifetime, or duration, of the fluctuations in $I_s(t)$. That is, once the interval t' between two sampled intensities exceeds the average width of a major fluctuation in $I_s(t)$, the two sampled intensity values will cease, on average, to be correlated.

For random diffusion of non-interacting particles, the autocorrelation function C(t') of the fluctuating scattered light intensity $I_s(t)$ is an exponentially decaying function of time t', as shown symbolically in Figure 26. This is described by the expression:

$$C(t') = A \exp(-t'/\tau) + B \quad (3)$$

where $A = \langle I_s^2(t) \rangle - \langle I_s(t) \rangle^2$ and $B = \langle I_s(t) \rangle^2$

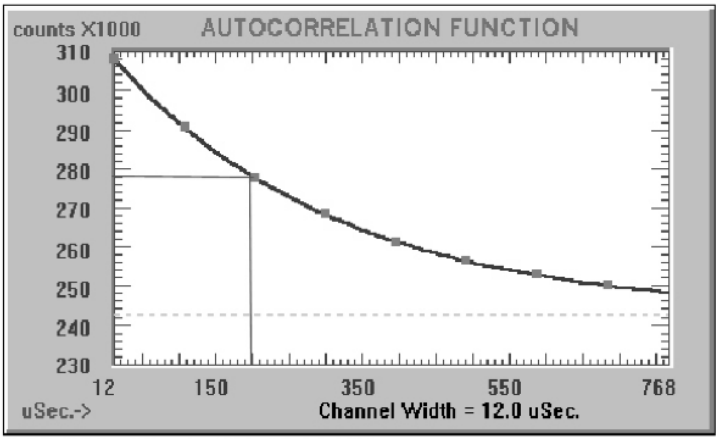


Figure 26 - Autocorrelation function C(t') for diffusion of uniform particles: exponential decay.

Variable τ is the characteristic decay time constant of the exponential function; τ characterizes quantitatively the speed with which the autocorrelation function C(t') decays toward the long-t' limiting value. In effect, the value of τ describes the characteristic lifetime, or duration, of a major fluctuation, in the scattered intensity I_s. Hence, the larger the particles, the slower the diffusivity and resulting fluctuations in I_s' and the longer the decay time constant τ.
It is possible to obtain the diffusion coefficient D of the particles from the decay constant τ; the precise relation is,

$$1/\tau = 2DK^2 \qquad (4a)$$

or

$$D = (1/2K_2)(1/\tau) \qquad (4b)$$

Here, the quantity K is called the "scattering wave vector". It is a constant that depends on the laser wavelength λ in the solvent and on the angle θ at which scattered light is intercepted by the PMT detector (λ= 632,8 nm; $\theta = 90^o$). In effect, K acts as an absolute calibration constant, which relates the time scale of the diffusion process to the distance scale set by the laser wavelength (making interference possible). Constant K is given by

$$K = (4\pi n/\lambda) \sin \theta/2 \qquad (5)$$

where n is the index of refraction of the solvent (e.g. 1.33 for water).
Summarizing, in this way, the scattered light (at a fixed angle) produced by an ensemble of particles suspended in a solvent is detected. The intensity fluctuates in time due to diffusion of the particles; there is a well defined characteristic lifetime

of the fluctuations, which is inversely proportional to the particle diffusivity.
We compute the autocorrelation function of the fluctuating intensity, obtaining a decaying exponential curve in time. From the decay time constant τ, it is possible to obtain the particle diffusivity D. Using the Stokes-Einstein relation (Equation 2), the particle radius R (assuming a sphere) is computed. The radius calculated from the diffusion properties of the particle is only indicative of an apparent size of the dynamic hydrated/solvated particle, hence the terminology hydrodynamic radius. The hydrodynamic radius therefore includes the effects of both the form and hydration.

1.5.1.1. Dynamic Viscosity η and Index of refraction n
Particles in solution can be interpreted as rigid spheres flowing through a liquid whose forces contrast the passage. The opposing forces of the solution can be represented as adjacent plans moving with different speeds in the opposite direction to that of the particle (Figure 27).

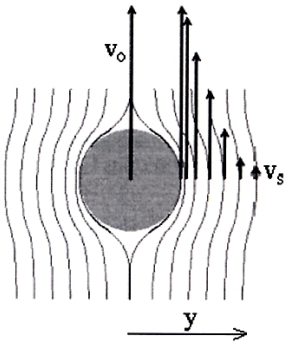


Figure 27 - Representation of the contrasting forces of a solution.

The extent of the creep resistance to these shear forces is evaluated through viscosity.
The force F_s, required to maintain a velocity gradient *dv/dt* between two layers of fluid of area A is given by the espression:

$$\frac{F_s}{A} = \frac{m}{A}\frac{dv}{dt} = \eta\frac{dv}{dy} \qquad (6)$$

where η ($Nm^{-1}s^{-1}$, centipoise:cp) is a constant called coefficient of dynamic viscosity.
As already said, in liquids viscosity decreases with increasing temperature since the cohesive forces between very close molecules decrease. That does not occur for gases, in which the increase of temperature causes the increase of viscosity. In this

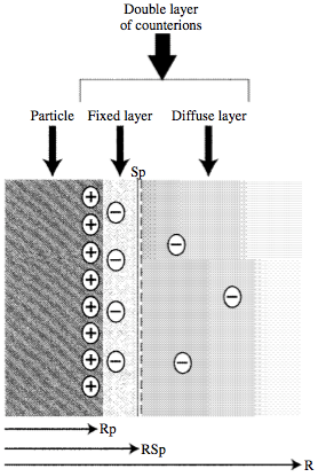
state as the temperature increases, the speed of particles increases, fostering the interchange between the molecules of various plans, leading to an increased viscosity (Cornetti, 1989).
The viscosity is closely related to the type of liquid, to which it refers and its concentration.
The index of refraction is the ratio between the speed of electromagnetic radiation in vacuum and the speed of electromagnetic radiation in a given medium (absolute index of refraction). Since the index of refraction varies with wavelength, it is necessary to specify the wavelength to which it refers, but this is not enough because the index is also correlated with temperature and alcohol concentration.
The relative refractive index n_0 is important when the radiation passes from one medium to another and is equal to the ratio between the speed of electromagnetic radiation in the first medium and the speed of electromagnetic radiation in the second medium.
Even if this technique is not so well known in the enology sector some researches on wine have already been done exploiting the DLS.
As already said, in 2002 Riou *et al.* used the DLS technique to study the capacity of some tannins fractions to form aggregates with a colloidal behavior in a model wine solution and to study the effects of some polysaccharides on this colloidal behavior of tannins. It has been observed that the ability of the polysaccharides of the wine to prevent the aggregation of tannin is not directly related to the density of their global charge, and that only the fractions presenting molecules with relatively high values of Mw are actually involved (particularly mannoproteins).
In addition, the mechanism involved in this "protective effect" is unclear. Two hypotheses can be proposed:

- Molecular association in solution between polysaccharides and polyphenols competes with the aggregation of tannins;
- The absorption of the formed tannins particles by the polysaccharide prevents the growth of the particles themselves.

This result shows that the addition of polysaccharide prevents the growth of tannin particles but not their formation, indicating that the latter hypothesis is the most reliable.
The same technique along with the measure of the streaming potential has been used to evaluate the evolution of size and surface electrical charge of the hydrocolloidal particles in a wine kept at 15 °C for 13 months (Celotti *et al.,* 2003).

1.5.2 Streaming potential

Particles in an aqueous solution are surrounded by ions. The particle/ion configuration is described
by the double-layer model (Figure 28). The charged surface of particles in contact with an aqueous phase are surrounded by a first layer of ions with the opposite charge. This layer, strongly bonded to the particle surface, is known as the fixed layer. This particle-fixed layer system is surrounded by a second layer of counterions, whose mobility increases in direct proportion to the distance from the particle. However, the fixed layer of counterions strongly bonded to the particle only partially compensates for the particle's initial charge. Residual charges are therefore responsible for the difference in potential at the solid/liquid interface (Sp), which decreases as the distance from the solid increases. The zeta potential, or electrochemical potential, ζ (mV), is defined as the potential at the plane that separates the fixed layer from the diffuse layer of counterions, known as the cut plane of the system (Figure 29).



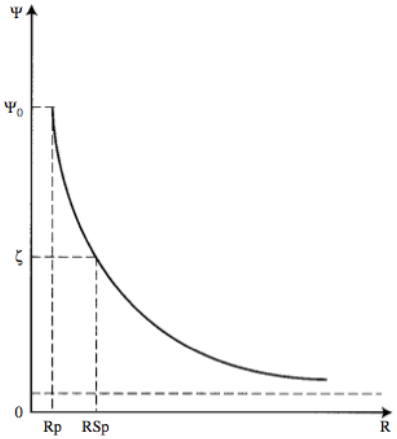


Figure 28 - Diagram of the double layer of a charged particle and the electrostatic phenomena. R = distance from the particle; Rp = radius of the charged particle; RSp = hydrodynamic radius (including the layer of strongly bonded counterions, i.e. the fixed layer); Sp = edge of the cut plane between the fixed and diffuse layers of counterions (Lagune, 1994).

Figure 29 - Changes in electrostatic potential in the vicinity of a double layer. Ψ = potential; Ψ_0 = surface potential; ζ = zeta potential (potential at the cut plane) (Lagune, 1994).

The zeta potential is involved in the interaction and adsorption mechanisms between particles and ions, as well as their coagulation, flocculation and sedimentation behavior.
It can be measured by

Electrophoresis: According to this technique, there is the presence of two phases. The first is a liquid or a gas and the second, dispersed in the first, consists of solid or liquid. By applying an electric field the charged particles are induced to move, while the liquid remains stationary. The speed of this movement offers us the possibility to calculate the zeta potential
Electro-osmosis: Charged particles remain stationary while the liquid phase is in motion for the action of an electric field. A necessary condition is the immobilization of the charged particles. From the speed or the volume of the liquid moved per unit of current applied, it is possible to calculate the zeta potential.
Sedimentation potential: in this case the liquid phase is stationary, while the electrically charged particles are in motion, under the influence of gravity or centrifugal force. Both generate a separation between the two phases and thus a potential change, which is also detectable in a measured electric field and can be related to the zeta potential.
Streaming potential: PE is the potential created between the particle-fixed layer system and the diffuse layer when it moves away from the particle due to an external force. It may be measured using a particle charge detector. The zeta potential depends on the streaming potential PE, but is independent of the conditions in the medium. The zeta potential is generally regarded as a remote effect of surface charge.
Where a measurable potential exists, its value can be used to show whether a positive (cationic) or a negative (anionic) charge is present. The value of the potential depends on different external factors:

- Electrical conductivity of the dispersion;
- Sapmle viscosity;
- Molecular weight, or specifically, particle size;
- Sample cell dimensions;
- Temperature.

Therefore, by measurement of the streaming potential, information is obtained only about the sign of the charge, and not about its quantity. To measure the amount of charge in a sample an electro-chemical titration using a polyelectrolyte is necessary.
PE is measured using a particle charge detector (PCD-O2, Müteck). This consists of a cylindrical polytetrafluoroethylene (PTFE) bath, equipped with two gold electrodes, located at the top and bottom and linked to an amplifier. A PTFE piston mounted in the bath oscillates vertically at a constant frequency, making the liquid flow along the sides of the bath (Figure 30). This apparatus is connected to an automatic titrator used to add polyelectrolyte. When a solution of ionic particles is placed in the detector, the particles are surrounded by a double layer of counterions (Figure 31 (a)). The Van der Waals force is then responsible for adsorption phenomena on the bath and piston surfaces.

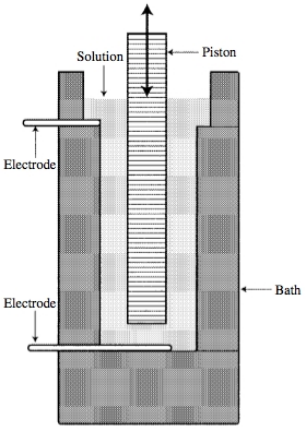


Figure 30 – Particle charge detector measuring system (Lagune, 1994).

A small space separates the cell wall from the piston. The oscillation of the piston (A), 4 Hz, streams the liquid phase along the walls, gathering counterions in the diffuse layer into a cloud that moves away from the particle-fixed layer system. A difference in potential is thus created between the diffuse layer cloud and the particle-fixed layer system. This is known as the 'initial streaming potential' (PEI). It is measured by the two electrodes built into the vat and expressed in mV. It indicates the charge of the particles under investigation.
The addition of ions with the opposite charge (polyelectrolyte) (B) neutralizes the charge and cancels out the PEI potential (Figure 31 (c)). The quantity of polyelectrolyte necessary to neutralize this charge is used to calculate the 'surface charge density', d, expressed in meq of polyelectrolyte g^{-1} or mL^{-1}. This is a characteristic of the system under defined conditions. If the system has a positive charge, the polyelectrolyte is anionic (sodium polyethensulfonate, or PES-Na). If the charge is negative, the polyelectrolyte is cationic (polyallyldimethylammonium chloride, or polyDADMAC). The titration with polyelectrolytes is based on the fact that the complexation for charge neutralization occurs at a 1:1 stoichiometry.

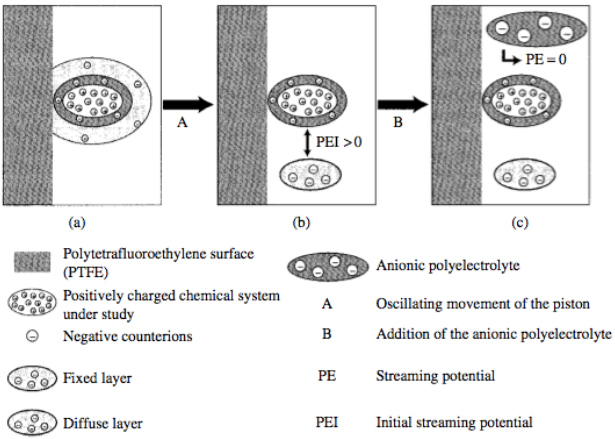


Figure 31 - Mechanisms operating in the solution of a positive species during titration (Lagune, 1994).

The optimum titration agent concentration depends on the charge density in the sample. Where the dilution of a sample with water has no influence on the charge density, a sample concentration which requires a weak concentration for the titrating reagent, since the reproducibility of the polyelectrolyte titration is better at lower concentrations.
The titration is represented by a curve (Figure 32). In the case of a negative system, V_0 (ml) is the volume of polyelectrolyte necessary to obtain PE = 0. This volume is used to calculate the surface charge density of the system, expressed in meq/L or meq/mL. according to the type of system.

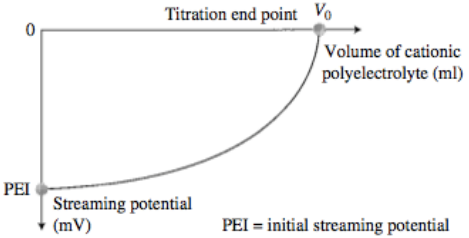


Figure 32 - Determining the surface charge density of a negative species by titration with a cationic polyelec- trolyte (Lagune, 1994).

The streaming potential is not an absolute value and, thus, depends on several parameters. The conductivity of the sample has a large influence on the streaming potential. The higher the conductivity, the lower will be the sample's resistance, therefore the lower the streaming potential.
Up to a conductivity of about 15 $mScm^{-1}$, however, this has no influence on the titration result.

It is also influenced by any parameter that changes the flow of the measuring cylinder, for example the size and shape of particles, the solid content or the viscosity of the dispersion. Other influences derive from the molecular weight distribution, the molecular structure and the charge density of the particles that are measured.
The measure of the streaming potential has already been proposed in some studies in the field of wine for the dosage of fining agents, for classification and selection of oenological adjuvants (Ferrarini *et al.,* 1996, Ferrarini *et al.,* 1998) and for the evaluation of products inhibiting colloidal precipitations (Celotti *et al.,* 1999).
In 1996, Verhnet used the streaming current detector to show how the steaming potential and thus the surface electrical charge of macromolecules present in wine is influenced by the pH.

2. Aim of the research

The endogenous and exogenous colloidal system remarkably influence the stability characteristics and the sensory profile of a beverage. Since the scientific knowledge on the stability and instability conditions of macromolecules involved in wine processing is restricted, it becomes important to deepen the knowledge on molecules and their chemical-physical interactions, as well as on the reactions dependent on the colloidal system of wines and other alcoholic beverages.
Thus, it is clear that the adjuvants management and the mastery of the technological processes in winemaking can not ignore the study of the colloidal system.
From these remarks arises the need to further explore some aspects related to the winemaking process and wine stabilization, particularly the management of adjuvants, to the technological processes and to the interactions between molecules and macromolecules that condition the product quality.
The use of oenological coadjuvants is without doubt one of the critical points in the alcoholic beverages production process, and in fact their use and compositional characteristics are correlated with risks for the stability of the products, the accidental presence of residues and, in general, the risk of not being able to guarantee the conformity of the product to the required specifications.
These risks are due to the inefficiency of the coadjuvant caused by its specific compositional and functional characteristics, an excessive or insufficient quantity used and their inappropriate use.
Thus, it is necessary to intervene both on the creation of a control system of the coadjuvants use (quantity and use instructions) and on the control of the constituent and functional characteristics.
Oenological adjuvants are principally used for their separating effect that leads to a limpid product, a constituent change of the medium and to an improvement of the product stability.
In order to evaluate the adjuvants characteristics, it is possible to refer to binding laws, non-binding reference regulations (O.I.V. Codex Oenologique International) and contractual terms.
These existing laws are essentially aimed at ensuring the quality of the coadjuvant in terms of health and cleanliness, but they do not set the minimum quality standards that have to be guaranteed for their use. Only the Codex Oenologique International identifies some constituent characteristics, partly correlated with the product performance, but the suggested methods are often difficult to standardize and insufficiently exhaustive.
As already pointed out, it should be remembered that in order to assess the actual functional characteristics of coadjuvants, it should be considered that their action takes place mainly with respect to macromolecules in colloidal dispersion, and they themselves have often colloidal behaviour or interact with the colloidal state of the medium.

It appears interesting to support the traditional techniques used for the study of colloidal substances, such as filtration, gel-chromatography and electrophoresis, with new techniques and tools to better assess some of the aspects that the former are not able to investigate.
For this reason some techniques are used to measure the particle size by diffraction of radiation at different wavelengths. For example by using laser light for the determination of the particle size through the dynamic light scattering principle (DLS).
Another very effective tool is the Streaming Current Detector (SCD), which allows to measure the streaming potential and surface electrical charge (SEC), providing useful information for the improvement of the study of the colloidal state. It appears, therefore, as a valid analytical support for the quality control of technological adjuvants.
Consequently, the aim of this research was, therefore, to set a standardized procedure for adjuvants analysis and their quality control. Moreover, to study the interactions and the colloidal phenomena in the wine system in order to optimize the stabilization processes with respect to the sensory quality.

3. Materials and methods

Part 1
Characterization of tannins and technological application in a cellar

3.1 Characterization of tannins

For the first part of this research fifteen different commercial tannin preparations for oenological use of different botanical origin were used (Table 4). They were provided by suppliers Esseco (Novara, Italy), EverIntec (Pramaggiore, Italy) and Ferrari (Verona , Italy) .

Code	Origin	Type
T1 (Chestnut)	Chestnut	Hydrolizable
T2 (Grape 1)	Grape	Condensed
T3 (Grape 2)	Grape seed	Condensed
T4 (Grape 3)	Grape skin	Condensed
T5 (Cherry tree)	Cherry tree	Hydrolizable
T6 (Oak 1)	Oak	Hydrolizable
T7 (Oak 2)	Oak	Hydrolizable
T8 (Oak 3)	Oak	Hydrolizable
T9 (Galla 1)	Galla nut	Hydrolizable
T10 (Galla 2)	Galla nut	Hydrolizable
T11 (Quebracho 1)	Quebracho	Condensed
T12(Quebracho A)	Quebracho	Condensed
T13(Quebracho S)	Quebracho	Condensed
T14(Quebracho E)	Quebracho	Condensed
T15 (Tara)	Tara	Hydrolizable

Table 4 – Tannins used and their origin.

3.1.2 Analysis for the characterization tannins

For experimental purposes, according to various tests, various concentrations of commercial tannins were solubilized in a tartaric alcoholic buffer also known as model wine solution (5 g/L of tartaric acid, dissolved in 12% ethanol and buffered to pH 3.5 with NaOH).

The characterization of tannins was made using the analytical methods described below.

Analysis of UV-VIS spectra

The UV-VIS spectra and other spectrophotometric determinations were carried out using spectrophotometer Jasco V-530 (Eston, Maryland, USA) except for the analysis of chain-breaking activity performed with spectrophotometer Shimadzu model UV-2501PC (Kyoto, Japan).

Tannins were analyzed at a concentration of 1 g/L in model wine.

In the case of VIS, spectra of the absorbance in the range of 350 nm and 700 nm were assessed. The measure was performed in a cuvette with optical path length of 10 mm, against distilled water .

As for the UV spectra the absorbance in the range between 350 nm and 210 nm was considered, and the sample solution was diluted 1:50 with water. Like for VIS spectra the measure was performed in a quartz cuvette with optical path length of 10 mm, against distilled water.

Colorimetric analysis

For the colorimetric analysis a Minolta colorimeter model CR-200 (Osaka, Japan) was used. The analysis was performed directly on the tannin powders using the CIELab system (Piracci and Spera, 1986). The system involves the use of three parameters: L *, b * a *. L * indicates lightness. its value may vary between the value 0, in the case of an opaque pattern and 100 in the case of a sample perfectly transparent.

While a * and b * are the chromaticity coordinates. The coordinate a * is a measure of a greater or lesser tendency to red or green colour. If a * is greater than zero prevails the red component, if it is less than zero prevails the green. The coordinate b * is a measure of a greater or lesser tendency to yellow or blue colour. If b * is greater than zero prevails the yellow component, if it is less than zero prevails the blue. The three CIELab coordinates together form a three dimensional colour space where each point represents a different colour. The coordinates L *, a * and b * are the three coordinate axes of the spaces. For this analysis the illuminant C was used. It represents the spectral distribution of daylight with a colour temperature of 6774 K.

Particle size

To measure the size of the colloidal particles the Nicomp^{TM} 380/ZLS Particle sizing/Zeta Potential system has been used (Santa Barbara, California, USA) (Figure 33), with a monochromatic He-Ne laser at a wavelength of 632.8 nm, 0.5 mW of power, able to measure hydrodynamic diameters from 5 to 5000 nm at intervals of correlation of 10 μs. Parameters suchs as viscosity, index of refraction etc were set before the measurements (Figure 34).

The diffusion method is dynamic with a single-detector and the angle of detection of the scattered light is 90 °C (RALLS=Right Angle Light Laser Scattering) (Figure 35).

Figure 33 - Nicomp™ 380/ZLS Particle sizing/Zeta Potential system.

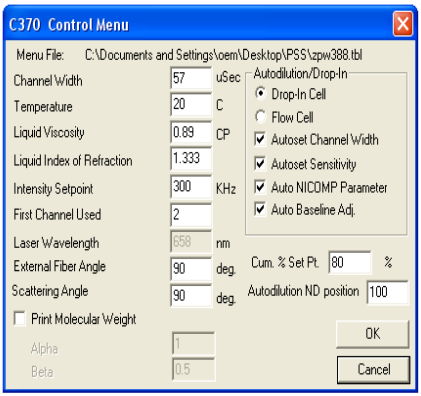


Figure 34 – Parameters set on the instrument.

The machine performs a single reading in a time interval (5 to 10 minutes) automatically required according to the criteria connected to selected and statistical parameters, automatically set. In case the optimal working frequency is not of 300 kHz or the minimum diameter is less than the measurable of 10 nm, the detection time increases to allow the analysis and a more reliable and comparable statistical distribution that is expressed with the response of the graphical distribution of mean diameter.

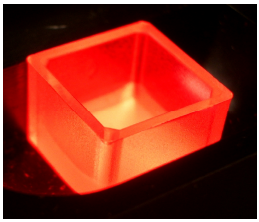


Figure 35– Light scattering of the particles of the solution in the cuvette during the measurement

It is necessary to highlight that most samples of practical interest differ appreciably from the uniform, "monodisperse" solutions (same diameter, shape and mass). Real samples usually contain a range of sizes, often of substantial width, and are so-called "polydisperse" solutions. Such a particle size distribution might be conceptually simple, consisting of a smooth, single-peak ("unimodal") population of well defined mean diameter and width. Or, the distribution might be qualitatively more complex, resembling two discrete peaks (a "bimodal" distribution), or an even more complicated shape.
Two different mathematical procedures have been developed to analyze the autocorrelation of specific parameters, which depend on the nature of the underlying particle size distribution. The management program of the instrument provides, through the graphical interface, two statistical interpretations of data, a standard Gaussian and the other named Nicomp-distribution. The software automatically selects the more appropriate of the two analysis procedures and provides the user with a running measure of the accuracy, of the computed distribution resulting from the particular analysis chosen (Figures 36, 37). The Gaussian distribution provides the detection of a single peak represented by the graph statistics. The statistical distribution process Nicomp can instead detect more peaks, corresponding to different size distributions, with a maximum of three.

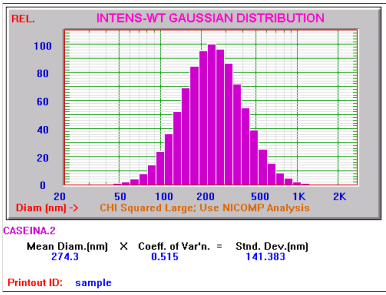


Figure 36 – Gaussian distribution example.

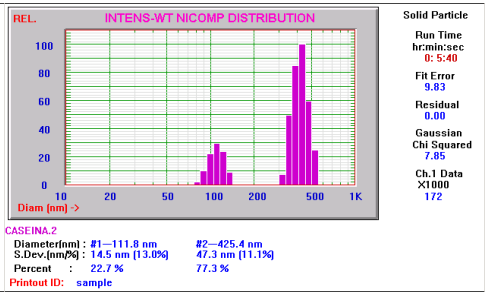


Figure 37 – Nicomp distribution example.

The dynamic diffusion of laser light technique provides the diameter data as a function of:
- Intensity;
- Number of particles with the same dimension;
- Volume of particles;

whatever the technique chosen, the data are provided taking into account the percentage of mass of particles. The procedure that was considered for this work involves the correlation of the data provided only in terms of diameter as a function of signal intensity.
The DLS technique has several advantages over other technologies based on laser light scattering. Firstly, it is an absolute measurement, so the knowledge of the

composition of the particles is not necessary. This can be useful if the optical properties of suspended particles are not known or if the suspension is composed of particles with different optical properties. Secondly, the DLS can measure particles down to 10 nm, which is ideal for providing measurements of nano particles.

Measure of Surface Electrical Charge (SEC)
The surface electrical charge measure has already been described in Paragraph 1.5.2. The titration of the samples was performed by coupling the instrument *Particle Charge Detector* (Mütek PCD-02, Mütek-GmbH, Kirschau, Germany) with an automatic titrator MICRO TT 2050 (Crison, Barcelona, Spain) The polyelectrolyte titrants used were in concentrations of 0.001 N.

Measure of turbidity
The turbidity measurements were conducted using a turbidimeter model TN-100 (Eutech Instruments-Thermo Fisher Scientific, Waltham, USA), which measures the clarity of a liquid in NTU (nephelometric turbidity units).
For the turbidimetric measurement the tannins powders were dissolved in model wine solution (tartaric alcoholic buffer) at a concentration of 1 g/L. This parameter is obtained thanks to an optical phenomenon caused by the presence of suspended particles that deflect the light from its normal trajectory, it is known as Tyndall effect. The measurement of clarness, therefore, in relation to the assessment of turbidity, depends on the number and size of particles in suspension (Ribéreau-Gayon, 2007).

Determination of antioxidant activity (chain-breaking activity)
For this analysis was used the method proposed by Brand-Williams *et al.* (1995), which is based on the discoloration of a relatively stable radical, the DPPH (2,2-diphenyl-1-picrylhydrazil radical), which occurs if in the medium there is a compound that acts as a radical scavenger.
A volume of 3 mL of a solution $6.1x10^{-5}$ M of DPPH dissolved in methanol was used for each sample. The reaction began with the addition of 10 µL of sample. The antioxidant capacity of the medium was evaluated considering the kinetics of decrease in absorbance at 515 nm of the reagent (3 mL), considering the negligible rate added to the same sample (10µL), at a temperature of 25 ° C for 20 minutes . The measure was performed with a 10 mm optic path length cuvette.

Determination of filterability index (IF)
The filterability index measurements were conducted using the instrument for the QFT - QUALITY FILTRATION TEST ® (Vason, Verona, Italy) based on what proposed by Descout *et al.* (1976) and Gaillard (1976). It is capable of automatically measure and calculate the filterability index (IF) that represents the actual fouling due to suspended material, the index of Modified Filterability (IMF) , that represents the fouling due to the material in solution (colloidal charge) and

Maximum Filterable Volume (Vmax) that represents the milliliters of filtered product with the membrane used. The optimal values for the first two indices are 0, best case, and 10, while the third index optimum is for values higher than 3000. For the tests a membrane of cellulose ester of 0.65 μm was used. The indices are calculated with the following formula:

$$IF = T2 - 2T1$$
$$IMF = (T3 - T1) - 2(T2 - T1)$$

where T1 is the time after the filtration of 200 mL of the solution, T2 is the time after the filtration of 400 mL of the solution and T3 is the time after the filtration of 600 mL of the solution

Phenolic Oxidizable Materials - test (POM-test)
This is a rapid browning test, proposed by Muller-Späth H. (1992). The sample was prepared by dissolving the tannin in white wine at a concentration of 100 mg/L. The sample was then placed in 2 tubes of 10 ml each: one of them was added with 50 μL of 3% H_2O_2 and both were held at 60 °C for 1 hour. After cooling, the absorbance was read at 420 nm with water as blank in a 10 mm optical path cuvette. The browning of polyphenols in this oxidative medium was calculated as increase percentage of absorbance.

$$\%\ \text{Incease} = \frac{\text{O.D.420}\ (H_2O_2) - \text{O.D.420}\ (H_2O)}{\text{O.D.420}\ (H_2O)} \times 100$$

Anthocyanins oxidizability index (A.O.I.)
Method proposed by Comuzzo (1998). This index is an adaptation for the red wines of the "POM-test" developed by Muller-Späth (1992) for whites.
Tests were conducted at three different concentrations of tannin. The sample was prepared by dissolving the tannin in red wine, respectively, at a concentration of 100-500-1000 mg/L.
Also in this case samples were placed in two tubes of 10 ml each. In one of the two tubes 50 μL of 3% H_2O_2 were added. Samples were held at 65 °C for 1 hour. After cooling, the absorbance at 520 nm with water as blank (1 mm optical path cuvette) for both tests was measured.

$$\text{A.O.I.} = \frac{\text{O.D.520} - \text{O.D.520}(H_2O_2)}{\text{O.D.520}} \times 100$$

The decrease in optical density at 520 nm by the addition of H_2O_2 was mainly due to oxidation of the anthocyanins.

3.2 Technological trials in cellar

3.2.1 Grapes used

For technological trials in winery the grape Corvina Veronese variety grown at the farm's vineyards Sabaini Adolfo Illasi (VR) and harvested in the months of September-October in the year 2009 was used. This grape was grown in plots located in the plain and partly on a hill, in a region within the municipalities of Illasi (VR), San Martino Buon Albergo (VR) and Tregnago (VR).
The grapes were monitored regularly for the choice of the optimal harvesting time. The Corvina Veronese is the main variety used for the production of Valpolicella and Bardolino wines. Unlike other varieties, both domestic and international, Corvina is one of those whose the extractable anthocyanins average content is lower.
Wines of more or less intense ruby red, with a sour taste, slightly tannic, medium-bodied and quite durable are produced using this variety.

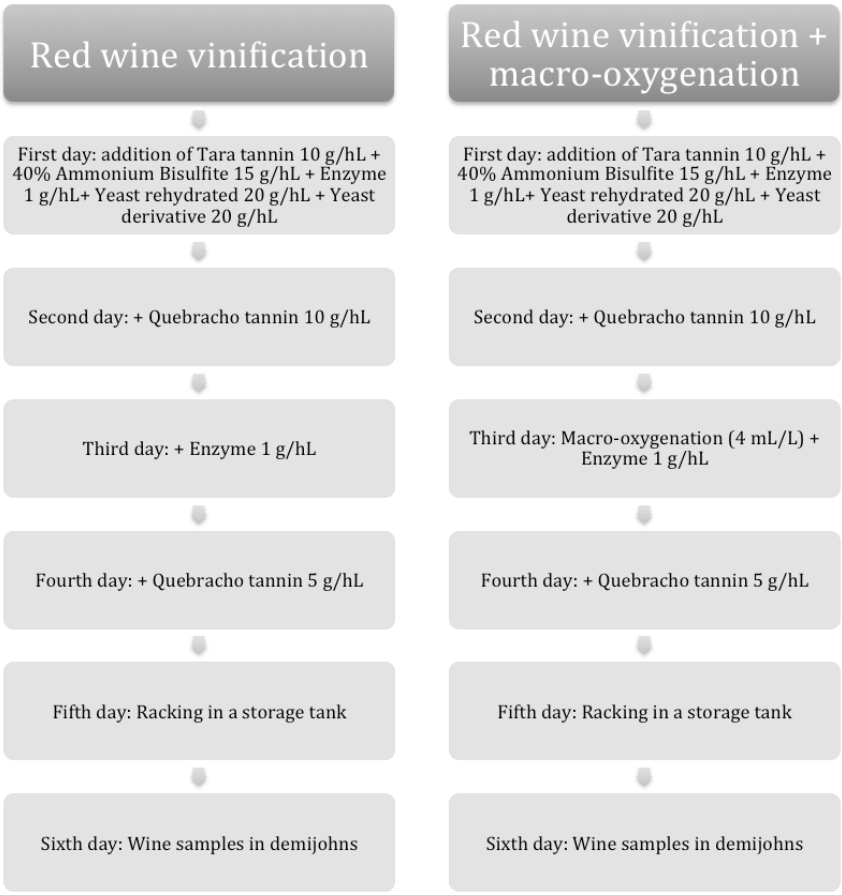
3.2.2 Experimental vinification

The technological tests were conducted in the cellar of the Sabaini Adolfo factory farm Illasi (VR) during the months of September-October-November 2009. Thirteen vinification (Figure 38) tests were carried out (300 quintals of grapes each). Among the fifteen previously investigated, four tannins were chosen to be used during maceration, as the only ones whose informations about the type of solvent used for the extraction were known. Specifically "Quebracho W" indicates that the solvent used was water, "Quebracho E" refers to ethanol as a solvent and "Quebracho S " to diethyl ether and “Tara” a tannin derived from tara.
Depending on the tests, as well as the addition of these tannins, on some of the samplex was also applied the macro-oxygenation. The control sample was the one with no addition of tannin and without macro-oxygenation (Table 5).

Test	Addition	Code
1 (Control)		Control
2	Qebracho S	QS
3	Qebracho E	QE
4	Qebracho W	QW
5	Qebracho S + O^2	QS+O
6	Qebracho E + O^2	QE+O
7	Qebracho W + O^2	QW+O
8	Qebracho S + Tara	QS+T
9	Qebracho E +Tara	QE+T
10	Qebracho W+ Tara	QW+T
11	Qebracho S + Tara + O^2	QS+T+O
12	Qebracho E + Tara + O^2	QE+T+O
13	Qebracho W + Tara + O^2	QW+T+O

Table 5 – Treatments and codes for red wine trials.

Figure 38 - Phases of red wine vinification:



The wine in demijohns was subsequently racked and 3 g/hL of potassium metabisulfite were added. The wine was then bottled in order to perform analytical measurements.

3.2.3 Analysis on bottled wines

Anthocyanins content
The method was proposed by Ribéreau-Gayon and Stonestreet, (1965).
It represents free anthocyanins and the anthocyanins combined with tannins that can be bleached by SO_2. Consists of a SO_2 bleaching procedure that requires the preparation of two samples, each containing 1 mL of wine, 1 ml of EtOH 0.1%HCl and 20 mL of HCl at 2% (pH 0.8). For the two samples, 4 mL of H_2O is added to 10 mL of the first sample, 4 mL of 20% sodium bisulfite solution is added to 10 mL of the second sample and the mixture is diluted by half. The difference, Δ, in OD at 520 nm is measured on a 10 mm optical path.

$$C\ (mg/L) = \Delta \times 875$$

where 875 is a value used as a constant in this analysis method. It derives from the analysis of a reference anthocyanins solution (Glories, 1978).

Optical Densities – O.D. 280, 320, 420, 520, 620 nm
O.D. 280 nm is an index related to the total polyphenols content. Red wine is diluted 1/100 with distilled water. The O.D. is measured at 280 nm on a 10 mm optical path

O.D. 280 nm = Abs 280 nm * dilution

The value is between 6 and 120.
Measuring absorption at 280 nm has some advantages like speed and reproducibility. However, certain molecules, such as cinnamic acids and chalcones, have no absorption maximum at this wavelength.
O.D. 320 nm indicates the hydroxycinnamoyltartaric acids content and like for the O.D. 280 it is measured after dilution of the samples evaluating the absorption at 320 nm on a 10 mm optical path cuvette

O.D. 320 nm = Abs 320 nm * dilution

O.D. 420 nm is the index for the yellow colour of wines, in red wines it is related to the presence of tannins and anthocyanins in the form of chalcone, at the wine pH.
O.D. 520 nm indicates the red colour of the wines, related to the presence of anthocyanins in the form of flavylium cation.
O.D. 620 nm is an chromatic index on the blue colour due to the fraction of anthocyanins present in the form of an anhydrous basis at the pH of wine.
For the measure of these optical densities the red wine can not be diluted, considering that the dilution would change the pH values of wine and then the

colour.
Consequently, spectrophotometric measurements must be made on a 1 mm optical path cuvette, using undiluted wine. The use of a 10 mm optical path cuvette would give values of optical densities that would be higher than the capacity of the instrument.

O.D. 420 nm = Abs 420 nm * 10
O.D. 520 nm = Abs 520 nm * 10
O.D. 620 nm = Abs 620 nm * 10

Colour Intensity and Hue
Colour intensity and hue, as defined by Sudraud (1958), only take into account the contributions of red and yellow to overall colour.
The current approach to colour analysis in winemaking requires optical density measurements at 420 (yellow component) and 520 nm (red component), with an additional measurement at 620 nm to include the blue component in young red wines (Glories, 1984).
Colour intensity (CI) represents the amount of colour. It varies from one wine and grape variety to another:

$$CI = O.D.\ 420\ nm + O.D.\ 520\ nm + O.D.\ 620\ nm$$

The hue (H) is a definition colour parameter of a wine that is subject to change during its evolution, so it gives information about the evolution degree of the colouring component. It is influenced by various factors such as intensity, the maturation of the grapes and the wine aging. The hue of the colour of a wine depends on the type of pigments present in wine, and varies according to its acidity, the oxidation state of the pigments and the evolutionary state of the wine.Young wines have a value on the order of 0.5–0.7 which increases throughout aging, reaching an upper limit around 1.2–1.3.

$$H = \frac{O.D.\ 420\ nm}{O.D.\ 520\ nm}$$

Catechin content determination
The evaluation of flavanols in wine is carried out according to a method proposed by Di Stefano *et al.* (1989), using of 4-(dimethylamino)-cinnamaldehyde (DAC).
To 1 mL sample diluted 1:25, there shall be added 5 ml of DAC solution (prepared with 1 g of DAC, 750 ml of methanol and 250 ml of HCl 37%), and then the maximum value of absorption at 640 nm against a blank prepared by substituting the sample with 1 mL 10% ethanol is measured. Catechins are expressed in mg/L, in reference to a calibration curve obtained with (+)-catechin in ethanol 10% (or multiplying by 30 and the number of dilutions).

Determination of polymerized pigments index

Method proposed by Glories, 1978. Represents an index of the contribution to the red colour of condensed tannins and polymerized forms of anthocyanins insensitive to bleaching by sulfur dioxide. To 5 mL of sample shall be added, in a beaker, 45 mL of tartaric buffer pH 3.2 (5 g/L of tartaric acid brought to pH 3.2 with 4N NaOH) and 0.2 mL of 20% solution of potassium metabisulfite; in a second beaker, 5 mL sample and 45 mL of tartaric buffer are added with 0.2 mL of water. After 5 minutes the optical density is read, using deionized water as blank, at 420 nm and 520 nm for both tests.

$$\text{Polymerized pigments index} = \frac{\text{O.D. 420 nm } (SO_2) + \text{O.D. 520 nm } (SO_2)}{\text{O.D. 420 nm } (H_2O) + \text{O.D. 520 nm } (H_2O)} \times 100$$

Ethanol index

Method proposed by Glories, 1978. 1 mL of sample is added, in a beaker, to 9 mL of 96% ethanol and 90 mL of distilled water, the O.D. 280 nm against water (d0) is measured. In a second beaker, 5 mL of sample are added to 45 mL of 96% ethanol. After 24 hours samples are subjected to centrifugation (3000g x 10') and after diluting the supernatant 10 times, the absorption at 280 nm against water (d24) is measured.

$$\text{Ethanol index} = \frac{d0 - d24}{d0} \text{ x } 100$$

This value indicates the fraction of tannins bound to polysaccharides and salts and thus present in the colloidal fraction of the wine. For young wines it is around 10-20% while for the old ones is 50%.

Gelatin index

This parameter is indicative of the astringent properties of tannin. It is based on the properties that have the so-called astringent tannins to combine in a stable manner with gelatin and to precipitate. This index is a modification of the one that was originally developed by Glories (1978). The affinity to gelatin is visible from the decrease in absorbance at 280 nm, observable after the reaction course.

To 10 mL of sample 1 mL of water is added and the O.D. at 280 nm against water is measured, after dilution 1:50 (d0). Simultaneously to other 10 mL of solution into a test tube 1 mL of gelatin solution is added, nitrogen is insufflated in the head space, and, once plugged, the test tube is placed at 10 ° C for 72 hours. After this period, the sample is centrifuged and the O.D. at 280 nm of the supernatant is measured, against water, after dilution 1:50 (d72).

$$\text{Gelatin Index} = \frac{d0 - d72}{d0} \times 100$$

A high value, above 60, indicates the presence of highly reactive tannins that may be responsible for toughness, or even astringency. A low value, below 35–40, indicates a lack of body and may be the reason for an impression of bitterness.

HCl index
This index is based on the instability of procyanidins in a concentrated HCl medium, where the precipitation speed depends on the degree of polymerization (Glories, 1978). The procedure required a sample consisting of 10 ml of wine, 15 ml of HCl (12 N) and 5 ml of water. After dilution to 1/30, the optical density (d0) at 280 nm was measured immediately on a 10 mm optical path. The same measurement was made after waiting 7 h and 24 h and centrifuging the mixture. Two new values (d1 and d2) were obtained.
The HCl index is given by the equations:

$$I_7(\mathrm{HCl}) = (d0 - d1)/d0 \times 100$$

$$I_{24}(\mathrm{HCl}) = (d0 - d2)/d0 \times 100$$

The values range is between 5 and 50. At values above 35–40, the tannins in wine precipitate, thus decreasing the value. At the beginning of barrel aging, very light wine has a low value, between 5 and 10. A wine suitable for aging has a value of 10–25 and a wine with a high concentration of highly polymerized phenolic compounds has a value >25.
The HCl index, reflects the state of polymerization of tannins in the wine, which, in turn, depends on the aging conditions.

A.O.I.
See paragraph 3.1.2.

Determination of total tannins content
The method, developed by Weinges and Nader (1982), is based on the properties of monomers and condensed 3,4-flavan-diols to oxidaze and give coloured anthocyanidins in an acid-alcoholic medium with the presence of hot temperatures. 2 mL of a sample diluted 50 times were added to a tube containing 6 mL of acid butyl alcohol (1 L of the solution was prepared by dissolving 150 mg of $Fe_2(SO_4)$ in 500 mL of n-butyl alcohol and 500 ml of HCl 37%) (tube A).
Half of the solution was poured into a Pyrex glass tube with a screw cap (tube B) and placed in a water bath at 100 °C for 30 minutes. After cooling the solution in tube B, the optical density at 550 nm for both solutions were measured, against water.

$$\text{Total tannins (g/L)} = (\text{O.D.B} - \text{O.D.A}) \times 0.1736 \times 50$$

Refrigeration test

Wine was placed in test tubes and left for a night at a temperature of -4 ° C controlling the possible formation of crystals of bitartrate (Sudario, 1975).

Statistical analysis

The correspondnce analysis between the treatments and the variables analyzed and the correlations between all the data obtained were performed using the form basic STATISTICA/W, version 7.0.

For chapter 6.4 means and standard deviations of all the data obtained were calculated, the analysis of variance (ANOVA) was performed, and significant differences (measured by $p < 0.05$) were determined by the 'Honest Significant Difference Test (HSD test) Tukey, using the form basic STATISTICA/W, version 7.0.

4. Materials and Methods

Part 2 . Interactions between tannins and polysaccharides

4.1 Materials and experimental plan

The experimental plan involved the assessment of the interactions between polysaccharides and tannins in wine and model wine solution (12% alcohol, 5 g/L tartaric acid, buffered at pH 3.5 with NaOH). It was structuredd as follows (Figures 39, 40, 41):

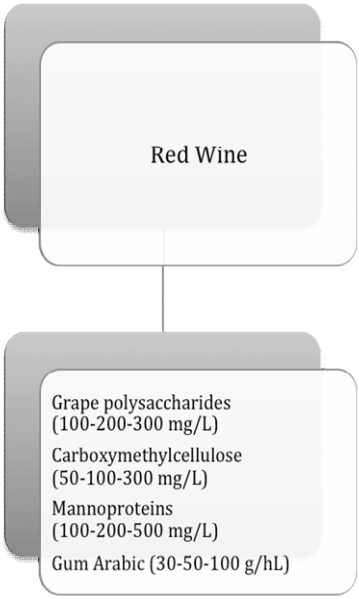


Figure 39 – Tests with red wine.

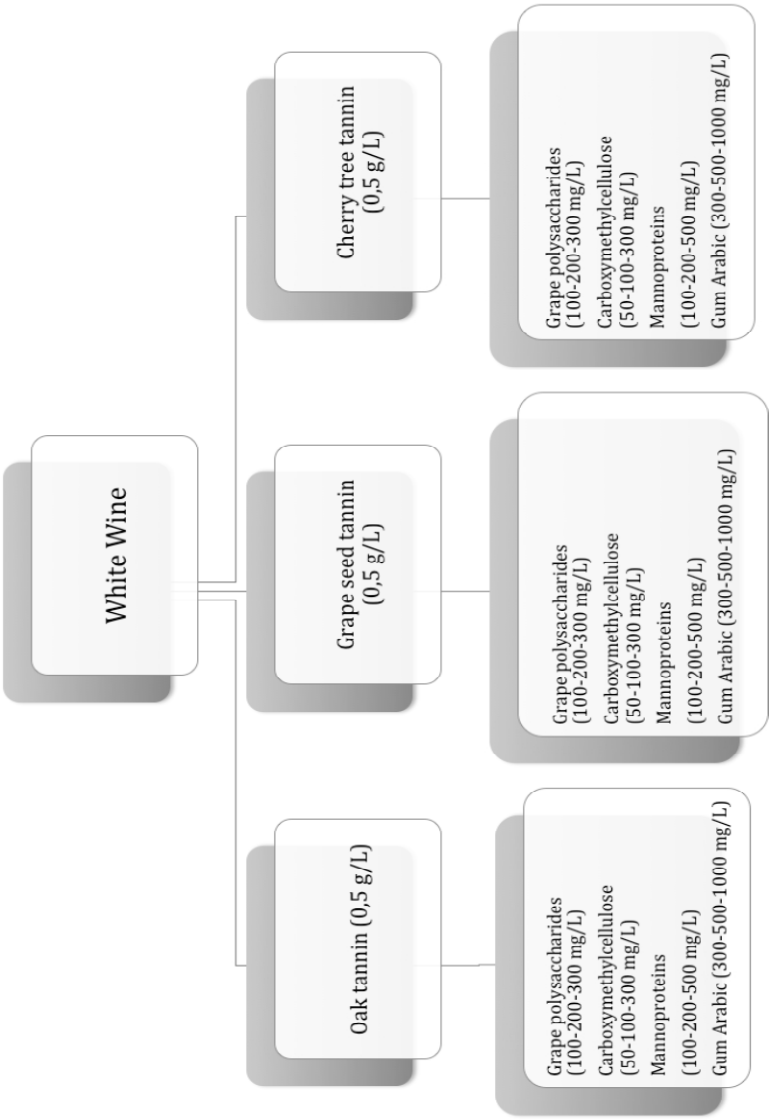


Figure 40 – Tests with white wine.

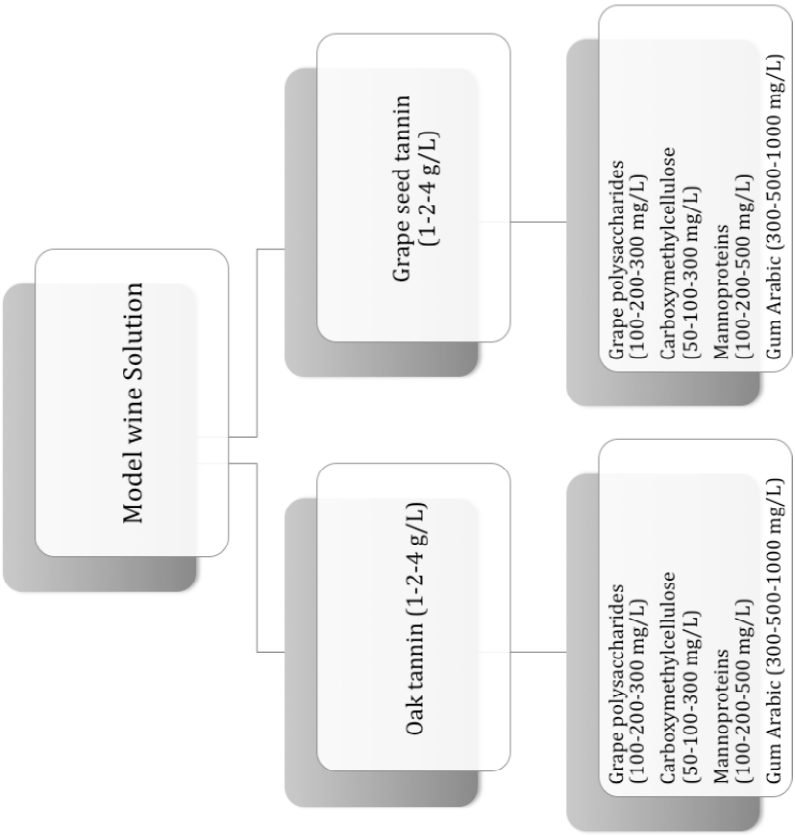


Figure 41 – Tests with model wine.

For this research it was chosen to use commercial tannins of different origins: oak tannin (1400 Da MW) extracted with ether and alcohol; grape seed tannin (1000 Da) extracted with water and cherry tree tannin.
Four different types of polysaccharides were used: mannoproteins, with a MW of about 17 kDa; grape polysaccharides (100-250 kDa); carboxymethylcellulose DP 150, DS 0,9 and MW 400 kDa and gum arabic (1000 kDa).
These polysaccharides were all commercial and experimental products not yet marketed and not purified, provided by Ever Intec and Enartis companies.
Besides the model wine solution, for this work it was used also a white wine (Friulano provided by Casarsa winery) with a pH 3,3, a total acidity of 5 g/L of tartaric acid, 50 ppm of total SO_2 and 12% vol of alcohol; and a red wine (Refosco provided by Paladin winery) with the same acidity of the white wine, pH 3,5, 12% vol of alcohol, total polyphenols (Abs 280 nm) 47, total tannins 4 g/L, IC 8 and free anthocyans 450 mg/L. It was chosen not to add tannins to the red wine as the matrix already had a high content of these compounds.
Red wines thus obtained were monitored for a period of 45 days (especially at the time 0, 7, 15, 30, 45 days) performing analyses such as the evaluation of: turbidity, colour intensity (Glories, 1984), colour hue (Sudraud, 1958), O.D. 280-320-420-520-620 nm, anthocyanins (Ribéreau-Gayon and Stonestreet, 1998), polymerized pigments index (Glories, 1978), A.O.I. (Comuzzo, 1998) and catechins (Di Stefano *et al.,* 1989), complemented with measures of particle size and surface electrical charge (SEC). During the monitoring period all samples were storaged at 20°C in a temperature controlled room.
All measurements of hydrocolloidal diameter (particle size) were made with laser source at 632.8 nm and using a detection angle of 90°. The diameter was measured with a variable angle only when necessary. The measurements were carried out at 25 °C setting the liquid viscosity at 0,89 cP and the liquid index of refraction at 1,333.
White wines were monitored for a period of 45 days performing analysis such as the evaluation of: turbidity, O.D. 280, 320, 420 nm, POM-test, SEC and particle size.
For model wine solutions the parameters considered were particle size, surface electrical charge, turbidity and total polyphenols (Abs 280 nm).
Since this is a new methodological approach, it was decided not to perform repetitions, but to perform a screening for different types of tannins and polysaccharides, to evaluate possible differences due to the different nature and origin of the compounds used.
In the last part of this research it was decided to perform three repetitions for the model wine solution added with oak tannin in all the three concentrations (1-2-4 g/L).

5 Results and discussion Part 1

5.1 Tannins characterizations

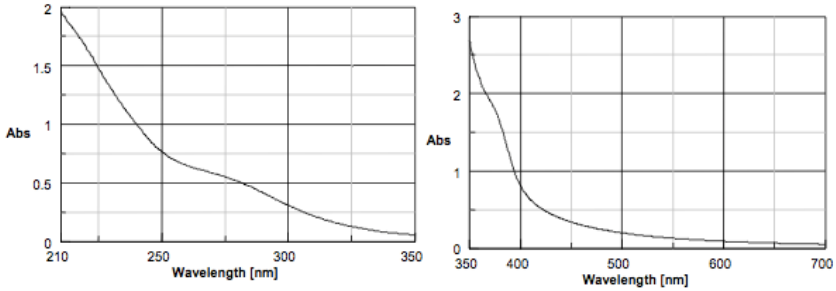
Analysis of UV-VIS spectra

The characterization of the tannins tested has allowed for several observations regarding the distinction between preparations of hydrolyzable and condensed nature. Interesting information can be obtained by UV-VIS spectra (Figure 42). In fact, it is possible to see how the condensed tannin-based formulations (grape, quebracho) exhibited higher absorption than hydrolysable tannins in the visible (chestnut, cherry, galla, oak and tara), whose spectra seemed to have a maximum peak pronounced in the UV. Only the tannin extracted from chestnut showed a slightly different behavior, given the low absorption also in the UV spectra.

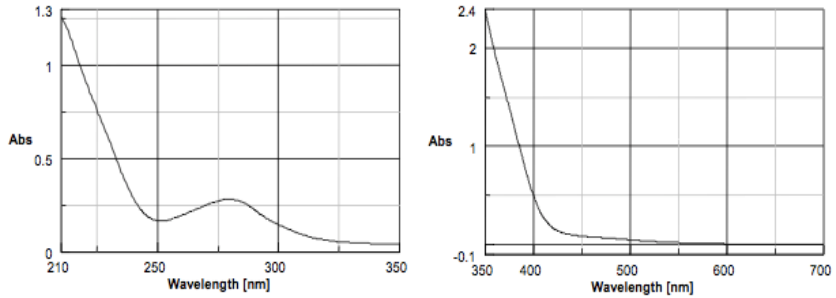
The curves confirmed what was reported by Chauvet *et al.,* (1992), finding that the UV spectra of tannins extracted from exotic woods such as quebracho and tara have a maximum between 270 and 280 nm. The grape tannins (Grapes1, Grapes2 and Grapes3) are characterized by two peaks, the first at 280 nm, the second at 450 nm, while the tannin of galla (Galla1 and Galla2) differs in having two maxima in the UV, at 220 nm and 270 nm respectively.

The UV-VIS spectra can also be replaced by simple absorbance measures at different wavelengths (250, 280, 420, 520, 620 nm), by determining the colour intensity or even by simple colorimetric measures (CIELab parameters).

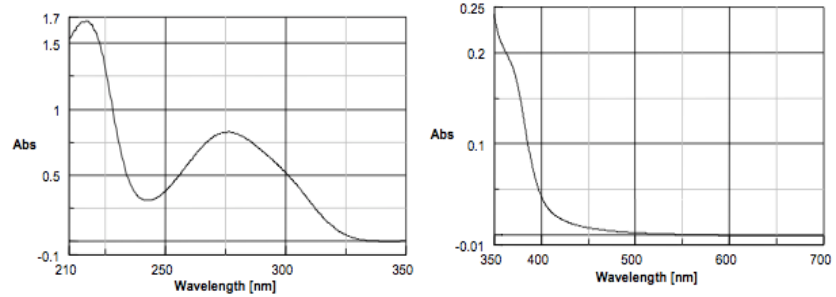
Chestnut



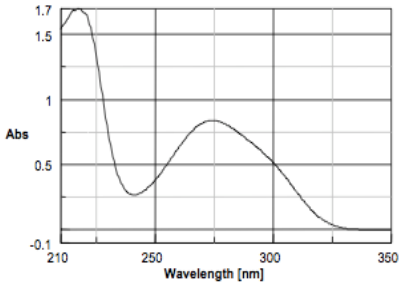
Cherry

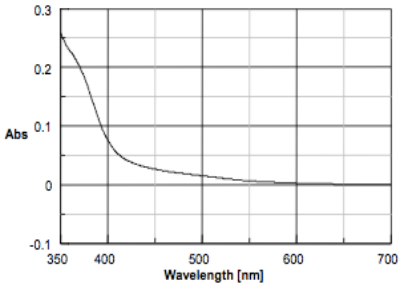


Galla1

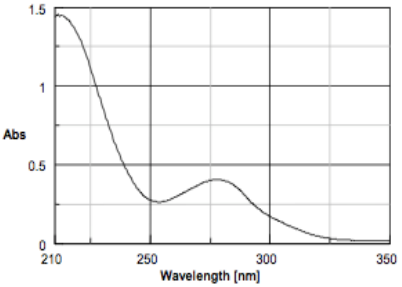


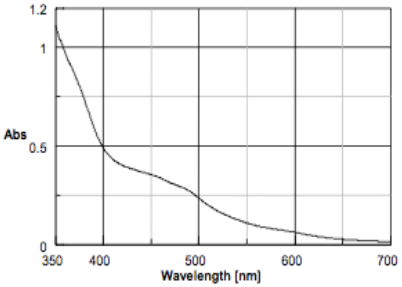
Galla2



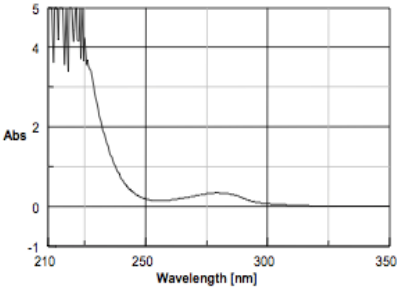


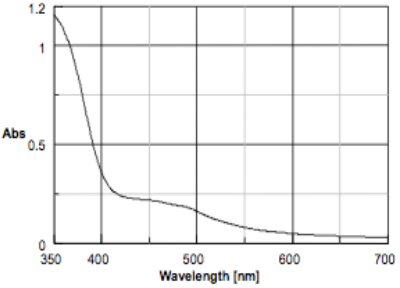
Quebracho 1



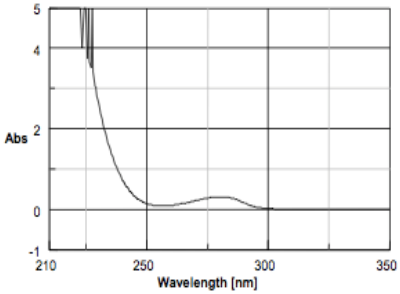


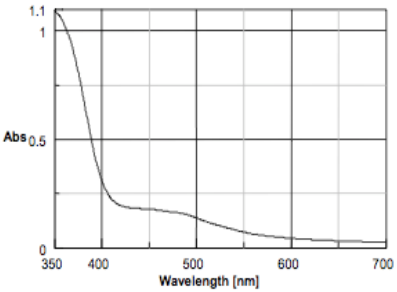
Quebracho W



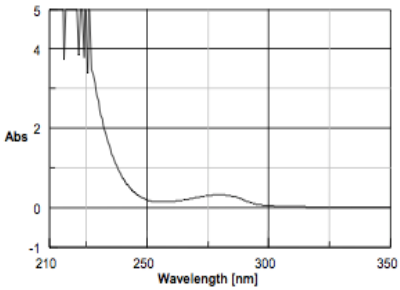


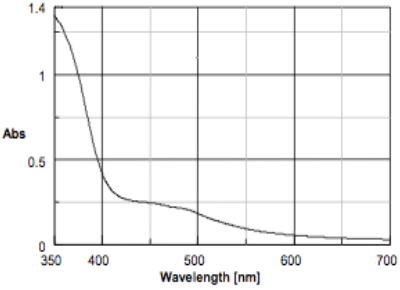
Quebracho E



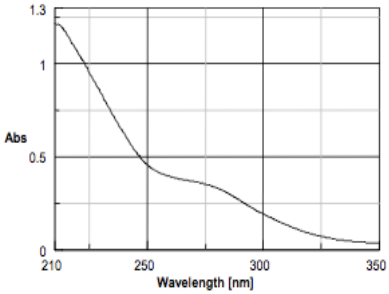


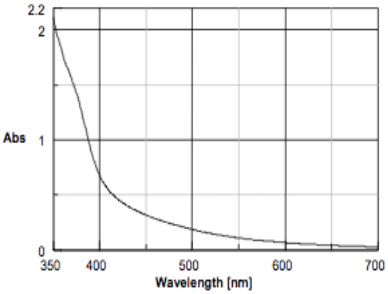
Quebracho S



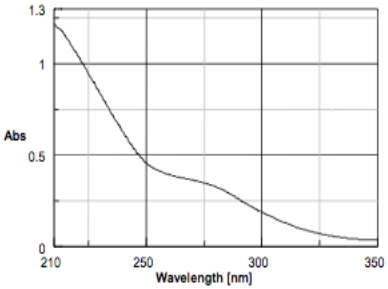


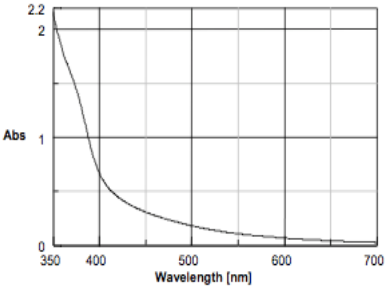
Oak 1



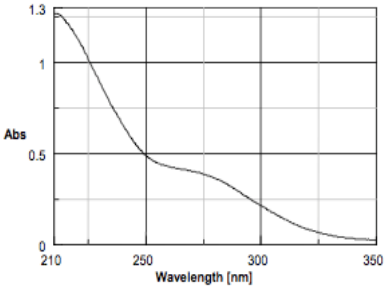


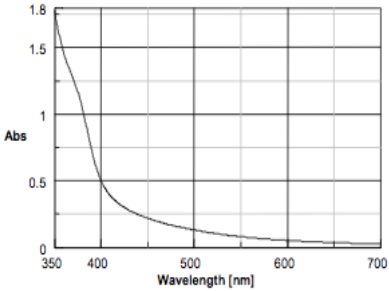
Oak 2



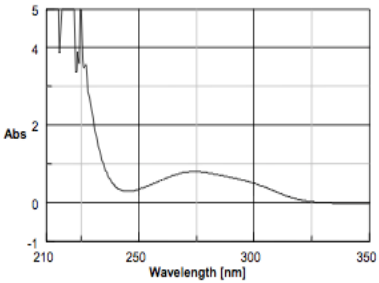


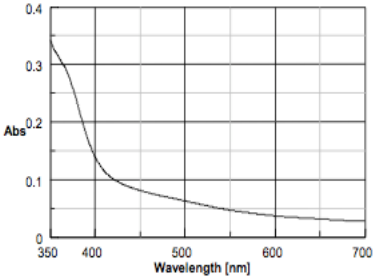
Oak 3



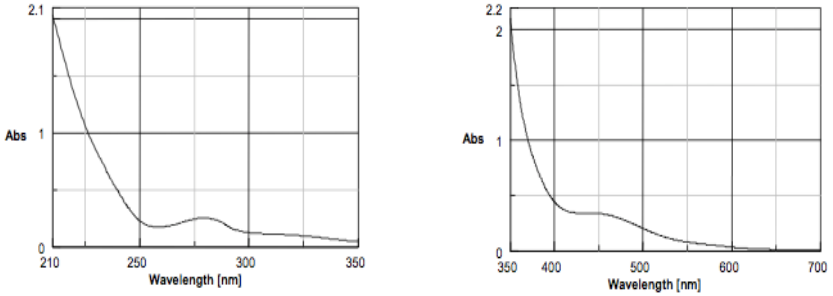


Tara

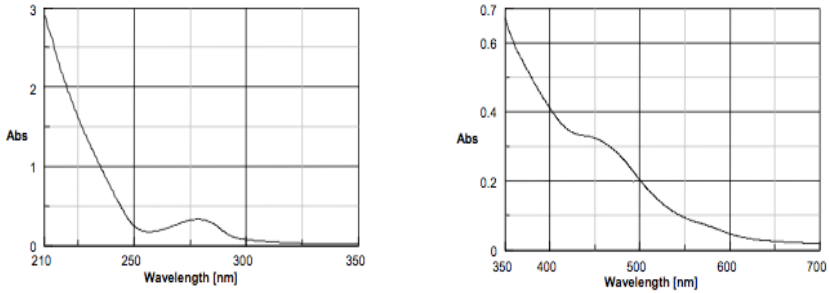




Grapes 1



Grapes 2



Grapes 3

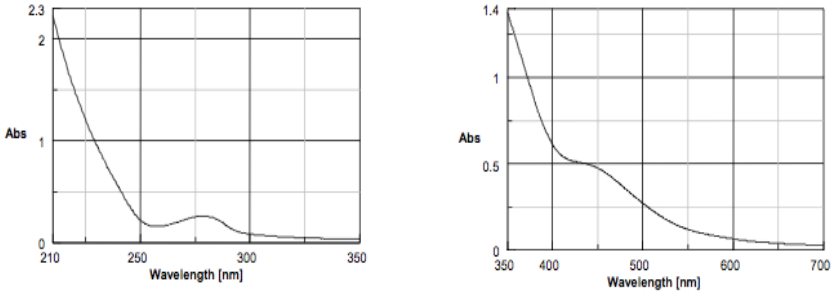


Figure 42 - UV-VIS spectra of the tannins analyzed at 1g/L in model wine. The graphs on the left refer to the UV spectra, while those on the right show the VIS spectra.

Measurement of Surface Electrical Charge (SEC)

Another parameter analyzed in this work was the surface electrical charge (SEC) (Figure 43).

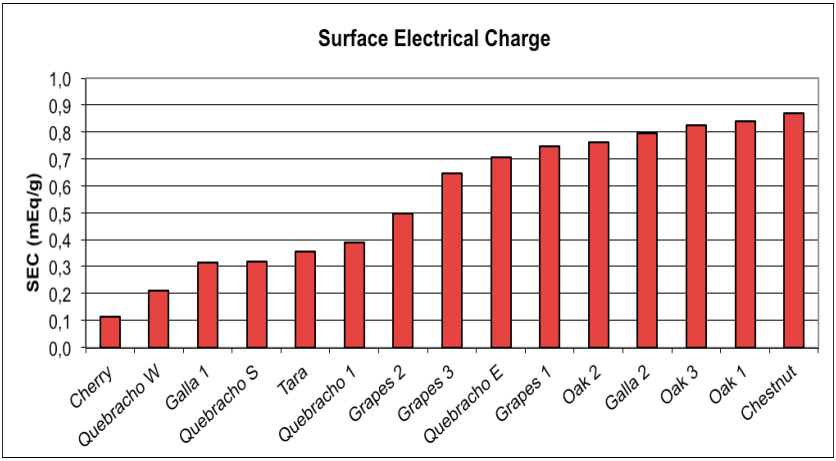


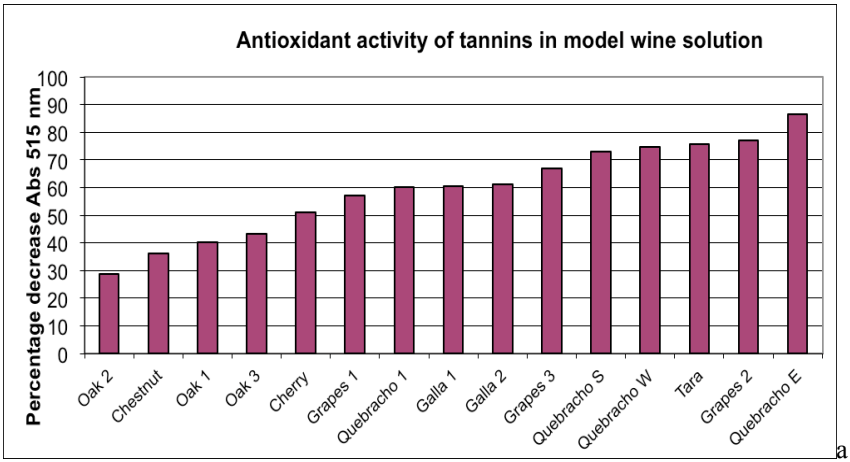
Figure 43 - Surface electrical charge (-) (mEq/g). Measurement conducted on model wine solutions added with tannins at a concentration of 1 g/L.

It is possible to notice how the tannins studied presented values significantly different both within the same type of product and between different categories, but such values are in general quite low, confirming that the interaction phenomena of this class of polyphenols with proteins are not mainly due to electrochemical phenomena, but rather to other forces (interface tensions, hydrogen bonds, etc..) (Vernhet et al., 1996). A tannin with a high electrical charge could be more appropriate as a clarifying agent. The statement of Vivas (1997), about the higher amount of charge and therefore the greater reactivity against proteins made from condensed tannins than hydrolysable ones are in part disregarded, because the charge values are equally divided between the two groups. The chestnut tannin showed the highest amounts of charge.

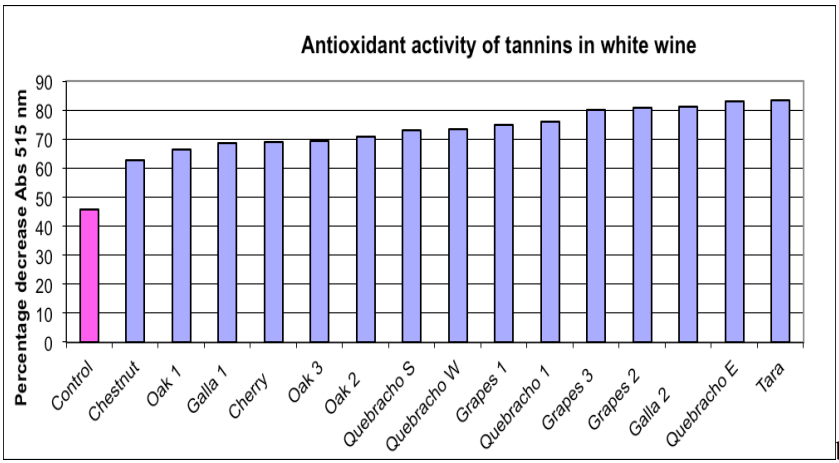
Determination of the antioxidant activity

As an additional parameter, the antioxidant activity (chain-breaking activity) of tannins (100 mg/L) in model wine, white and red wine, was taken into account (Figure 44 a, b, c). The results of the analysis confirmed the antioxidant effect of tannins, specifically, among the hydrolysable ones, the tara tannin was the one with the highest activity. Furthermore, the main differences between the different preparations were found in the tests with samples prepared in model wine solutions (Figure 44a), where there was no interference of wine polyphenols. Differences were also found between the tannins of the same botanical origin, but extracted with different methods. It should be noticed how in red wine samples (Figure 44c),

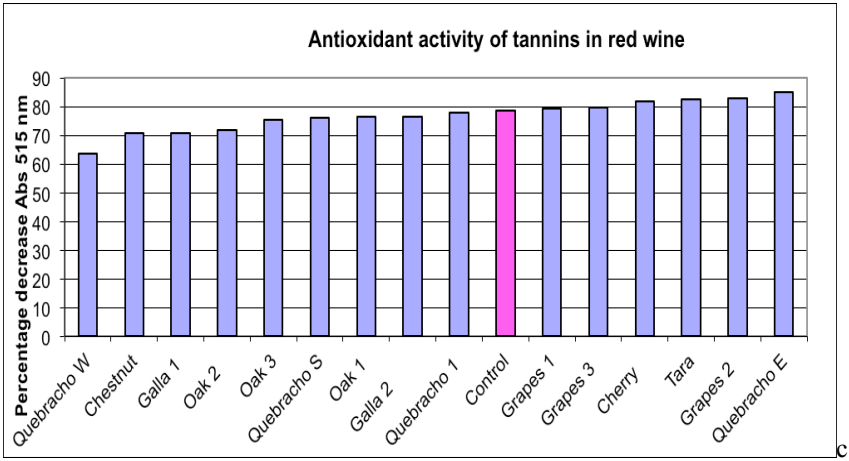
the antioxidant capacity (chain-breaking activity) was only slightly increased by the presence of tannin compared to the white wine and model wine samples. That could be explained by the fact that red wine, already has its own polyphenols that attenuates the protective effect of exogenously added tannin. For the tests with the red wine, it was also noted that the control showed intermediate values of antioxidant activity compared to the ones of the samples added with tannins. This, as already noted above, can be related to different interactions of the tannins with the constituents of the wine and leads to infer that probably adding tannins to the wine will not always improve the antioxidant power and that the addition of these tannins has different effects on different wines (white wine vs. red wine). Further insights are needed to understand the causes of this difference in behavior.
From the performed tests it is possible to see how the higher activity of hydrolysable tannins in blocking free radicals with respect to the condensed ones can not be granted. This assumes significance when considered in relation to the various stages of use of tannin in the wine cellar.



a



b



c

Figure 44 a, b, c - Antioxidant activity of tannins (100 mg/L) in model wine solution (a), white wine (b) and red wine. (c).

Determination of POM-test and "Anthocyanins Oxidizability Index" (A.O.I.)
From the analysis it is possible to notice that POM-test is positively correlated with the antioxidant activity of tannins in the model wine solution examined (Figure 49). This means that the tannin added participates in wine darkening, contrary to what would be expected, while in red wines (AOI) there is a negative correlation that explains the role of tannins as antioxidants towards anthocyanins (Figure 50). These results confirm what was previously observed by other authors (Marchinu, 2009, Toffoli, 2010).

With reference to the POM-test analysis, it showed great variability between the different preparations (Figure 45). In particular the highest values were found for the quebracho tannin, highlighting the differences also in reference to the extraction methods (Quebracho S, Quebracho E and Quebracho W). Only two tannins, more precisely Oak 2 and Grapes 3, showed values of POM-test lower than the control, while all the other tannins tested showed values similar to or greater than the control. This seems contrary to what would be expected, given the protective effect of the added tannin, with respect to the control. We can explain this result with the fact that probably tannins added participate in wine darkening, confirming what was stated before.

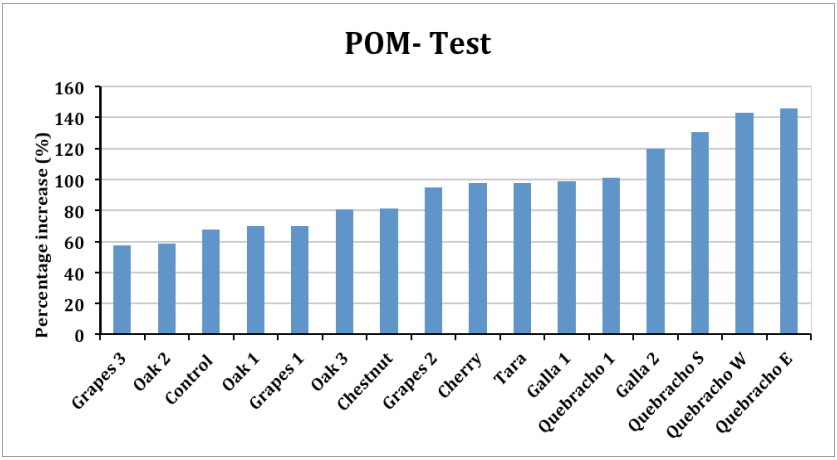


Figure 45 – POM-test of tannins added on white wine at 100 mg/L.

The anthocyanins oxidizability index (Figure 46) showed that tannins extracted from the wood of quebracho and tara provided greater protection against oxidation of anthocyanins, in particular Quebracho W, Quebracho E and Quebracho S tannins, while not showing specific differences depending on the type of solvent used for extraction. It also appeared that the concentration of tannins used in the tests only partially affected their role as antioxidant agents for anthocyanins (Figure 46). In fact, tests with a tannin concentration of 1000 mg/L (highest concentration among those tested) showed values similar to the ones obtained in tests with the lowest concentration of tannins added (100 mg/L), thus, not highlighting such differences in behavior to justify a greater cost in their practical use. The major differences in behavior among the tannins tested and compared to the control can be seen at a concentration of 500 mg/L, a concentration which is already very high for normal use of tannin preparations in the cellar. When these products are used under normal conditions (100 mg/L), no big differences in the value patterns are detected, both regarding the tannins tested and the control, with the exception of quebracho and tara tannins

Comparing the POM-test data with the AOI of tara and quebracho tannins, it can be noticed that the latter presented low values for the AOI, while they had the highest values in the POM-test. This reversal in the value patterns passing from POM-test to AOI testing occurs in a minor way for the tara tannin, showing more aptitude in the protection of wines from oxidation, according to these parameters.
From these results it seems that, in practice, the use of tara tannin in combination with quebracho tannin would give the best results in terms of protection of anthocyanins from oxidation, especially in the early stages of fermentation.

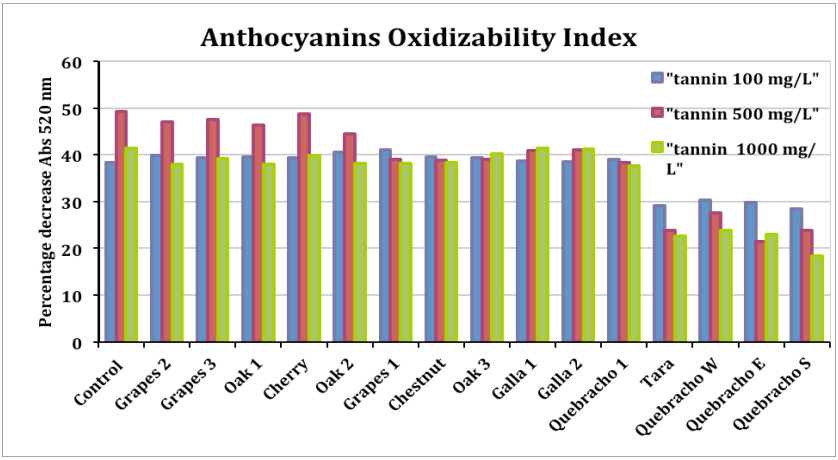


Figure 46 – Anthocyanins oxidizability index of tannins in red wine at 100-500 and 1000 mg/L.

Turbidity

Another parameter evaluated was the analysis of the turbidity expressed in NTU (Figure 47). The differences between the various preparations are remarkable, further confirming the importance of knowing the characteristics of the formulations purchased prior to their use in the cellar. In fact, ellagitannins were, on average, the ones with the highest values of turbidity, unlike gallotannins and condensed tannins, in particular those derived from Quebracho wood. The only exception among condensed tannins is the one obtained by extraction from the grape skins that has the highest value of NTU. The differences observed in turbidity show different behaviors at the level of preparation in the cellar.

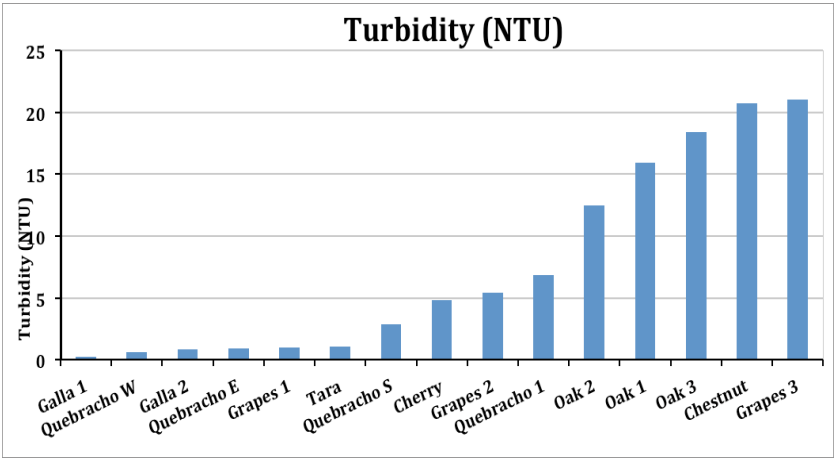


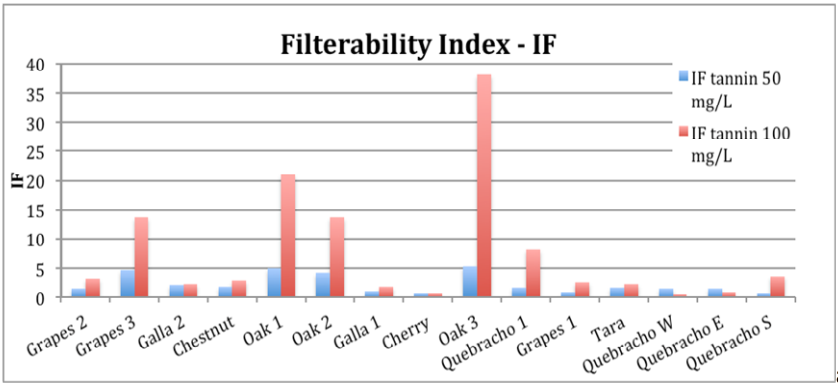
Figure 47- Turbidity of tannins. 1 g/L of tannin in model wine solution.

Determination of the filterability index

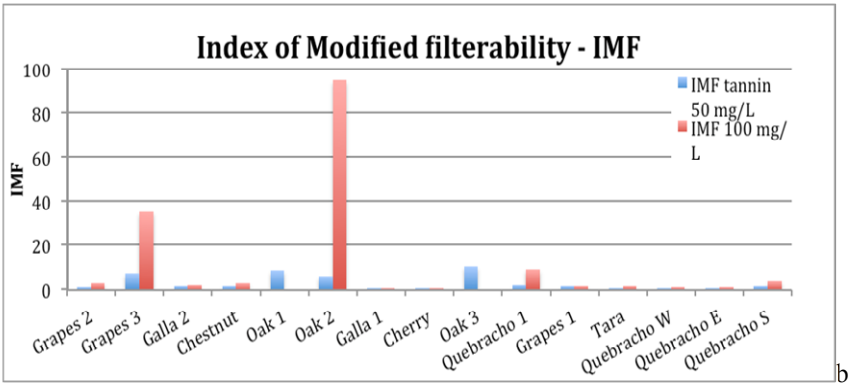
An important parameter for practical use in the cellar is the determination of the filterability index through the calculation of three indices: IF, IMF and Vmax (Figure 48 a, b, c). The filterability index (IF) represents the fouling of a membrane due to the filtration of 400 mL of a solution, the index of modified filterability (IMF) represents the fouling due to the filtration of 600 mL of a solution and the maximum volume filterable (Vmax) reproduces the milliliters of product that can be filtered with a 0.65 µm membrane. The optimal values for the first two indices are 0 and 10, while for the third index they exceed 3000. From Figure 48 it is evident that the solution with oak tannin is the most difficult to filter, showing the highest values for the IMF and IF and the lowest values for Vmax index, especially at a concentration of 100 mg/L, where it shows both values above 10 for the IF and MFI and lower than 3000 for Vmax index.

For Oak1 and Oak3 tannin solutions it was impossible to calculate IMF at the concentration of 100 mg/L due to the premature fouling of the filter membrane, at 525 mL and 475 mL respectively. The sample with Grapes 3 at the concentration of 100 mg/L could cause problems in the filtration phase (values above 10 for IF and IMF and lower than 3000 for Vmax index). This could be related to the high turbidity values of tannins found for the four ones just mentioned.

The significant differences found on the filterability index confirm the importance of a rational management of endogenous tannins, but mostly of the exogenous ones that could be used during the aging of wines or just before bottling.



a



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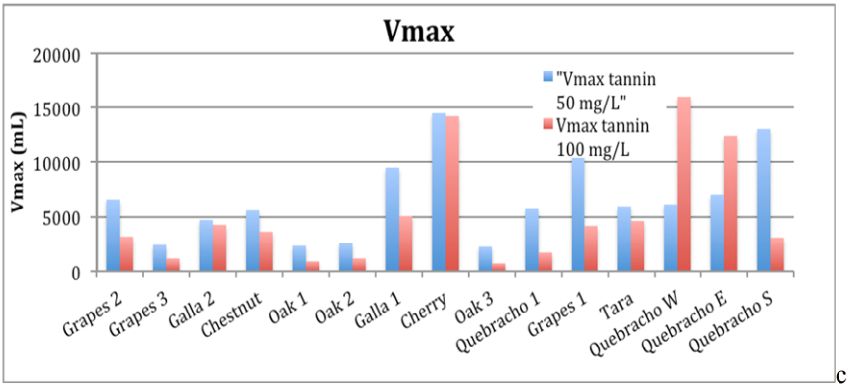


c

Figure 48 a, b, c – Filterability index (a), Index of modified filterabilityc (b), Vmax (c) for tannins at 50 and 100 mg/L.

Colorimetric analysis

Even the colorimetric analysis of the tannin powders (Table 6) gave different results between the various tannins, confirming the usefulness of this parameter for rapid evaluation of the quality of tannins. To confirm this, it was possible to highlight a negative correlation between the parameter b* with the AOI (r = -0.6) and the antioxidant activity in white wine (r = -0.6) and a positive correlation for the same parameter with the POM-test (r = -0.56). This could be helpful in practical use as a quick reference for the technician.

Taking into account the luminosity (L* parameter), Galla and Tara tannins showed the highest values, while Chestnut and Grapes 3 tannins had the lowest values. Cherry, Grapes 1 and Quebracho E are the tannins that showed a rather more reddish chromaticity (a* parameter). Quebracho 1, Chestnut and Grapes 3 show the greatest tendency toward blue (-b* parameter).

Tannin	L*	a*	b*
Cherry	97,6	5,4	-4,4
Chestnut	86,4	1,1	-17,1
Galla 1	111,4	-0,9	-1
Galla 2	100,7	2,3	-5,3
Grapes 1	94,6	6,6	-7,4
Grapes 2	87,2	3,4	-16,2
Grapes 3	86	1,2	-17,6
Oak 1	89	1,8	-14,4
Oak 2	89,2	2,6	-13,8
Oak 3	91,8	2,3	-11,7
Quebracho 1	86	1,5	-17,9
Quebracho E	97,7	5,1	-3,8
Quebracho S	94,4	3	-7,1
Quebracho W	97,7	4,8	-3,4
Tara	104,6	4,5	2,2

Table 6 – Values of colorimetric analysis.

Particle size (colloidal diameter)

This analysis allows to measure the dimension of the hydrocolloidal particles. The instrument used for the present work has as an optional multi-angle module in order to make DSL measurements of certain particle sizes more sensitive by changing the detection angle between 14,4 and 170,1 degrees. This way, it is

possible to optimize the measure by varying the scattering angle. In fact, different measures can be performed by changing the scattering angle in order to correct the interference due to different types of particles present in different amounts of a complex distribution of particles. Specifically, besides the measurement at 90°, a second measurement at 14,4° allows to correct interference due to possible large diameter particles, while a second measure at 170,1° allows to correct interference due to possible small diameter particles.

Most of the tannins analyzed (dissolved in model wine solution at the concentration of 1 g/L) showed variable values depending on the species from which they were extracted and the type of extraction. The diameters range was between few nanometers, like in the case of Quebracho W tannin (10 nm) and 4548 nm in the case of Galla 2 tannin (Tables 7, 8, 9).

It is also interesting to note that the Grapes 2, Galla 2, Cherry, Quebracho 1, Oak 1 and Oak 2 tannins are composed of particles with different diameters (more than one peak). This evidence may be relevant for a reasoned choice to use exogenous tannins in the cellar according to the targets set.

It is, then, clear that the tannins must be used in different stages of vinification depending on the size and consequently on the greater or lesser ability to bind the colloidal fraction present in wine. The hydrocolloidal diameter could be a useful tool for monitoring the quality of the tannins and to monitor them during wine aging.

Particle Size 90° (nm)						
Tannin	1 peak	%	2 peak	%	3 peak	%
Cherry						
Chestnut	99	4,7	147	95,3		
Galla 1						
Galla 2						
Grapes 1						
Grapes 2	55	6,6	660	40,4	2156	53,1
Grapes 3	2758	100				
Oak 1	52	0,9	236	5,9	1304	93,2
Oak 2	55	3,7	1417	96,3		
Oak 3	701	100				
Quebracho 1	55	3,1	697	30,4	1886	66,5
Quebracho E						
Quebracho S						
Quebracho W						
Tara						

Table 7 – Particle size at 90°.

Particle Size 14,4° (nm)		
Tannin	1 peak	%
Cherry	322	100
Chestnut	221	100
Galla 1	885	100
Galla 2	237	100
Grapes 1	611	100
Grapes 2	672	100
Grapes 3	116	100
Oak 1	262	100
Oak 2	126	100
Oak 3	340	100
Quebracho 1	522	100
Quebracho E	594	100
Quebracho S	534	100
Quebracho W	265	100
Tara	311	100

Table 8 – Particle size at 14.4°.

Particle Size 170,1° (nm)				
Tannin	1 peak	%	2 peak	%
Cherry	28	88,9	902	11,1
Chestnut	567	100		
Galla 1	238	100		
Galla 2	227	32,7	4548	67,3
Grapes 1	306	100		
Grapes 2	55	67,6	3259	32,4
Grapes 3	621	100		
Oak 1	554	100		
Oak 2	407	100		
Oak 3	1489	100		
Quebracho 1	863	100		
Quebracho E				
Quebracho S	611	100		
Quebracho W	10,3	100		
Tara				

Table 9 – Particle size at 170.1°.

Correlations

It was also considered useful for a better understanding of the results to see if there are linear relationships between the various analytical parameters considered, using the statistical investigation with Pearson's r. r is a dimensionless index between -1 and 1, which reflects the extent of a linear relationship between two data sets. The surface electrical charge (Table 10) is positively correlated with turbidity (r = 0.53). This could be interesting at the operational level in the cellar when a tannin has to be chosen, so that a quick and easy evaluation of the turbidity of a tannin could give an overview of its surface electrical charge.

As previously explained, a positive linear relationship between the antioxidant activity of tannins measured in model wine solution and the POM-test (r = 0.69) could also be observed (Figure 49), while it is negative with the anthocyanins oxidizability index (r =-0.68) (Figure 50).

Looking at the trend of the particle size, it is negatively correlated with turbidity (r =-0.57) and the index of filterability (r = -0.57) while it is positively correlated with Vmax (r = 0.52). This type of correlation confirms once again the usefulness of measuring the particle size as a parameter for the characterization of commercial preparations of tannins. In this specific case it could give useful indications of the tannin capacity to foul the filter membranes.

The antioxidant activity of tannins in model wine solutions is negatively correlated with turbidity (r = -0.63) and with the index of modified filterability (IMF) (r = -0.54).

Observations of the correlation values of the filterability test show how it has a negative linear relationship with the POM-test (r = -0.60). Moreover, also the turbidity follows this trend in correlation with the POM-test (r = -0.68).

As it might be expected, a positive correlation between turbidity and filterability index (IF) (r = 0.77) and the index of modified filterability (IMF) (r = 0.76) in both concentrations of tannins tested (Figure 51) is evident. Instead, the correlation between turbidity and the Vmax (r = -0.60) is also negative, in both concentrations of tannins tested (Figure 52).

Correlations Marked correlations are significant at p< ,05000 N=15 (Casewise deletion of missing data)																						
Variables	SEC	AOI tannin 100 mg/L	AOI tannin 500 mg/L	AOI tannin 1000 mg/L	PCM-test	Particle size tannin 5 g/L	Chain-breaking activity in model wine	Chain-breaking activity in red wine	Chain-breaking activity in white wine	Turbidity	IF tannin 50 mg/L	MF tannin 50 mg/L	Vmax tannin 50 mg/L	IF tannin 100 mg/L	IMF tannin 100 mg/L	Vmax tannin 100 mg/L	a*	b*	L*
SEC	1,000	0,080	-3,006	-0,031	-0,329	-0,412	-0,450	-0,438	-0,434	0,631	0,507	0,522	-0,352	0,467	0,511	-0,399	-0,123	-0,350	-0,395
AOI tannin 100 mg/L	0,080	1,000	0,918	0,964	-0,753	-0,087	-0,679	-0,025	-0,396	0,465	0,375	0,395	-0,248	0,344	0,321	-0,435	-0,329	-0,600	-0,403
AOI tannin 500 mg/L	-0,005	0,918	1,000	0,901	-0,717	-0,190	-0,584	0,013	-0,348	0,483	0,405	0,412	-0,213	0,301	0,309	-0,354	-0,336	-0,557	-0,379
AOI tannin 1000 mg/L	-0,031	0,964	0,901	1,000	-0,664	-0,071	-0,624	-0,048	-0,358	0,428	0,362	0,367	-0,262	0,333	0,294	-0,394	-0,425	-0,466	-0,225
PCM-test	-0,329	-0,753	-0,717	-0,664	1,000	0,327	0,668	-0,053	0,338	-0,679	-0,599	-0,595	0,387	-0,495	-0,476	0,665	0,279	0,557	0,445
Particle size tannin 5 g/L	-0,412	-0,087	-0,190	-0,071	0,321	1,000	0,427	0,268	0,116	-0,564	-0,575	-0,458	0,523	-0,319	-0,341	0,085	-0,013	0,282	0,405
Chain-breaking activity in model wine	-0,450	-0,679	-0,584	-0,624	0,668	0,427	1,000	0,378	0,775	-0,625	-0,514	-0,535	0,301	-0,506	-0,584	0,421	0,335	0,442	0,341
Chain-breaking activity in red wine	-0,438	-0,025	0,013	-0,048	-0,053	0,268	0,378	1,000	0,581	-0,131	-0,105	-0,076	0,173	-0,070	-0,119	-0,037	0,366	0,023	-0,068
Chain-breaking activity in white wine	-0,434	-0,396	-0,346	-0,358	0,338	0,116	0,775	0,581	1,000	-0,459	-0,192	-0,290	0,061	-0,307	-0,409	0,096	0,382	0,254	0,157
Turbidity	0,631	0,465	0,483	0,428	-0,679	-0,564	-0,625	-0,131	-0,458	1,000	0,772	0,769	-0,558	0,678	0,613	-0,529	-0,460	-0,763	-0,706
IF tannin 50 mg/L	0,507	0,375	0,405	0,362	-0,599	-0,575	-0,514	-0,105	-0,192	0,772	1,000	0,982	-0,824	0,876	0,869	-0,543	-0,370	-0,514	-0,461
IMF tannin 50 mg/L	0,532	0,395	0,412	0,367	-0,595	-0,458	-0,535	-0,076	-0,290	0,769	0,982	1,000	-0,668	0,947	0,922	-0,537	-0,333	-0,509	-0,463
Vmax tannin 50 mg/L	-0,352	-0,248	-0,213	-0,262	0,387	0,523	0,301	0,178	-0,061	-0,596	-0,824	-0,668	1,000	-0,610	-0,595	0,480	0,463	0,476	0,405
IF tannin 100 mg/L	0,467	0,344	0,301	0,333	-0,495	-0,319	-0,506	-0,070	-0,307	0,678	0,876	0,947	-0,610	1,000	0,937	-0,547	-0,303	-0,437	-0,391
IMF tannin 100 mg/L	0,511	0,321	0,309	0,294	-0,476	-0,341	-0,584	-0,119	-0,408	0,613	0,869	0,922	-0,555	0,937	1,000	-0,433	-0,244	-0,354	-0,326
Vmax tannin 100 mg/L	-0,399	-0,435	-0,354	-0,394	0,665	0,085	0,421	-0,037	0,096	-0,529	-0,543	-0,557	0,480	-0,547	-0,463	1,000	0,527	0,584	0,422
a*	-0,123	-0,329	-0,336	-0,425	0,279	-0,013	0,335	0,366	0,352	-0,466	-0,370	-0,333	0,403	-0,303	-0,244	0,557	1,000	0,391	0,079
b*	-0,350	-0,600	-0,557	-0,466	0,557	0,282	0,442	0,023	0,254	-0,763	-0,514	-0,509	0,473	-0,437	-0,354	0,584	0,391	1,000	0,932
L*	-0,355	-0,403	-0,379	-0,225	0,445	0,405	0,341	-0,068	0,157	-0,706	-0,461	-0,463	0,405	-0,391	-0,326	0,422	0,079	0,932	1,000

Table 10 – Correlations between the analytical parameters. Values statistically significant are in red.

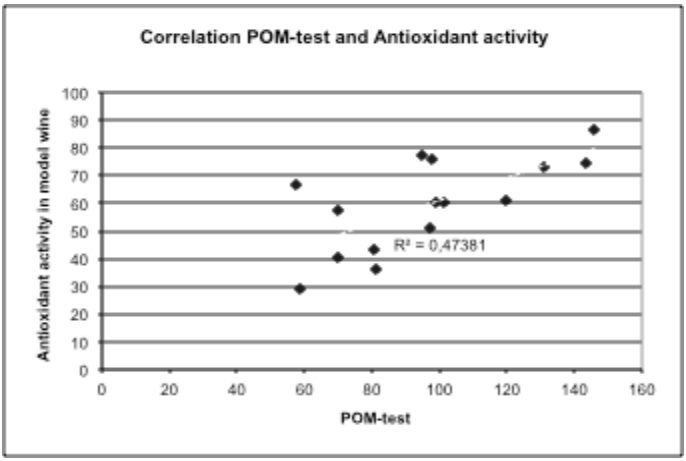


Figure 49 – Correlation between POM-test and antioxidant activity.

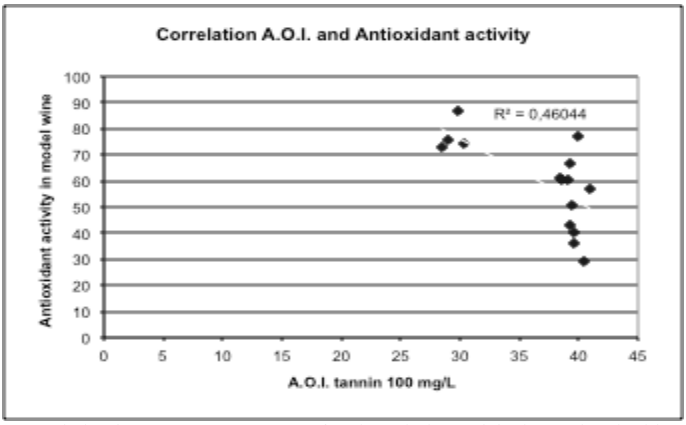


Figure 50 – Correlation between AOI at 100 mg/L of tannin in model wine and antioxidant activity in model wine.

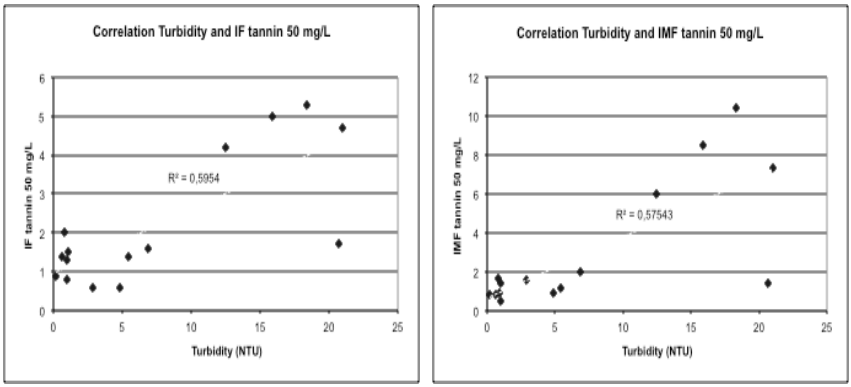


Figure 51 – Correlations between turbidity an IF and IMF of tannins at 50 mg/L.

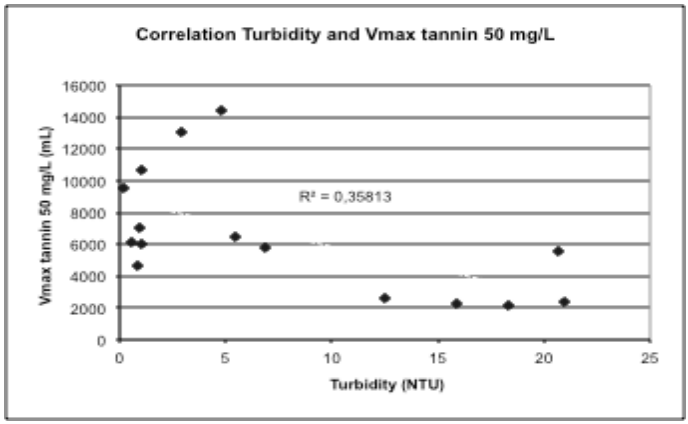


Figure 52 – Correlation between turbidity and Vmax of tannins at 50 mg/L.

5.2 Analysis on bottled wines form the cellar trial

Polymerized Pigments Index

Looking at the patterns of the polymerized pigments index values (Figure 53), which expresses the colour red contribution of condensed tannins and of the most polymerized forms of anthocyanins, in most of the tests values lower or similar to the control were found, demonstrating that when the analysis was performed the effect of the addition of exogenous tannin in maceration was not yet evident on polymerization and consequently on the stabilization of anthocyanins, an effect that probably could have been noticed during the storage of wine. The highest values of the free forms (Figure 54) confirm the minor presence of anthocyanins in combined form compared to the control at the time of the analysis, resulting in a greater sensitivity to thermal and oxidative degradation.

To confirm what was stated above, it can be noted how the samples QW and QE + T, among the other samples with the lowest values in terms of polymerized pigments index (Figure 53), were those with the highest values in reference to the anthocyanins oxidizability index (Figure 64).

Samples QS, QS+T and QW+T did not show these values, in fact they presented higher values in terms of polymerized pigments index and in terms of content in anthocyanins, compared to the control (Figure 53), this pattern can be justified by an important phenolic content, which is confirmed by the high total polyphenol values for these samples (Figure 56).

Anthocyanins

The analysis of the anthocyanins content highlighted the positive effects of the practice of macro oxygenation in combination with exogenous addition of condensed tannins compared to other combinations of technological variables analyzed and compared to the control (Figure 54). Moreover, the samples with the tannin Quebracho S showed on average higher values in anthocyanins, a further confirmation of the existing differences in behavior depending on the extraction methods used. In this case, the diethyl ether is confirmed as the best type of extraction solvent.

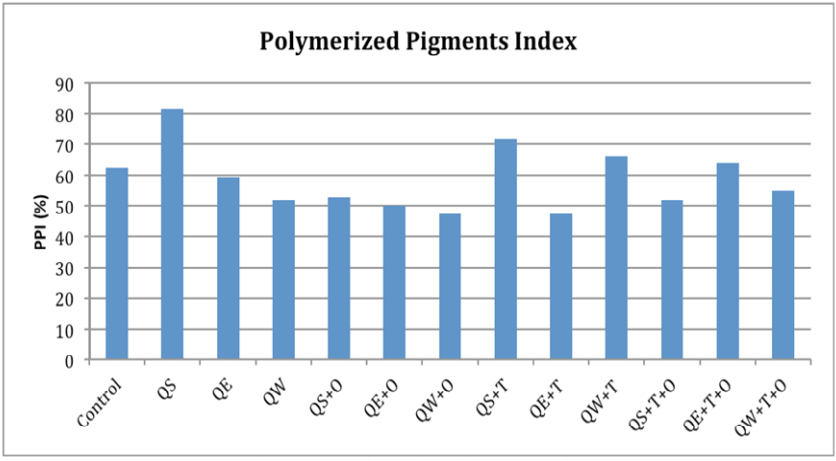


Figure 53 - Polymerized Pigments Index.

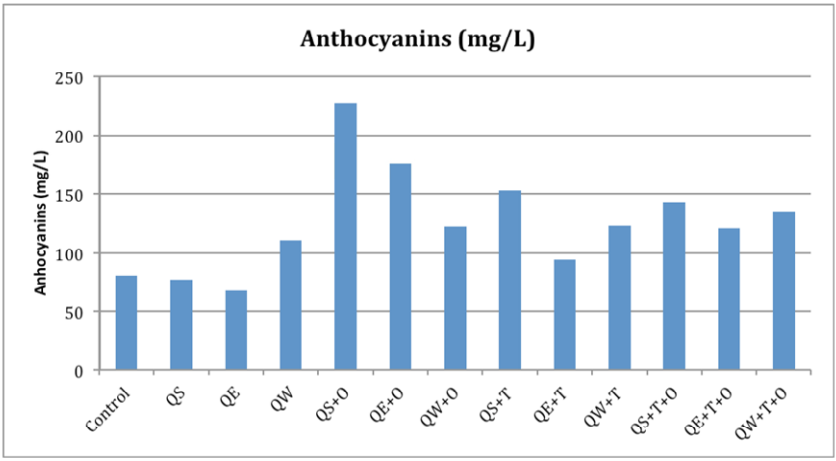


Figure 54 - Content in anthocyanins (mg/L).

HCl index

The HCl index (Figure 55), an indicator of the state of polymerization of tannins, generally showed higher values for the control than for the other samples, where a low polymerization of tannins was found, lower for QS+O, QE+O and QW+O, perhaps due to the oxygenation in maceration.

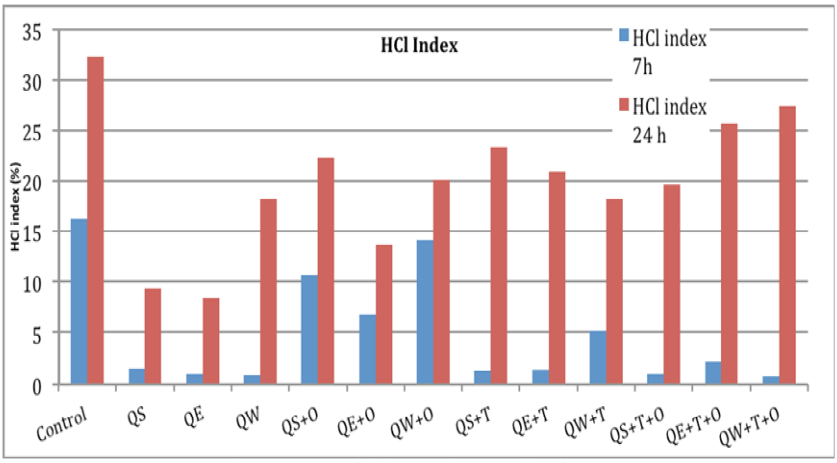


Figure 55 – HCl index at time 7 and 24 hours.

Total Polyphenols index and OD 320 nm

Samples added with quebracho tannin extracted with diethyl ether (QS) had higher values in terms of total polyphenols (Figure 56) and hydroxycinnamoyl tartaric acids (Figure 57), compared to the samples added with quebracho tannin extracted using ethanol or water. The results showed, once again, how the various types of extraction of tannins are decisive to the final oenological results.

As it would be expected, the addition of tara tannin besides the quebracho one, helped in preserving the polyphenols (Figure 56) and hydroxycinnamoyl tartaric acids (Figure 57), thus increasing the stability and maintaining the phenolic content.

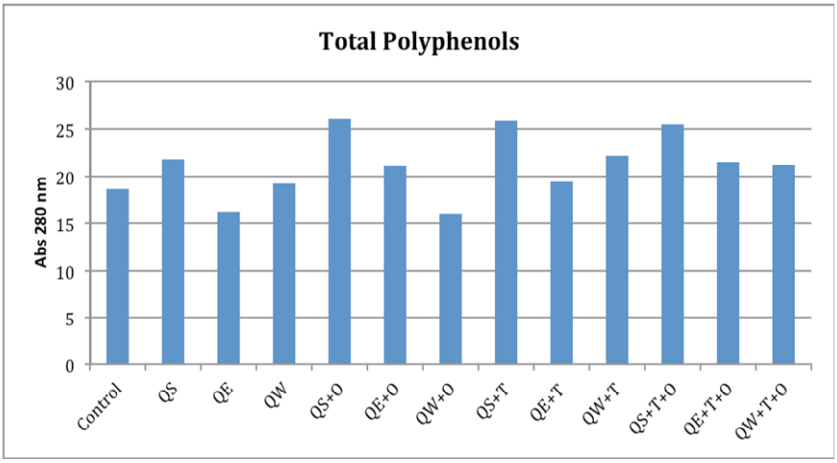


Figure 56– Total Polyphenols Index (OD 280 nm).

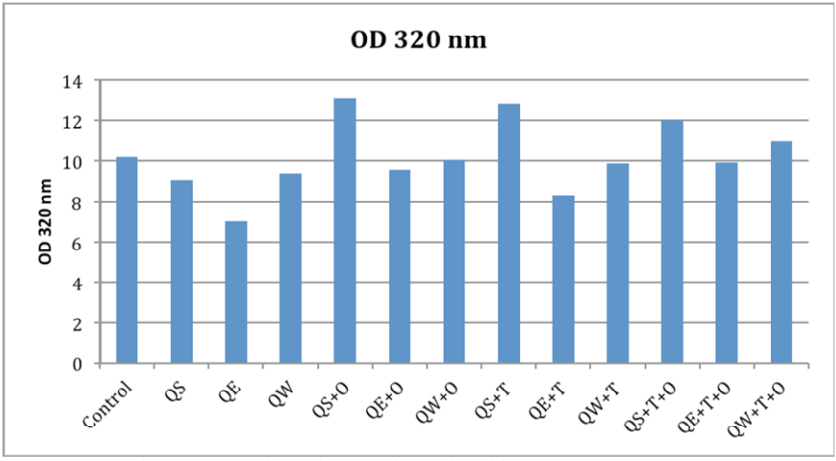


Figure 57 – Hydroxycinnamoyl tartaric acids content (OD 320 nm).

Gelatin index

The gelatin index (Figure 58) highlights the ability of tannins to react with the proteins (gelatin), this reactivity is involved in the astringent sensation that is felt at the time of tasting of a red wine. From the results overall low values emerged, indicating a low astringency of the wine. Among the various samples, it is possible to note slightly higher values for the samples with quebracho tannin extracted with diethyl ether and the ones with all the treatments present simultaneously (quebracho tannin-tara tannin-oxygenation), compared to the control.

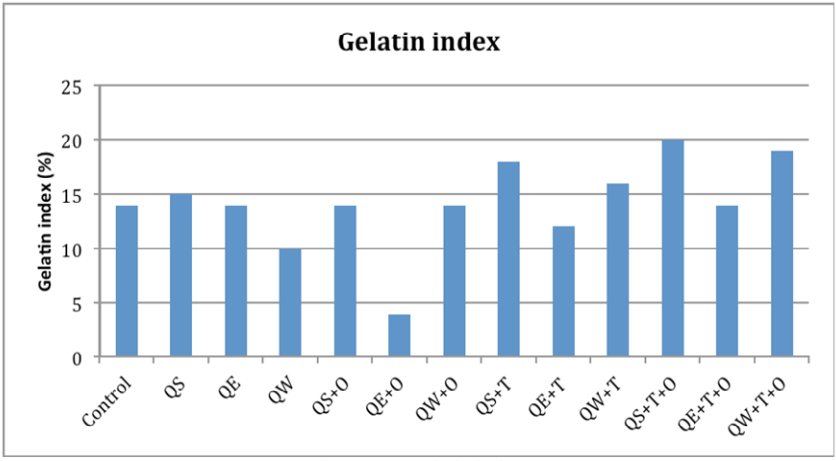


Figure 58 - Gelatin index.

Total Tannins, Catechins and Ethanol index.

The analysis of total tannins (Figure 59) highlighted the highest values for the samples added with Quebracho S and Tara tannin, with and without oxygenation. For the other tests similar values to the ones of the control were noticed. To explain this observation it should not be forgotten that a fraction of the tannin added in maceration is adsorbed on solids fractions or on the cell wall of yeasts, or it will be found on the lees after fermentation.

With reference to the analysis of the content of catechins in wines (Figure 60), higher values were found for the samples that were added with quebracho tannin extracted with diethyl ether than for the other samples and for the control. The samples added with the other tannins showed average values similar to the control. It reiterates the importance, also given the results of these two last analysis, of the difference of the effects on wine between the various tannins of quebracho depending on the extraction solvents used.

The ethanol index (Figure 61), which is representative of the fraction of tannins bound to polysaccharides and salts, and thus, present in the colloidal fraction of the wine, showed values lower than 12, further confirming that the analysis of this work were performed on very young wines where the fraction of tannins bound to polysaccharides and salts, mainly present in more mature wines was poorly represented.

The highest value of the samples was for the QS+T one; it is explained by the fact that it is also the sample with the highest value in total tannins (Figure 59).

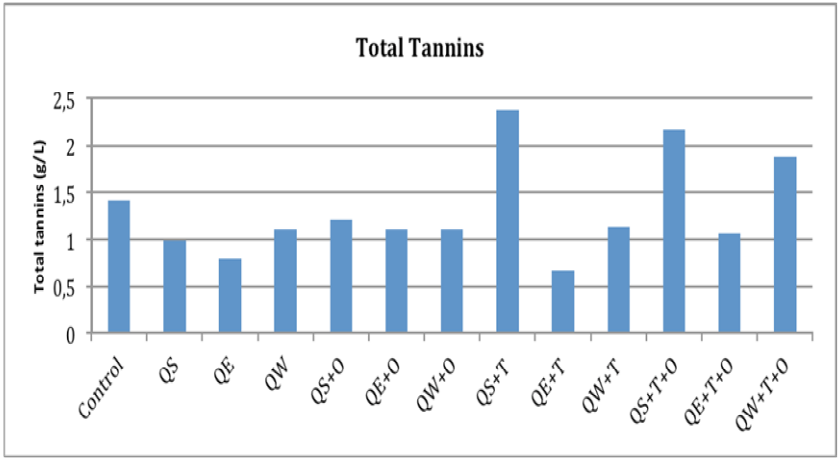


Figure 59 - Total Tannins (g/L).

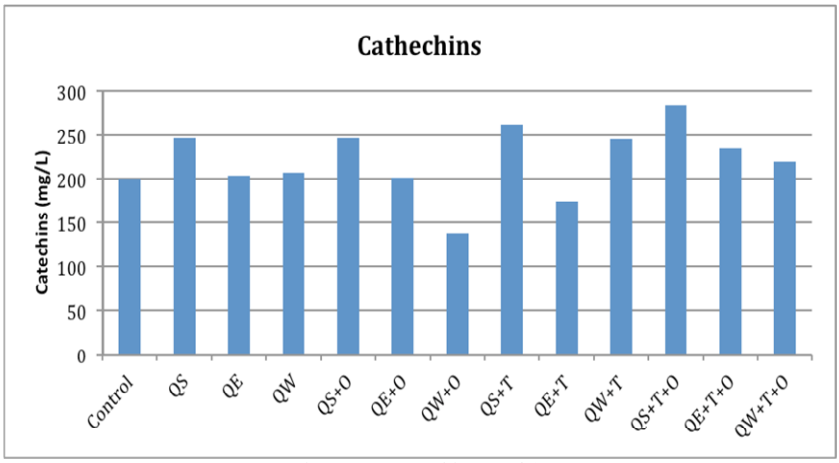


Figure 60 - Catechins (mg/L).

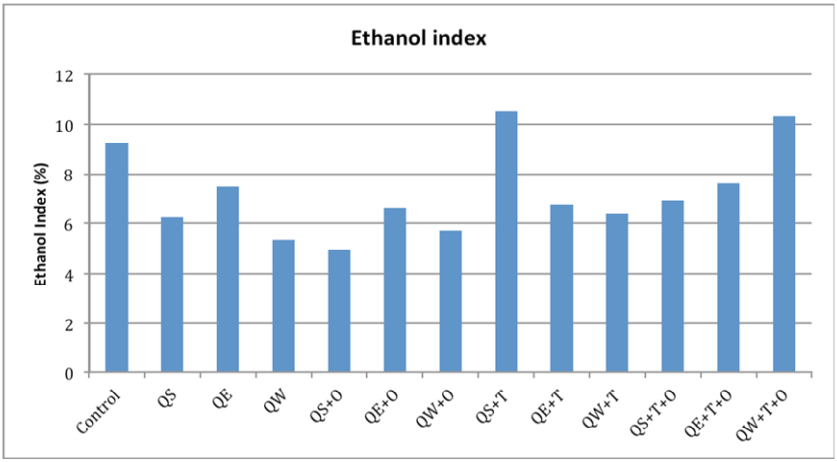


Figure 61 – Ethanol index.

Colour Intensity, Hue and AOI.

In Figure 62, representing the values of colour intensity, it soon becomes evident that in all tests except for the QE sample, the addition of condensed tannin resulted in an increase in colour intensity compared to the control. Analyzing specifically the QE test, it emerged how the lowest colour intensity observed with respect to all the other samples, including the control, may be due to the low values in terms of total content of tannins (Figure 59) and anthocyanins (Figure 54) found for the same sample.

With the same treatment conditions it is clear how the samples added with Quebracho S tannin (extracted with diethyl ether), showed higher values than the other tests.
This fact highlights the influence of the type of extraction of tannin on the colour intensity as well as underlining the importance of this aspect in the final evaluation of a tannin preparation.
The greater effectiveness of this preparation may be due to a greater dissolution of this tannin during maceration compared to the other types of quebracho tannin extracted with different solvents.
The combination of condensed tannin and macro-oxygenation has allowed a significant increase in colour intensity compared with the control and the other samples. The hydrolysable tannins, which was added in the wines, moderately positively influenced the values of colour intensity (Figure 62).
The hue of the wines (Figure 63) corresponds to the level of evolution of the colour towards orange colour and tends to increase during wine aging. The values, for all of the samples, except for QS and QE, appeared to be normal values for young wines, and still minor than the control one.
With reference to the anthocyanins oxidizability index (Figure 64) all the thesis seemed to have anthocyanins more susceptible to oxidation than the control. This trend can be justified taking into consideration the content of anthocyanins (Figure 54): the thesis with the highest values in the anthocyanins are also those that showed the greatest values of AOI and higher values in terms of colour intensity (Figure 62).
Considering the OD at 620 nm (Figure 65), which represents the blue colour of young wines related to the combinations anthocyanins-tannins via acetaldehyde, it is possible to see how the samples where the Quebracho S tannin was present were those with the highest value for this parameter as evidence of the oenological characteristics differences of a tannin according to the type of solvent used for extraction. High values, in reference to the samples in this study, were also found for tests in which all three variables appeared together.

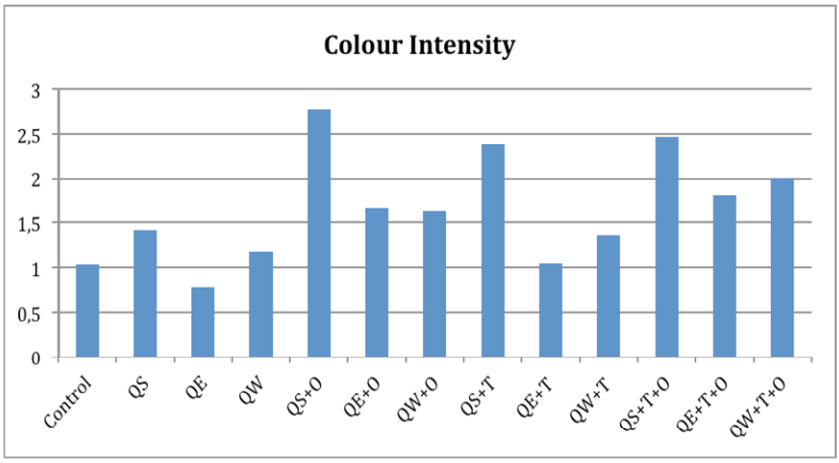


Figure 62 – Colour Intensity.

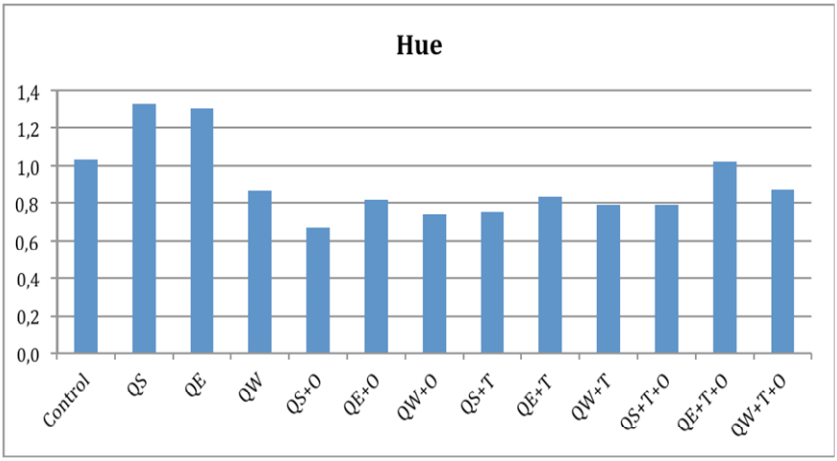


Figure 63 – Hue

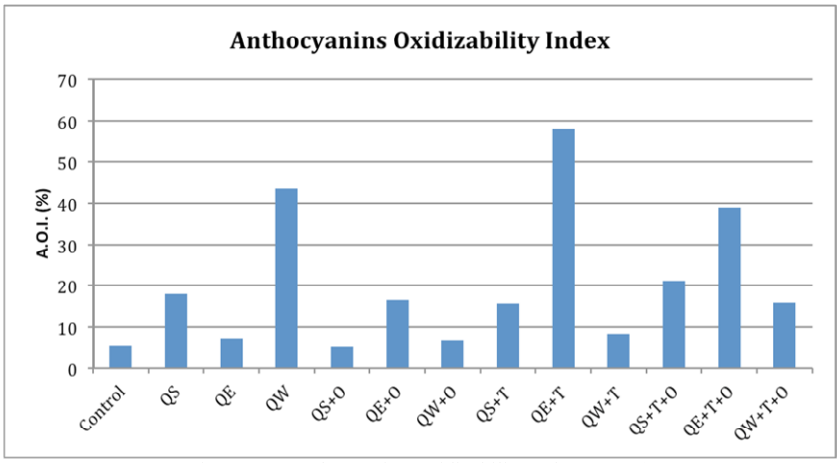


Figure 64 - Anthocyanins Oxidizability Index (A.O.I.)

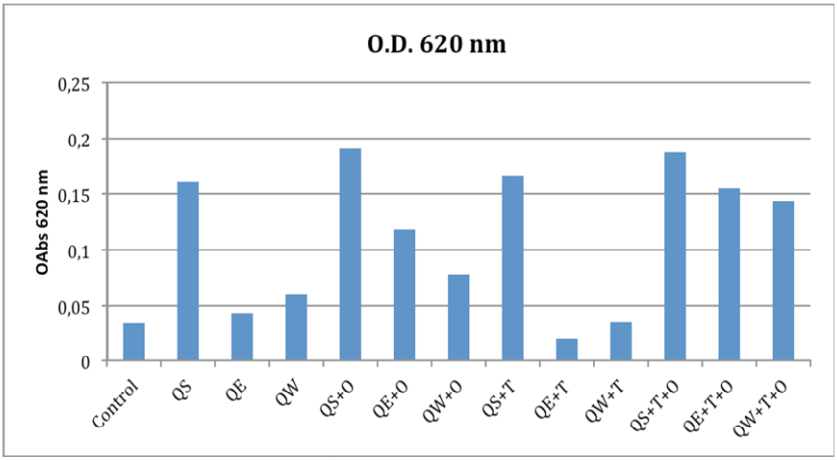


Figure 65 – OD 620 nm

In reference to the results of the refrigeration test, no formation of crystals of potassium bitartrate was found, thus confirming the tartaric stability of these wines.

Correlations

It was decided to create a graph (Figure 66) representing all the correlations between the different treatments and the analytical parameters evaluated in these part of the work.

The first thing to be highlited is the correlation between the colouring intensity and the samples with the quebracho tannins and the macro-oxygenation (QS+O,QW+O, QE+O), confirming the positive effects of these treatments on the

wine colour. The different response of the different tannins on the same grape and the same winemaking technique is clear. This confirms the need to characterize the exogenous tannins in order to optimize their technological use.

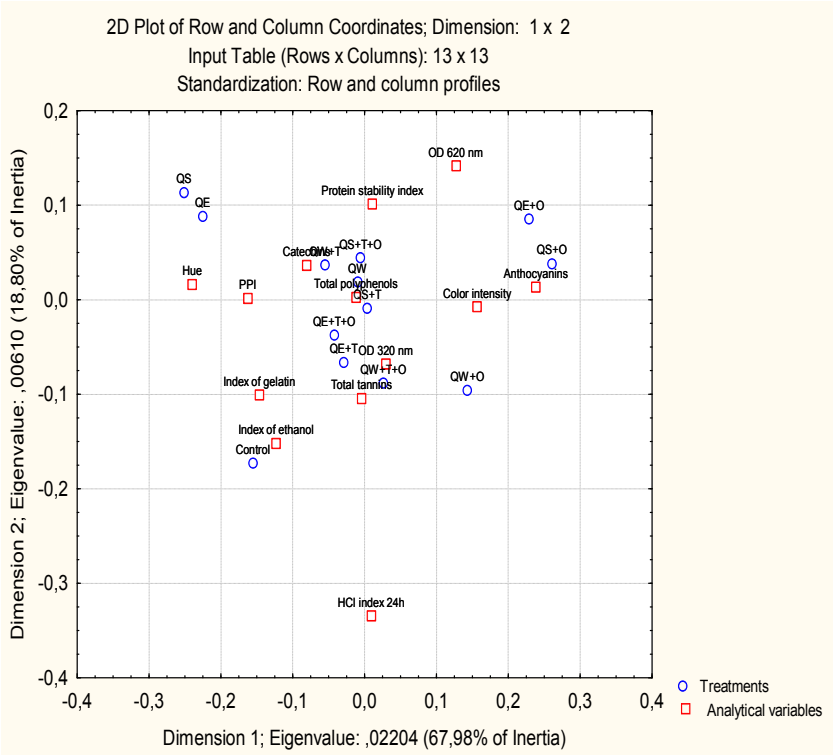


Figure 66 – Correspondence analysis between the different wine treatments and the analytical variables considered in this research.

6. Results and discussion Part 2

For this part, samples showed in the next graphs will be indicated in the following way:
"Control wine": wine with no additions;
"Control wine CT/OT/GT": wine added with cherry tannin oak tannin or grape skin tannin;
"CMC 50/100/200": addition to the wine+tannin of carboxymethylcellulose at 50/100/200 mg/L;
"GP 100/200/300": addition to the wine+tannin of grape polysaccharides at 100/200/300 mg/L;
"GA 300/500/1000": addition to the wine+tannin of gum arabic at 300/500/1000 mg/L;
"MP 100/200/500": addition to the wine+tannin of mannoproteins at 100/200/500 mg/L.

6.1 Analysis on red wine added with polysaccharides

Anthocyanin content
With regard to the analysis of the content of free anthocyanins in red wine added with various polysaccharides in the three different concentrations, a particular trend was noted: in the first 15 days of monitoring, the value decreased, but increased in the remaining 20 days.
All the samples, including the control, followed the same trend. Anyway, the addition of polysaccharides seemed to affect more or less significantly this parameter (Figure 67). Particularly at the time 45 days the control had the highest content of free anthocyanins (316 mg/L), compared to that of all other samples, suggesting that polysaccharides can contribute to the reduction of this parameter (even -75 mg/L). The lowest values were those of the samples of gum arabic added at 1000 mg/L (241 mg/L) and mannoproteins at 500 mg/L (266 mg/L).
The reduction of the free anthocyanins content can be related to their oxidation over time or to their tendency to combine with tannins. This second explanation can be corroborated by the decrease in the catechin content over time (Figure 71) the increase in the polymerized pigments index (Figure 68) and the increase in the colour intensity (Figure 77).
Although the timing is tight, it would seem that the anthocyanins in the first 15 days have participated in a sort of polymerization that did not occur in the second part of the monitoring.

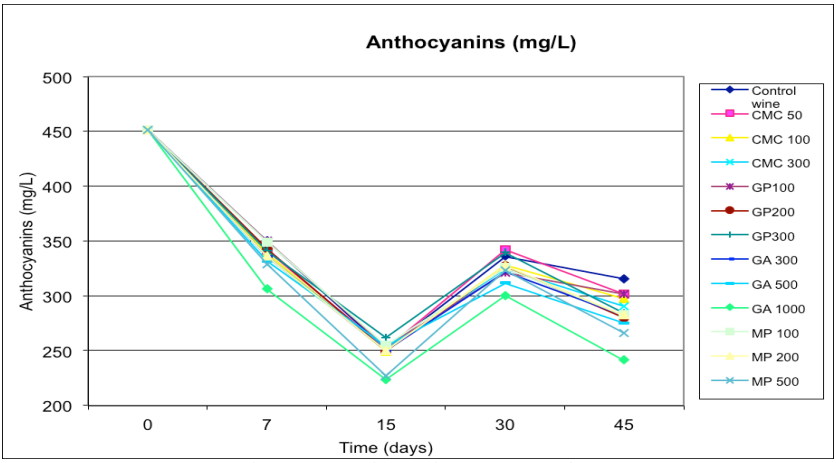


Figure 67 - Anthocyanins content (mg/L).

This parameter is usually linked to the trend of the parameter polymerized pigments index. It indicates the percentage of polymerized anthocyanins with tannins. As it can be seen in the Figure 76, with the decrease of the content of free anthocyanins, increases the percentage of polymerized pigments and vice versa. The two parameters therefore seem related. The anthocyanin-tannins interactions lead to an improved colour stability, the drop that can be noticed at 45 days could be a sign of greater stability for all samples compared to control. It could be possible to hypothesize an indirect effect of the polysaccharides added on the anthocyanins-tannins interactions.

Polymerized pigments index

As already stated, the polymerized pigments index presented an initial increase, up to 15 days, and then decreased in the remaining 20 days (Figure 68). In this case, this index has presented differences in final values less apparent. The control presented a value very similar to other samples. Only the sample containing gum arabic at the content of 300 mg/L was slightly different with a PPI of 69.7% at 45 days while the control showed a PPI of 62.6 % and the lowest value was the one of the sample with CMC at 50 mg/L (61.30 %). In any case this parameter is a percentage index and should be considered with due caution with respect to absolute parameters, such as the anthocyanin content.

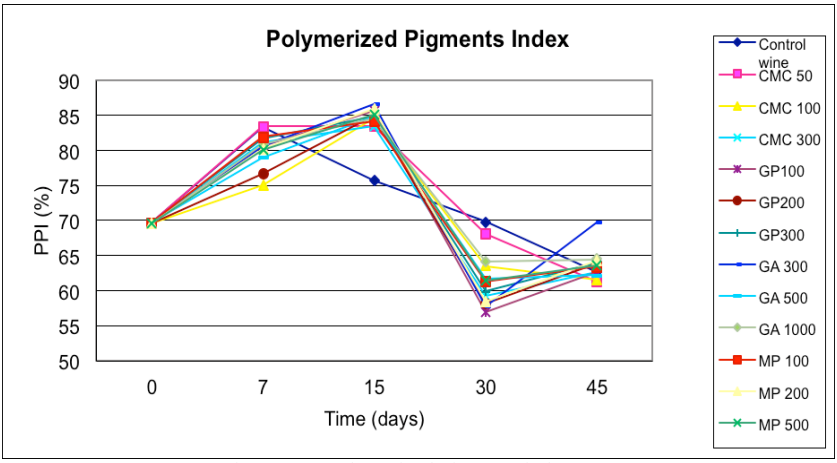


Figure 68 – Polymerized Pigments index (%).

Anthocyanins Oxidizability Index

The anthocyanins oxidizability index presented increasing values over time from 18% of the wine at time zero to a maximum of 56% for the sample containing gum arabic at a concentration of 1000 mg/L at 45 days (Figure 69).

For this parameter, even more than for the others so far evaluated, it was noticed a remarkably similar pattern for all samples, and the addition of polysaccharides to the samples did not seem to influence this parameter giving values higher up to a maximum of 4 % comapred to the control (53,5%).

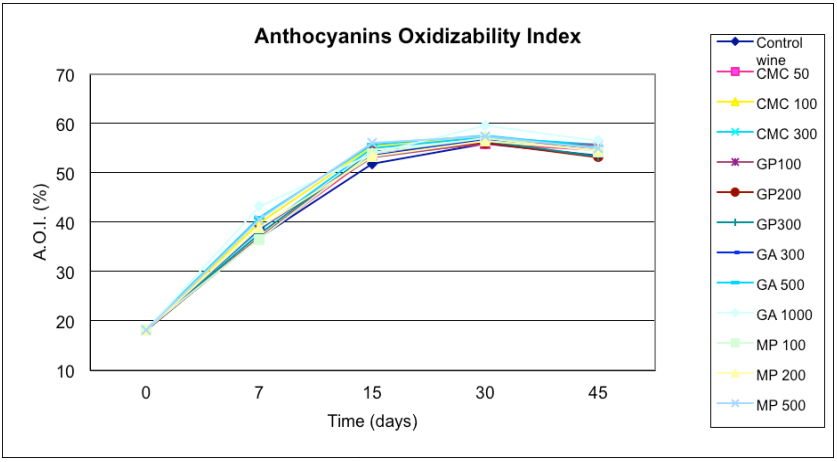


Figure 69 – A.O.I.

It was shown a positive correlation between AOI and colour intensity. Even if the colour intensity is higher at 45 days and the free anthocyanins content decreased over time probably due to interactions with tannins (Figure 70). This correlation could be explained thinking that the remaining free anthocyanins in solution, are more susceptible to oxidation, causing the A.O.I. increase, since it refers to the percentage of oxidizability of anthocyanins.

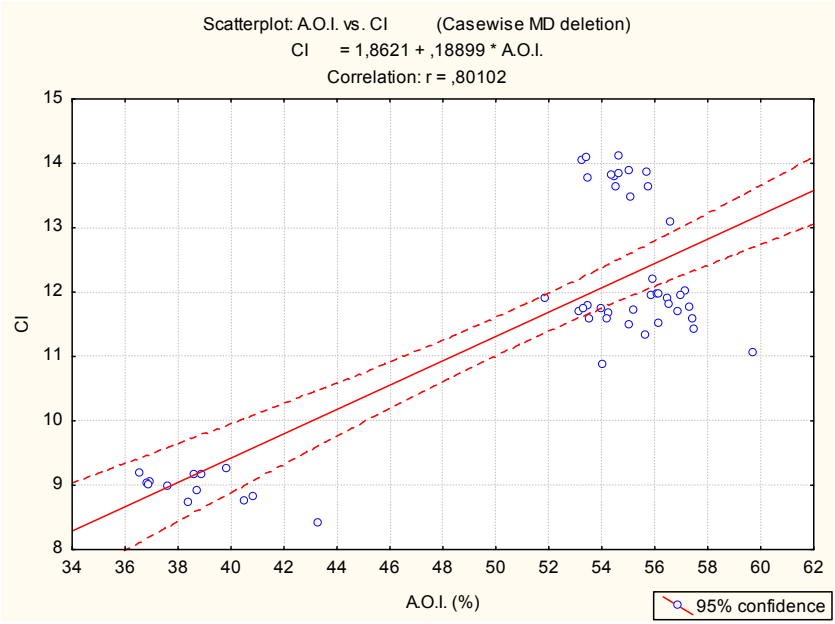


Figure 70 – Corralation between AOI and colour intensity.

The AOI represents the tendency of anthocyanins to oxidize, and tannins have antioxidant power, and thus the ability to protect anthocyanins from oxidation. Due to this, it would have been interesting to note differences between the samples to assess whether the interactions between tannins and polysaccharides could negatively affect their antioxidant activity.

Cathechins

The content of catechins showed a decrease in 45 days with a similar trend for all samples, including control (Figure 71). Since this is a parameter that evaluates the presence of non-polymerized catechins, the decrease could be associated to the tendency to the polymerization of these compounds.

That was more marked for most of the tests compared to the control (322 mg/L), reduction up to 47 mg/L for the sample added with gum arabic at 1000 mg/L (274 mg/L). These results can suggest that there is an indirect effect of the addition of

polysaccharides on the reduction in cathechin content, maybe favoring the tannin-anthocyanins interaction or tannin polymerization.

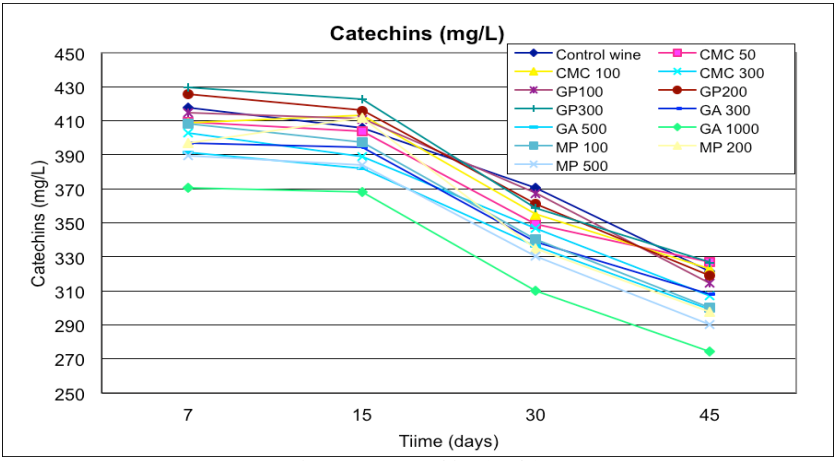


Figure 71 – Catechins (mg/L).

Turbidity

The addition of polysaccharides, generally, increased the initial value of turbidity comapred to the one of the control.

The turbidity before mixing the samples (Figure 72), showed an increase that could be related to the fact that the sediment particles were not completely settled back after every previous mixing. Anyway, it is possible to notice that the control turbidity (BM) at 45 days was higher than most of the ones of the other samples, with the exception of the samples with mannoproteins at 500 mg/L and CMC at 300 mg/L.

Over time there has been the formation of precipitate that increased the value of this parameter assessed after mixing the sample, especially at the time 30 and 45 days (Figure 73).

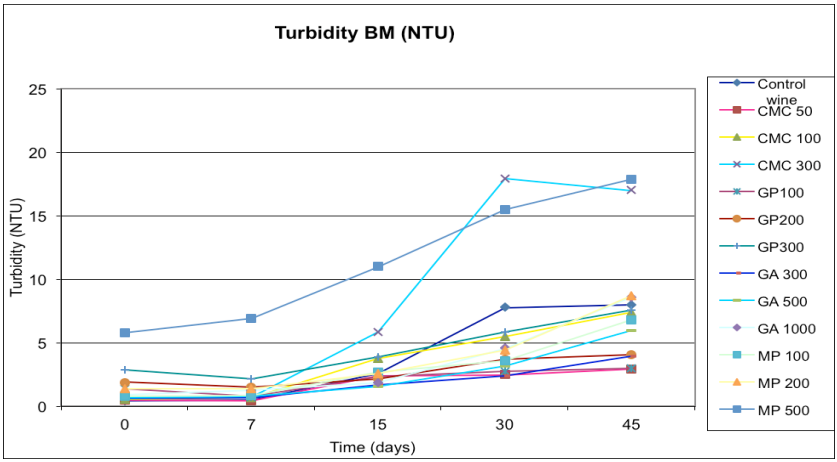


Figure 72 – Turbidity before mixing the samples. BM = Before Mixing.

At 30 and 45 days, however, the biggest differences in turbidity, especially after mixing the samples were noticed (Figure 73). It can also be noticed, how the polysaccharides added at higher concentrations affected this parameter giving the highest values.

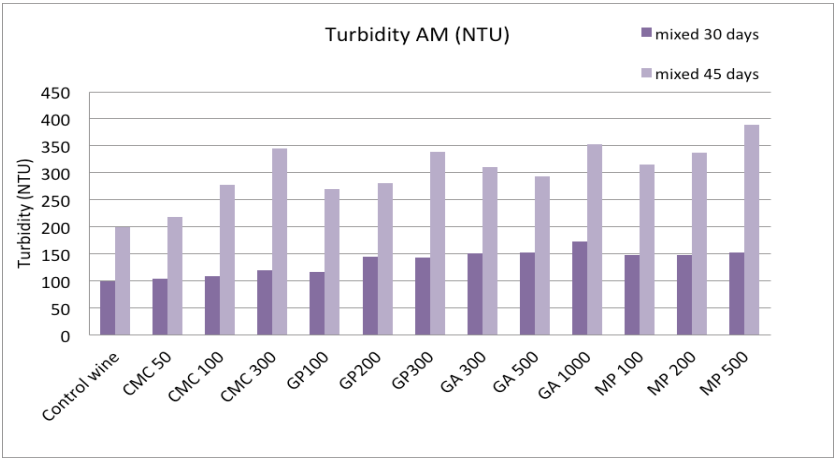


Figure 73 – Turbidity after mixing the samples at 30 and 45 days (NTU). AM = After mixing.

Particle Size

Considering the measure of particle size, it can be seen that all the samples presented a single distribution of the particle dimensions. This allowed to be able

to show the trend visually in figure 74. Missing data for MP 100 at 30 days for GA 300 and GA 1000 at 45 days are due to the fact that values were off scale for the instrument, indicating the presence of particles whose size was greater than the capabilities of the instrument. It was also noticed that this parameter is not correlated to turbidity. In fact, higher values of turbidity not always match the presence of large particles. In general only the addition of CMC to red wine, appeared to have maintained low values of particle size. All the other polysaccharides added appeared to have contributed to the formation of large particles.

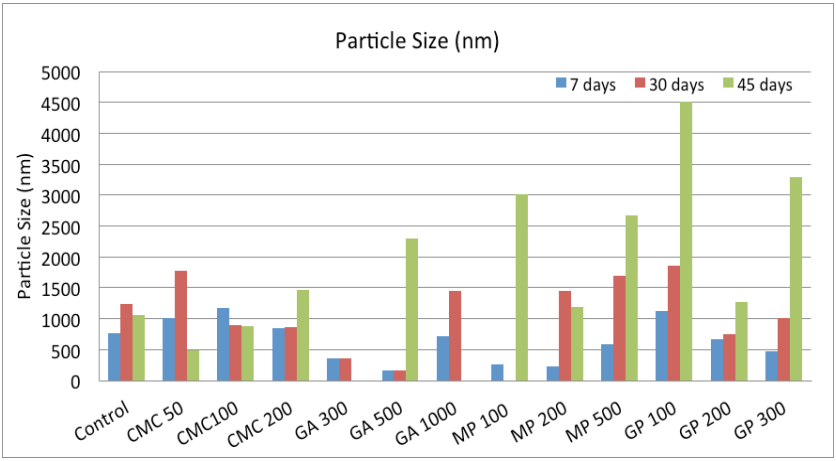


Figure 74 – Particle Size of red wine samples (nm).

The size of the macromolecular particles is an important factor to consider because it is linked to the colloidal stability of a wine over time, large particles could be separated easily from the system and give turbidity or sediment.

Surface Eletrical Charge

The measurement of surface electrical charge at 45 days showed no significant differences due to the addition of polysaccharides, only the addition of gum arabic has led to an increase in negative charge (Figure 75). What it is surprising is the fact that the measure of the charge before and after mixing the samples provides about the same result, leading to the conclusion that the precipitate does not appear to contribute to the total surface electrical charge.

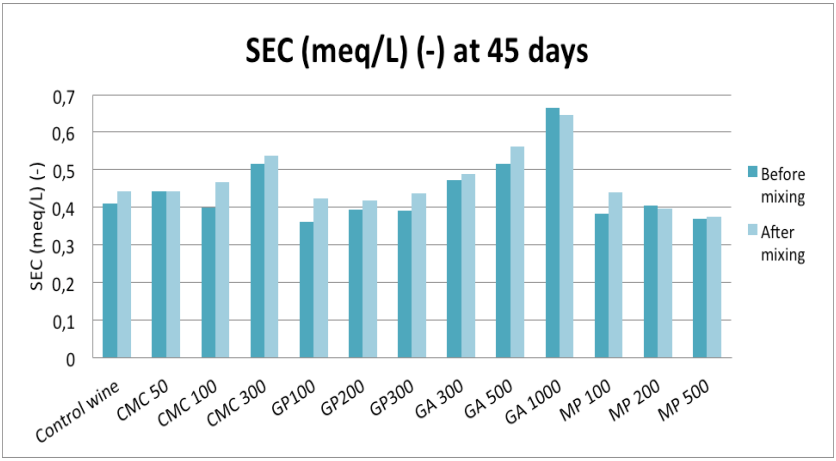


Figure 75 – Surface electrical charge (meq/L of solution) (-).

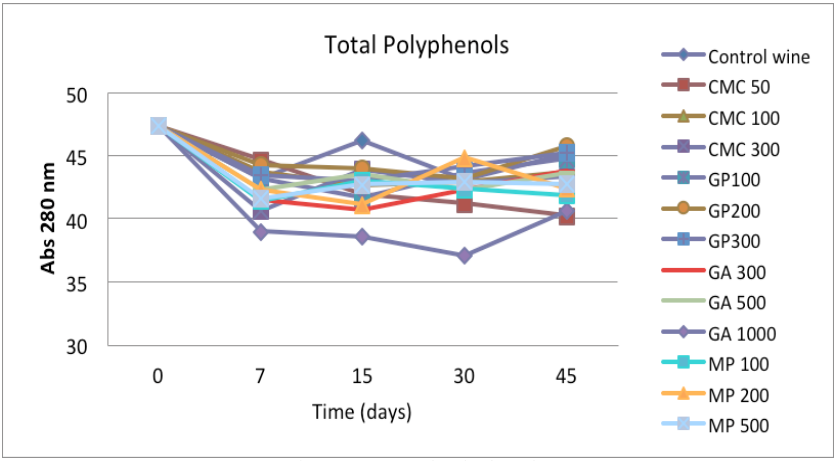


Figure 76 – Total polyphenols.

Total polyphenols

The total polyphenols index (Abs 280 nm) showed only a slight decrease in the value for all samples, probably due to a normal reduction over time in red wine caused by oxidation or precipitation (Figure 76). In particular samples added with CMC at 50 mg/L (40.2) and with gum arabic at 1000 mg/L (40.6) showed the lowest values compared to the control (45.1). The addition of polysaccharides in these conditions maybe caused a decrease in total polyphenols if compared to the wine with no additions.

Colour Intensity

The colour intensity showed an increase over time for all samples, from a value around 8 to about 14 (Figure 77). This increase is not related to an increase of the OD 420 nm but to the increase of the value of the OD 520 nm, which is, numerically, an unexpected result. Thus, these data are hard to be explained since they cannot be related to the oxydation of the colouring compounds of the wine.

In conclusion, the addition of polysaccharides did not result in significant differences on the parameters of colour, this allows, therefore, to use the different polysaccharides for their other stabilizing functions without any loss of colour.

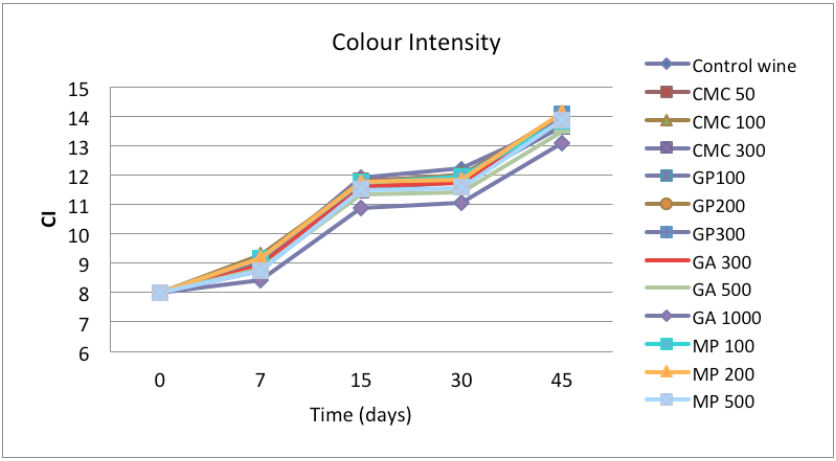


Figure 77- Colour Intensity

In red wines, the addition of polysaccharides slightly influenced positively the colour component. This is an interesting information in anticipation of their use as stabilizers products. However, in red wines it is difficult to define the colour trends because they are governed by kinetics that are generally longer than the 45 days evaluated and because they are difficult to predict.

6.2 Analysis for white wine added with tannins and polysaccharides

Total Polyphenols

The assessment of total polyphenols carried out on samples of white wine with tannins and polysaccharides added, clearly showed an increase in absorbance at 280 nm compared to the control, that is due to the addition of the tannin and the polysaccharides. The trend of this value in 45 days, however, does not show substantial differences as can be seen in Figures 78a, 79a and 80a related to the measurement of total polyphenols for samples containing the three types of tannin. While the range of values was similar for the tests with the three different tannins, a small difference in the pattern of samples added with grape skin tannin was noted, in fact, they presented a more pronounced decrease in the first 15 days.

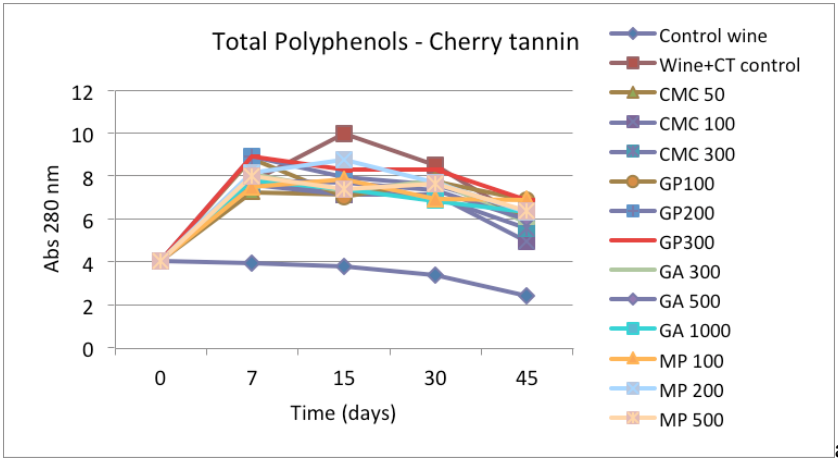
Since it was hard to show all the differences in figures 78a, 79a and 80a, it was chosen to use other figures representing the same parameter for the control and the control added only with the tannin and for each graph the values related to only one of the polysaccharides at the three concentrations. These figures are useful in comparing the data obtained and in showing if there is a polysaccharide concentration effect on the parameters evaluated. Graphs of the same type will be used throughout the remaining of this chapter and in chapters 6.3 and 6.4.

From figures 78 b, c, d and e it is possible to confirm what said before. After the initial increase of the values due to the addition of tannins and polysaccharides, more pronounced for samples containing grape polysaccharides, there was a devrease for all the values over time. This decrease is more evident for samples containing CMC at the three concentrations (Figure 78b), showing values lower than the control wine and the control wine added only with the tannin.

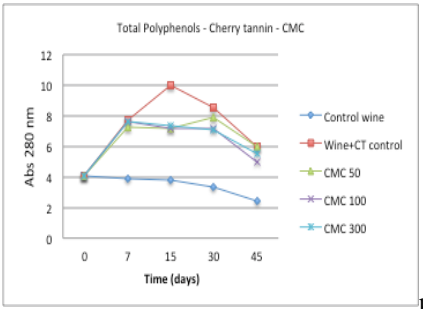
These additions bring a better polyphenolic structure to the wines compared to the control and even if there is a decrease in the values that indicates precipitation of the wine polyphenols, the decrease is smaller.

As for the samples containing the oak tannin, the same behavior of the ones with cherry tannin was noted (Figure 79a). In this case the addition of grape polysaccharides (Figure 79c) gave higher values only with the highest concentration, but the final values were all higher than the control wine and the control wine added only with the oak tannin The addition of CMC (Figure 79b) gave different results compared to the samples with cherry tannin, giving a higher decrease at 45 days only when added at the concentration of 50 mg/L.

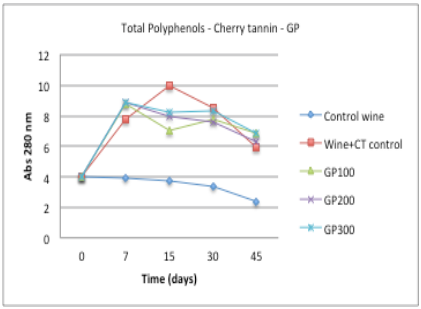
As already stated, samples with grape skin tannin showed the same behavior for the initial additions, but then they had a more rapid decrease of the values already after 15 days (Figure 80a). At 45 days the values obtained were similar to the ones of the samples added with the other tannins.



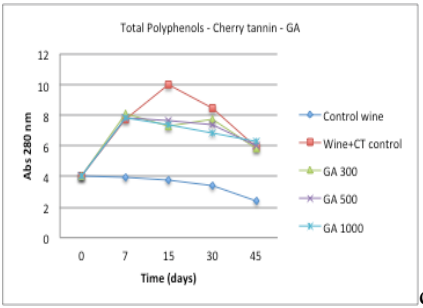
a



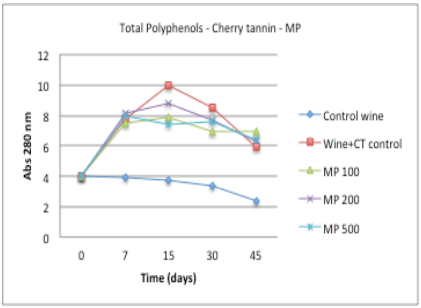
b



c

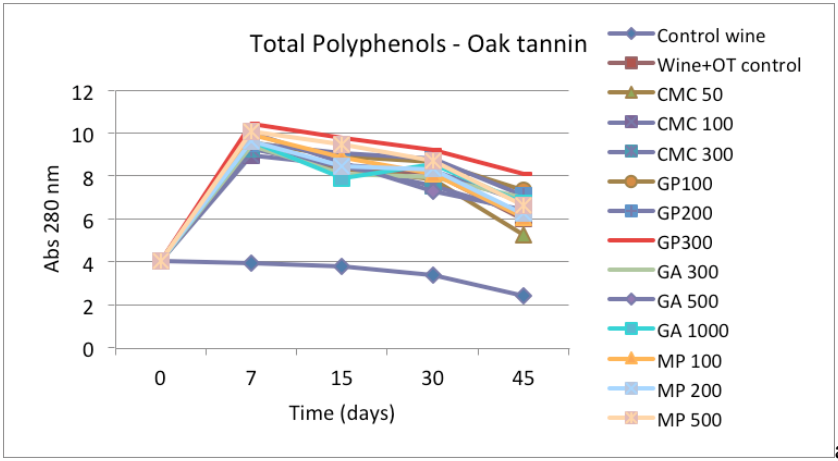


d

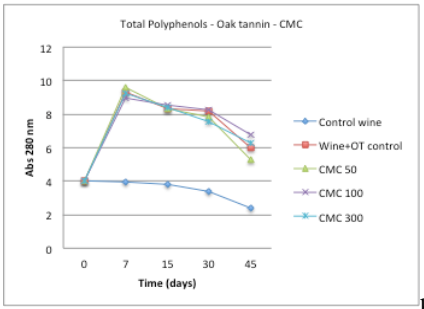


e

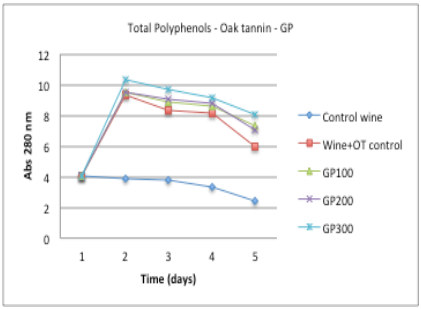
Figure 78 a b c d e - Total polyphenols in white wine added with Cherry tannin and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.



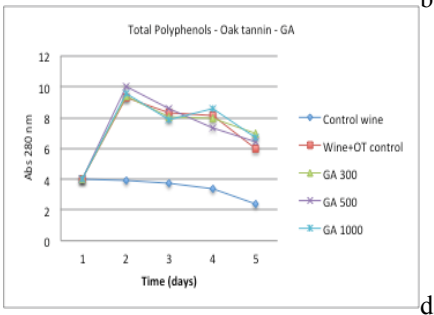
a



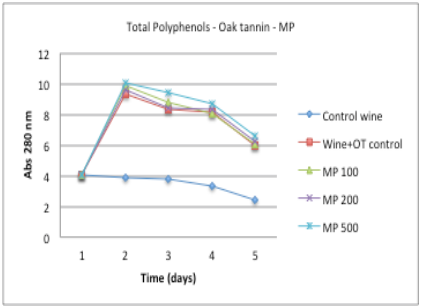
b



c

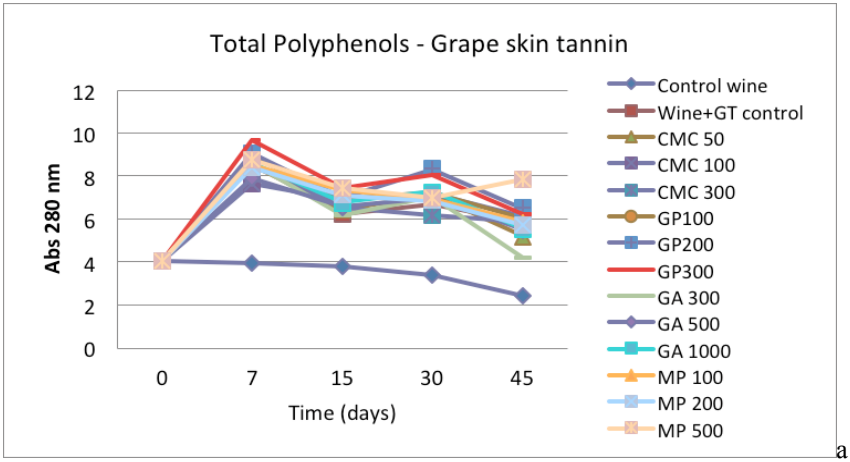


d

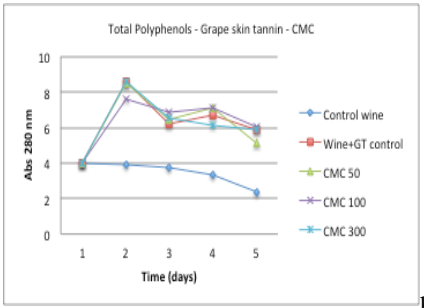


e

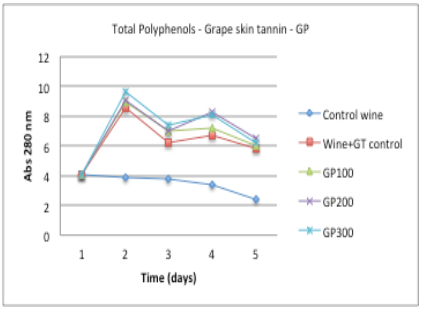
Figure 79 a b c d e - Total polyphenols in white wine added with Oak tannin and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.



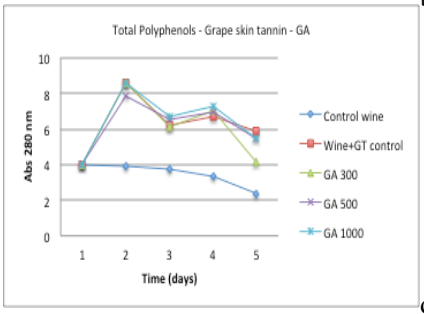
a



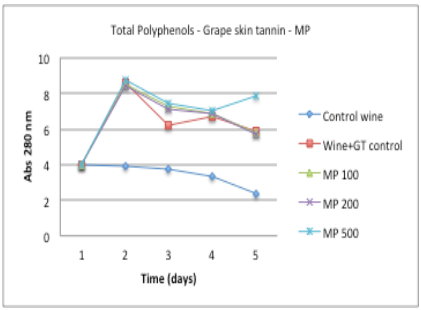
b



c



d



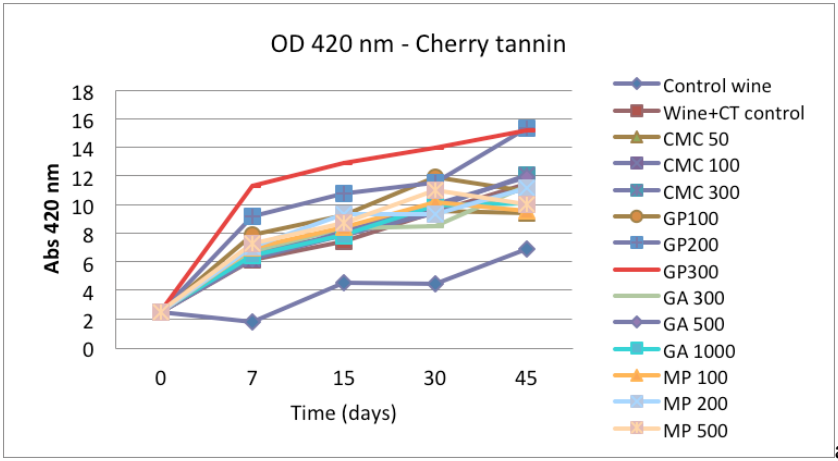
e

Figure 80 a b c d e - Total polyphenols in white wine added with Grape skin tannin and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.

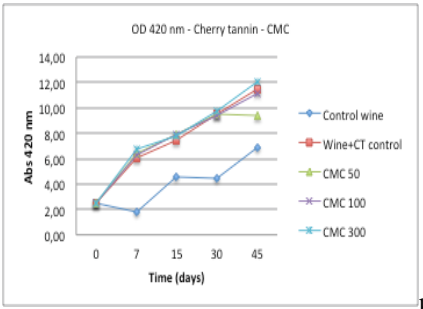
OD 420 nm

All the samples showed an increase of the value of this parameter after the addition

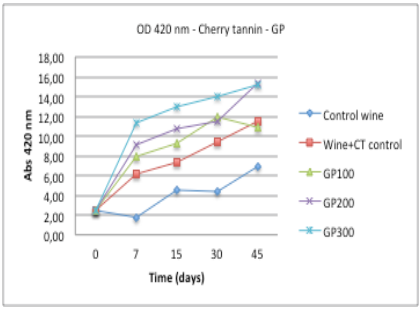
of tannin and polysaccharides. The values also increased over time (Figure 81a, 82a, 83a).
The addition of grape polysaccharides to the wine with cherry tannin, gave higher values of the absorbance at 420 nm compared to the control and the samples added with the other polysaccharides
In the case of the wine with oak tannin and grape skin tannin the values were higher than the ones of the samples with cherry tannin already after the addition, due to the darker colour of the first two tannins. In every case, generally, the behavior of the samples addes also with the different polysaccharides was similar. With oak tannin and grape skin tannin, in fact, the highest values were the ones of the samples added also with grape polysaccharides (Figures 82c and 83c)
This increase in absorbance at 420 nm for all samples with added tannin and polysaccharides compared to control, and the increase of this value over the 45 days may be associated with oxidation of the samples under storage conditions.



a



b



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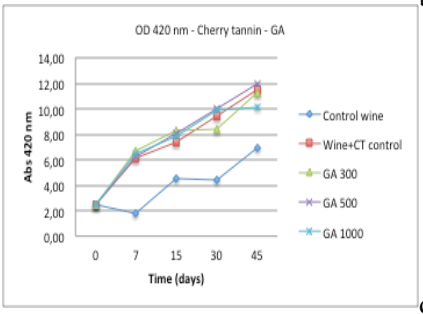

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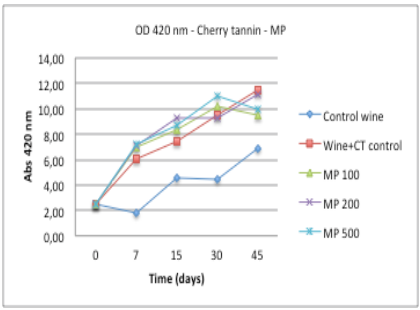
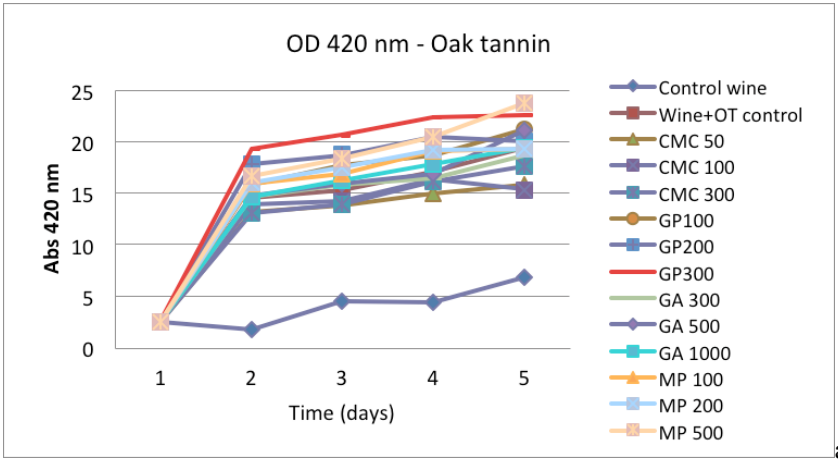
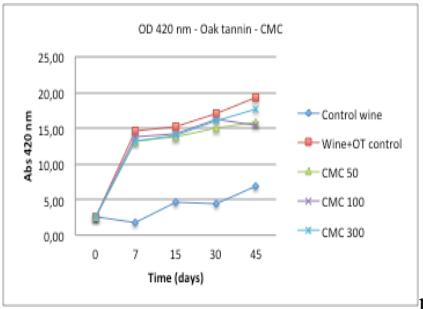


e

Figure 81 a b c d e – OD 420 nm of samples with Cherry tannin and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.



a



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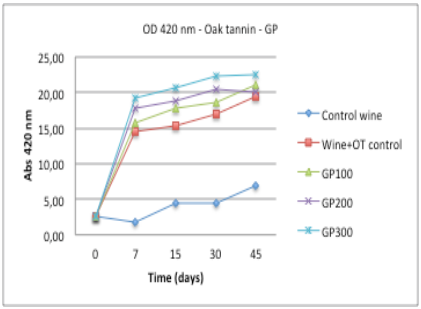

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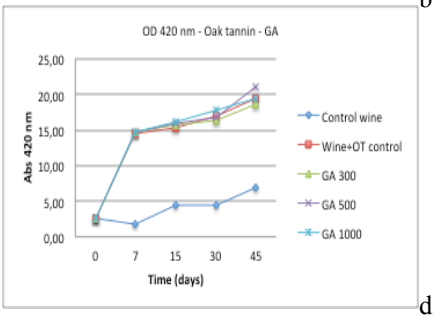
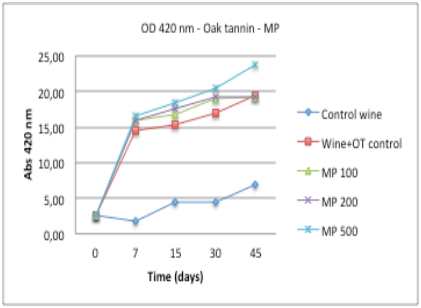
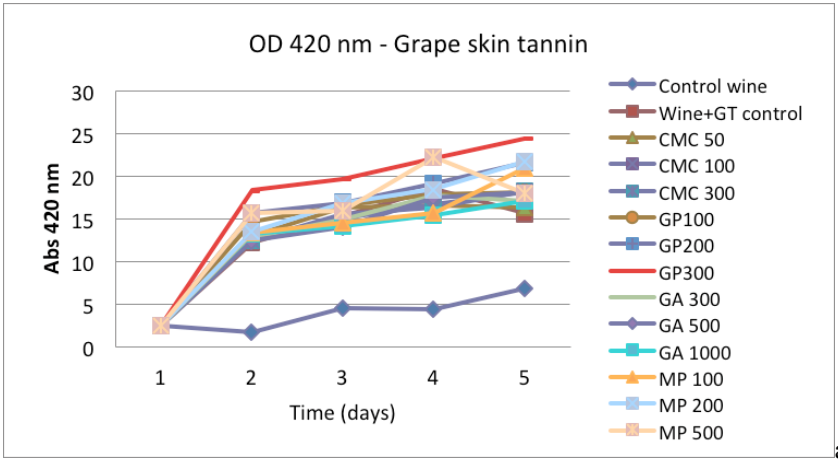


d

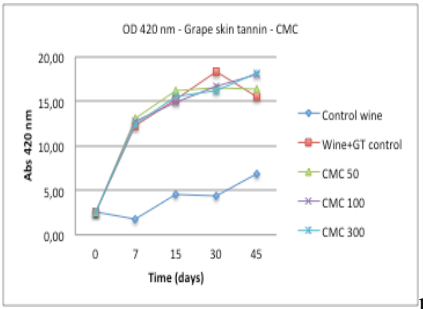


e

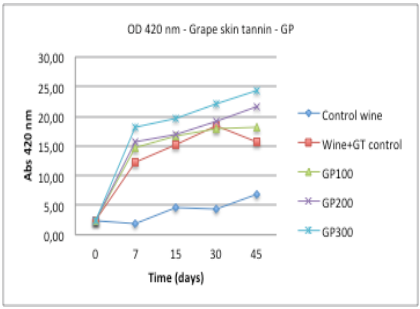
Figure 82 a b c d e – OD 420 nm of samples with Oak tannin and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.



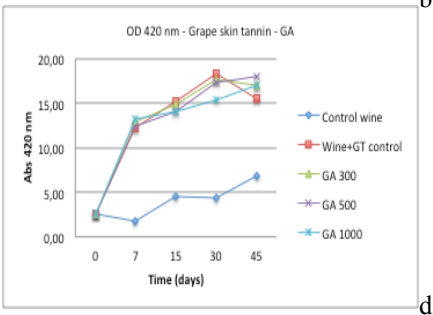
a



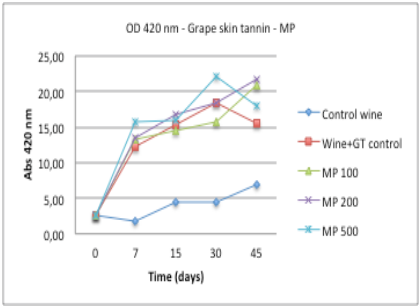
b



c



d



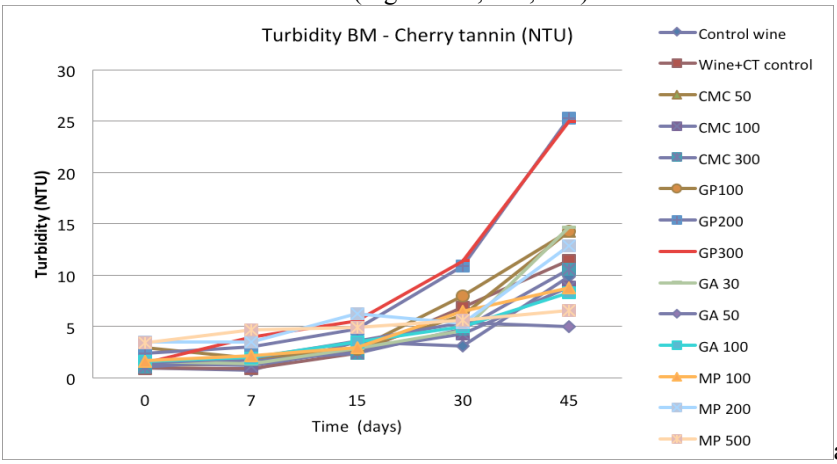
e

Figure 83 a b c d e – OD 420 nm of samples with Grape skin tannin and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.

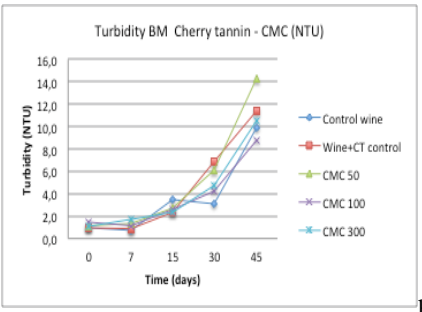
Turbidity

The turbidity parameter was considered for all samples both before and after mixing the samples. In order to check the possible increase in turbidity due to sediment presence. Regarding the sample before the mixing, in general it is

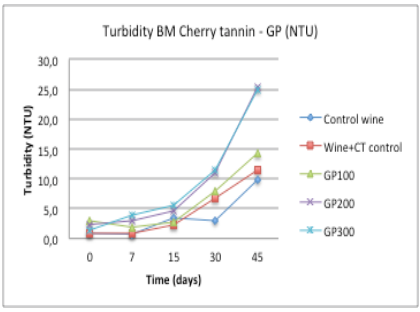
possible to affirm that the addition of oak tannin has made an initial increase in turbidity greater than the grape skin tannin and the cherry one, which actually does not seem to differ from the control (Figure 84a, 85a, 86a).



a



b



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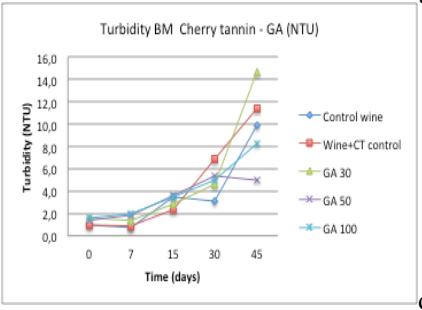

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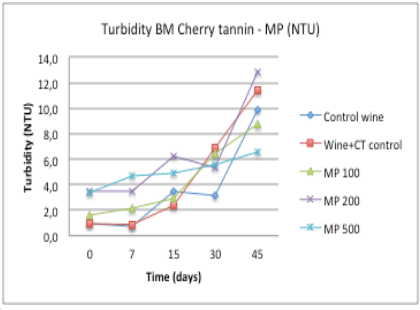
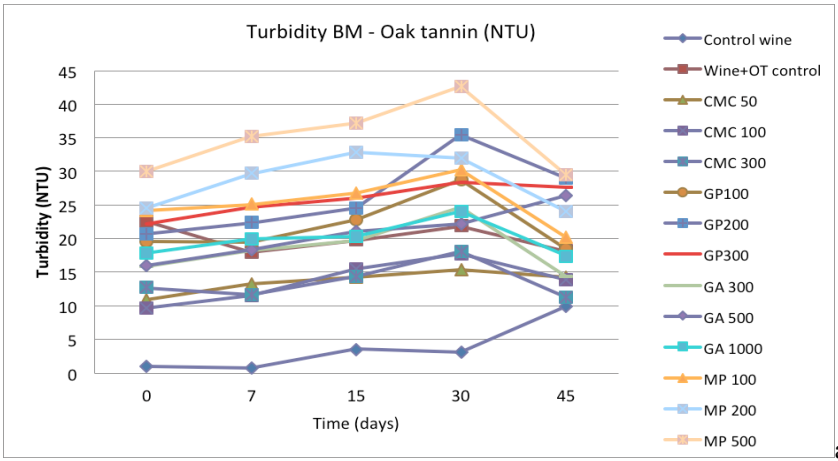
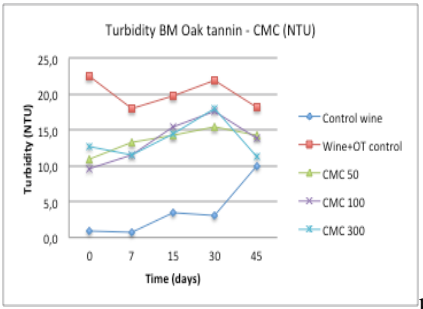


e

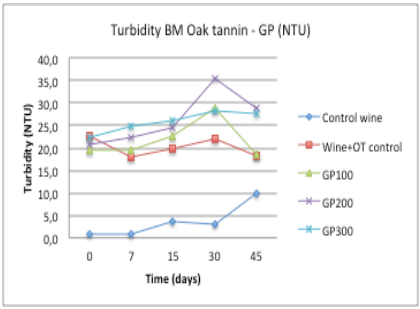
Figure 84 a b c d e – Turbidity (NTU) before mixing the samples with Cherry tannin and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP (BM = before mixing).



a



b



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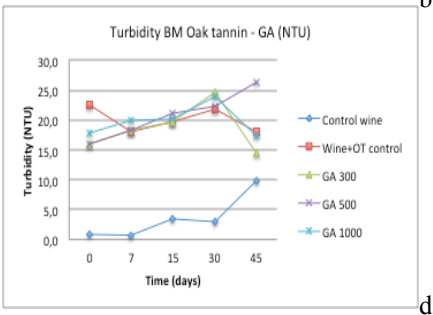

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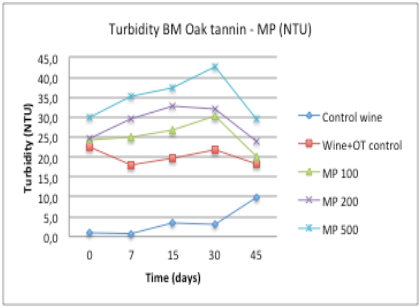
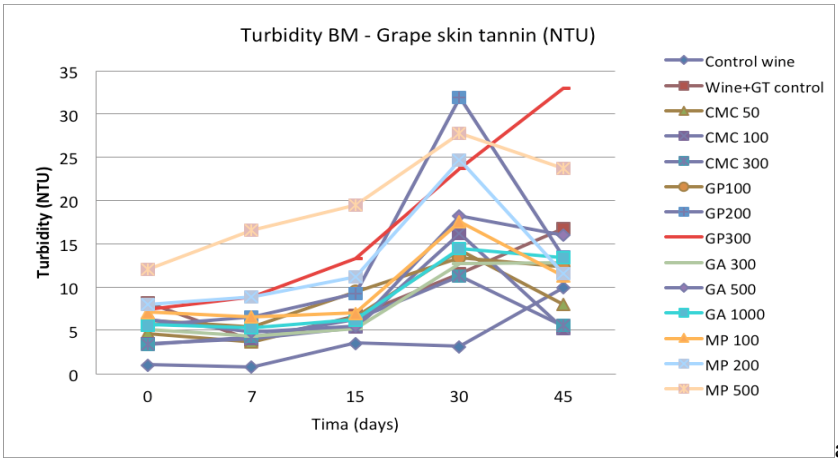
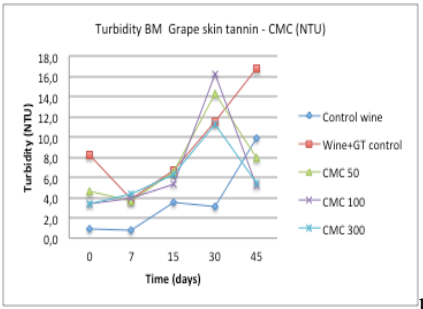


e

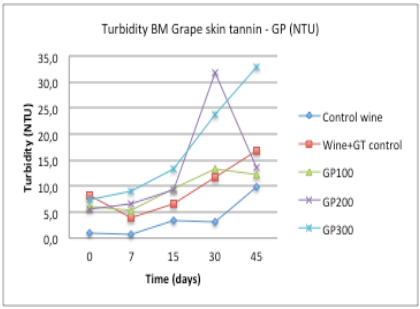
Figure 85 a b c d e – Turbidity (NTU) before mixing the samples with Oak tannin and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP (BM = before mixing).



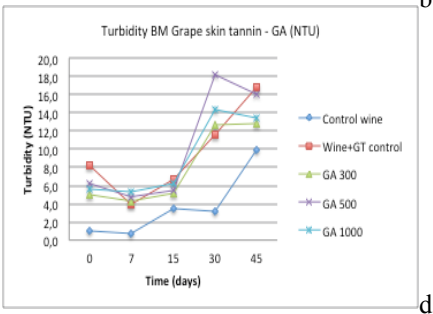
a



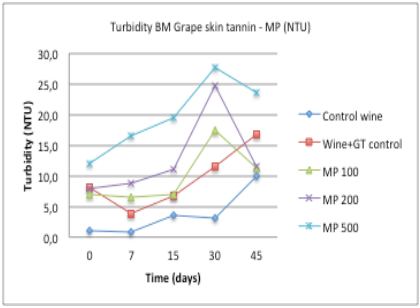
b



c



d

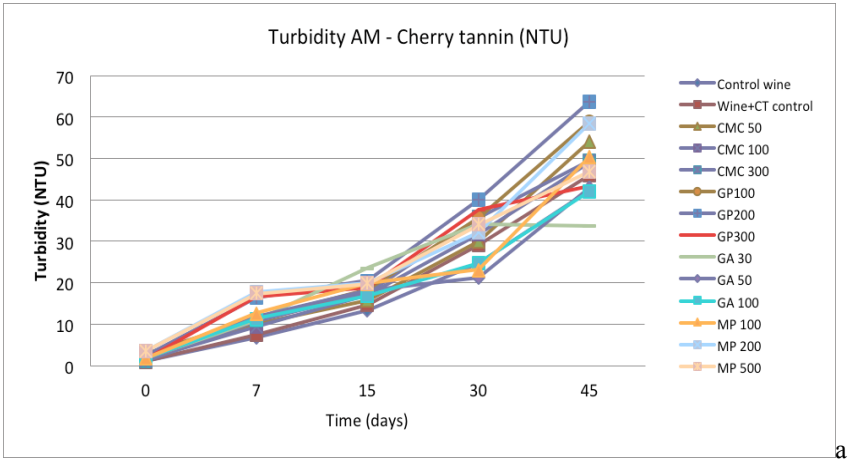


e

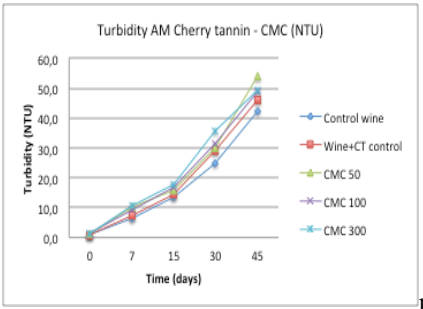
Figure 86 a b c d e – Turbidity (NTU) before mixing the samples with Grape skin tannin and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP (BM = before mixing).

The wine with cherry tannin and the different polysaccharides did not show big changes in turbidity (84 b, c, d, e) indicating that this type of tannin had a lower

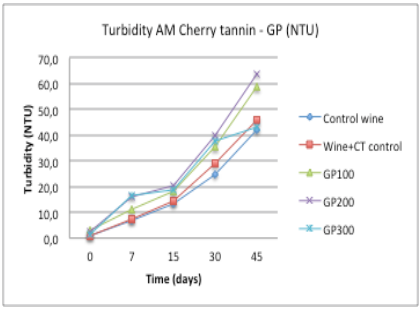
tendency in interacting with polysaccharides and in polymerizing.
For wine with oak tannin, the highest values of turbidity before mixing the samples were obtained from the ones added also with mannoproteins (Figure 85e).
Wines with grape skin tannin (86 a,b,c,d,e) slightly increased the values of turbidity and also in this case the highest values were related to the samples with mannoproteins (86e) and only with the sample with highest concentrations of grape polysaccharides (86c).
For all samples there is evidence of increased turbidity after shaking the sample, indicating that during the 45-day monitoring there was formation of sediment (Figures 87, 88, 89). In particular, the highest values of turbidity were noted in samples added with oak tannin (Figure 88). The tests with the addition of cherry tannin showed a similar pattern over time. At 45 days, samples with grape polysaccharides (Figure 87c) and mannoproteins (Figure 87e) were different by providing the highest values of turbidity. Even for samples with oak tannin the highest final turbidity was provided by samples containing grape polysaccharides (Figure 89c), while for samples with grape skin tannin the highest values were found in the samples added with mannoproteins (Figure 89e).
The difference with the control wine without additions is quite clear in samples with oak and grape skin tannin (figures 88 and 89).



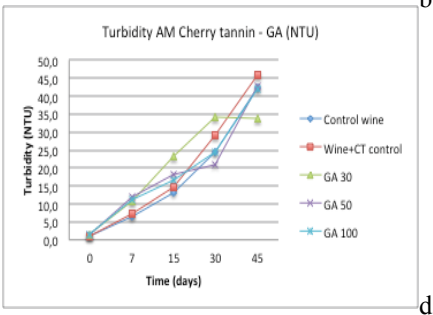
a



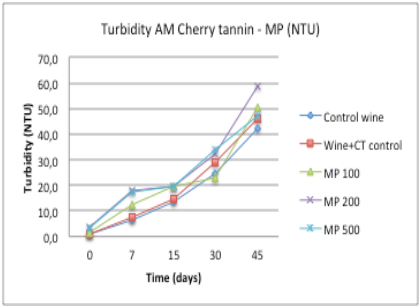
b



c

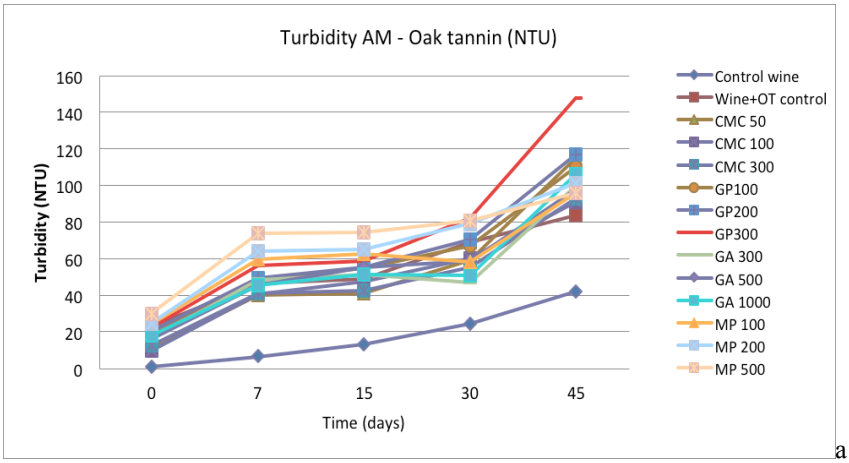


d

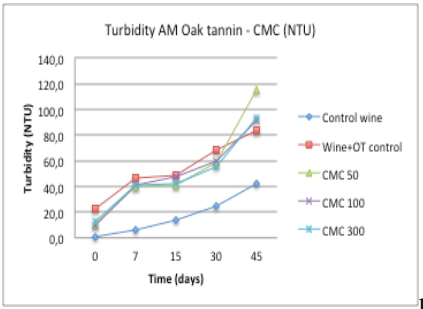


e

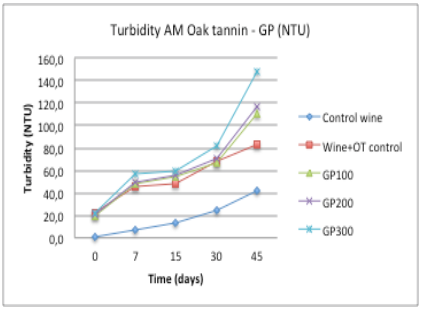
Figure 87 a b c d e – Turbidity (NTU) after mixing the samples with Cherry tannin and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP (AM = after mixing).



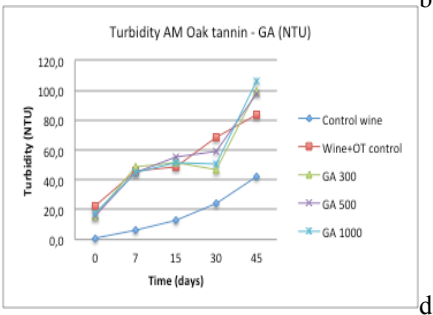
a



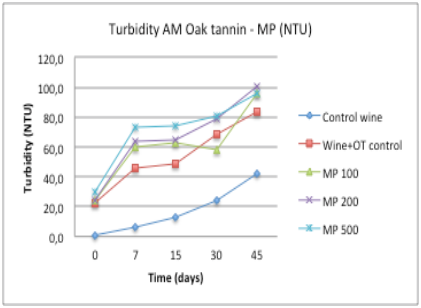
b



c

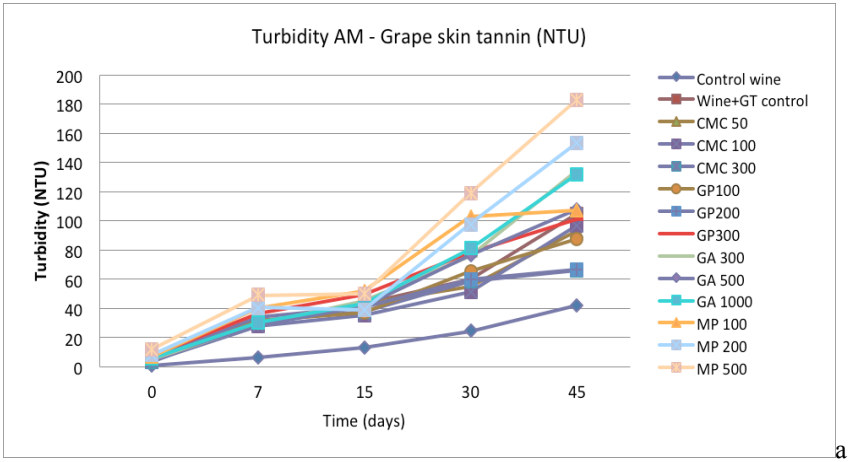


d

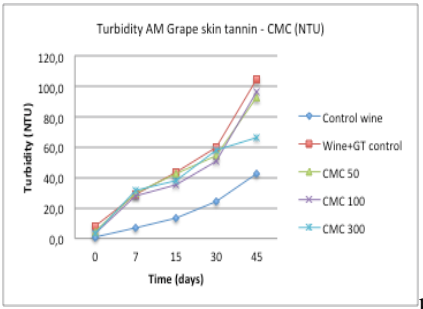


e

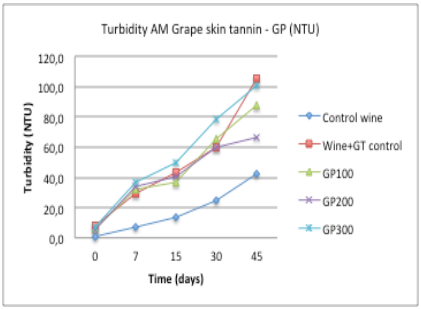
Figure 88 a b c d e – Turbidity (NTU) after mixing the samples with Oak tannin and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP (AM = after mixing).



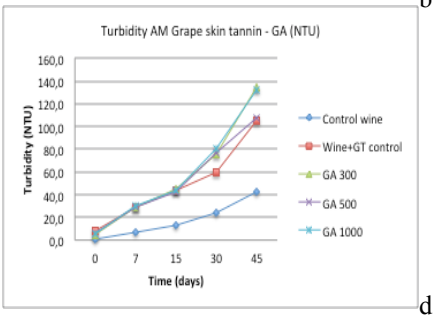
a



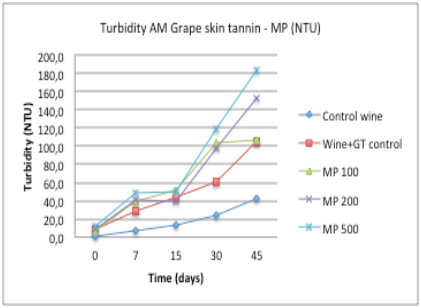
b



c



d



e

Figure 89 a b c d e – Turbidity (NTU) after mixing the samples with Grape skin tannin and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP (AM = after mixing).

The increase in turbidity after shaking is evidence of colloidal precipitation, confirming what was said in reference to polyphenols, while the increase in turbidity before mixing the samples could be associated to the formation of stable colloids in colloidal solution.

POM-test

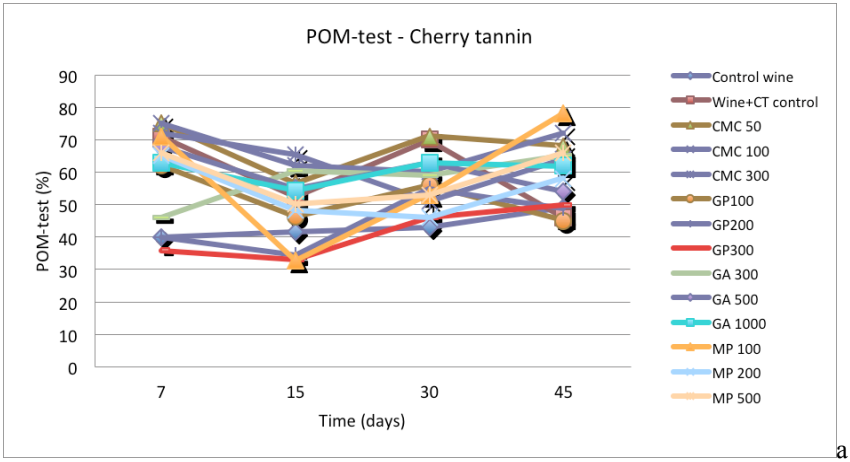
The evaluation of the parameter POM-test showed that the oak and grape skin tannins along with the polysaccharides can protect the white wine from oxidation (Figure 91 and 92). On the contrary, the addition of cherry tannin did not seem to provide an antioxidant effect (Figure 90). For samples with oak tannin, but especially for those with grape skin tannin there is a decrease of this value in the first 15 days followed by an increase, in particular for samples with oak tannin and MP 100, GA 300 and 1000 or CMC 50 and for samples with grape skin tannin and CMC 50 and 100, MP 100 and GA 300 and 500.

The results here obtained confirm what was found in the analysis on the chain-reaction activity of tannins in Part 1 of this research work. Cherry tannin in white wine was the one that provided less protection compared to oak and grape skin tannin. This also indicates that the polysaccharides added, did not interfere with the antioxidant activity of tannins.

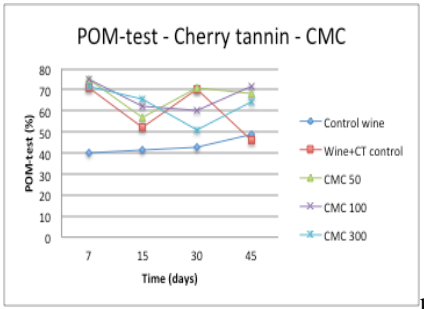
In the case of cherry tannin, the values obtained are variable, depending on the polysaccharide added. Samples with CMC (Figure 90c) showed values higher than the control and the wine added only with cherry tannin, indicating that this polysaccharide did not help in protecting from oxydation.

And the same result was obtained from the addition of gum arabic (Figure 90d) and mannoproteins (Figure 90e). Only the grape polysaccharides (Figure 90c) added to the wine with cherry tannin gave values similar to the control, showing nor a positive neither a negative effect.

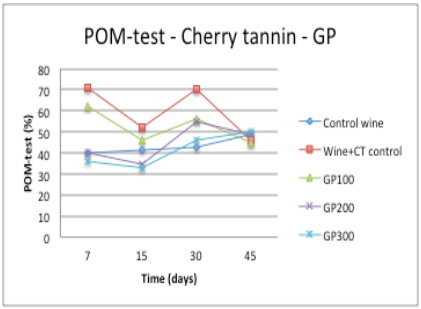
Regarding the samples added with oak tannin, the addition of grape polysaccharides (Figure 91c) gave the lowest final values, demonstrating that in this case its addition had a positive effect in the protection from oxydation, maybe due to a synergistic effect with the oak tannin. For the other polysacchairdes added, the protection from oxydation seems provided more by the oak tannin than by their addition (Figure 91 b,d and e).



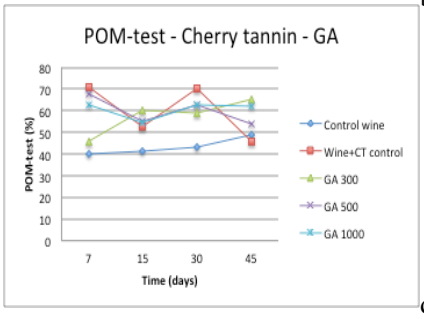
a



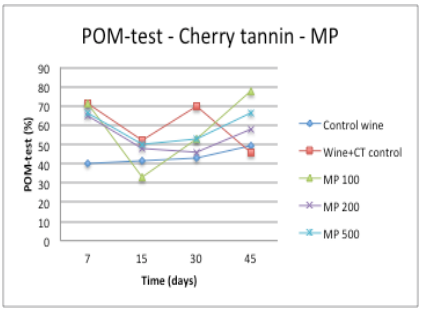
b



c

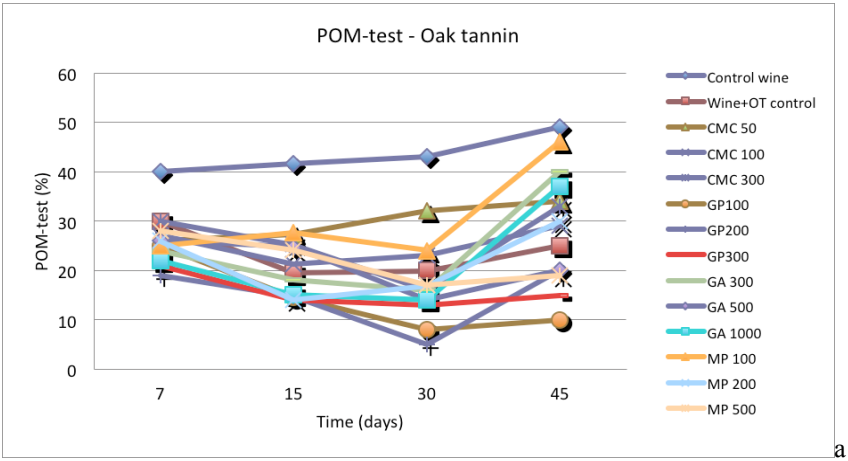


d

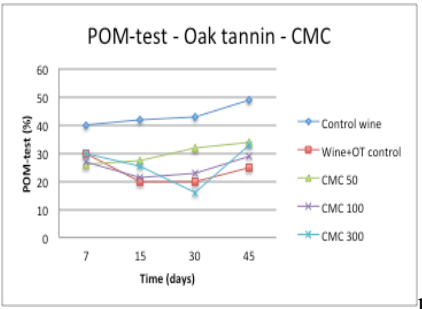


e

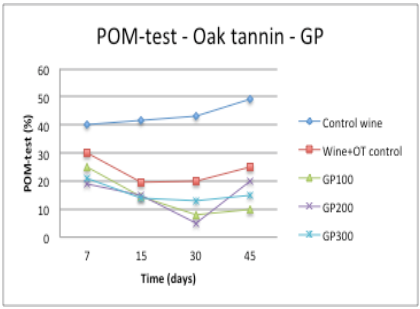
Figure 90 a b c d e – POM-test for the samples with Cherry tannin and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.



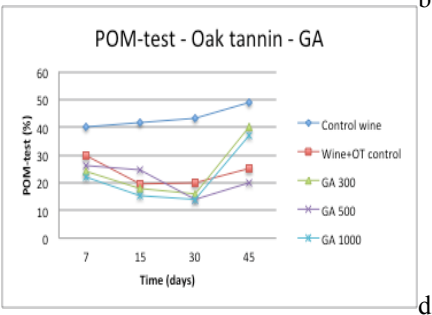
a



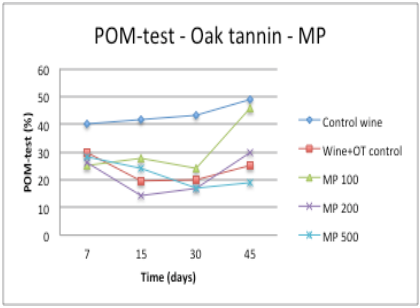
b



c

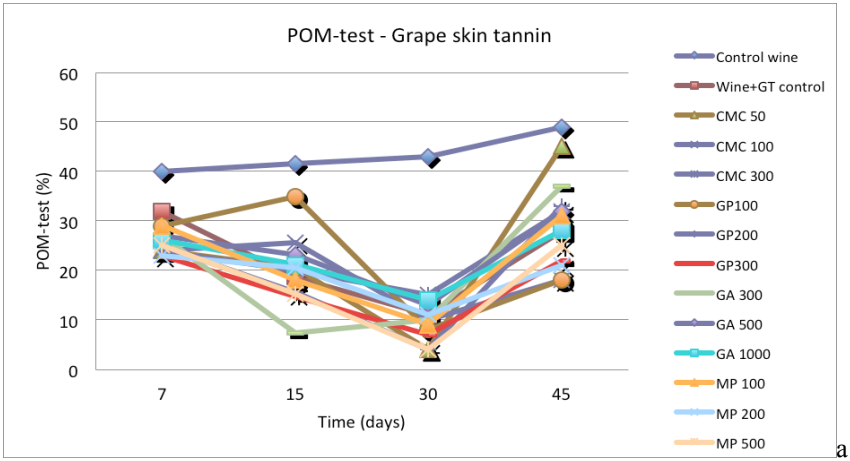


d

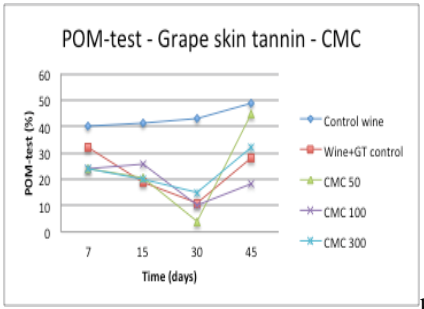


e

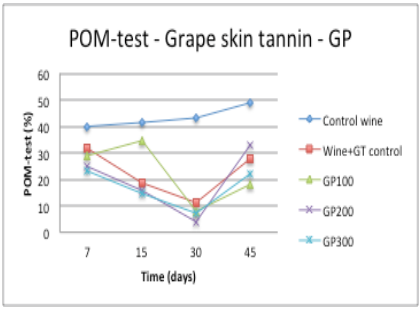
Figure 91 a b c d e – POM-test for the samples with Oak tannin and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.



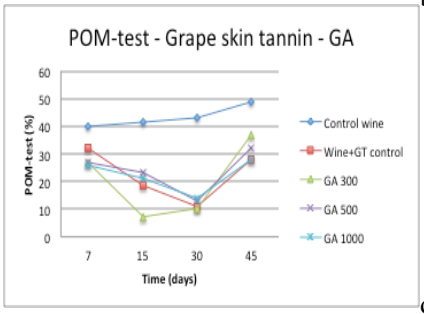
a



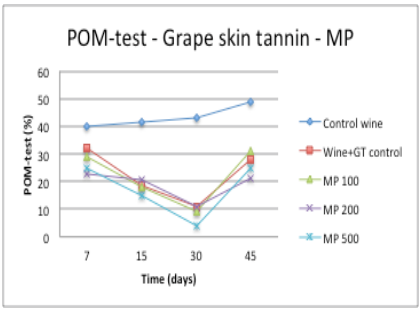
b



c



d



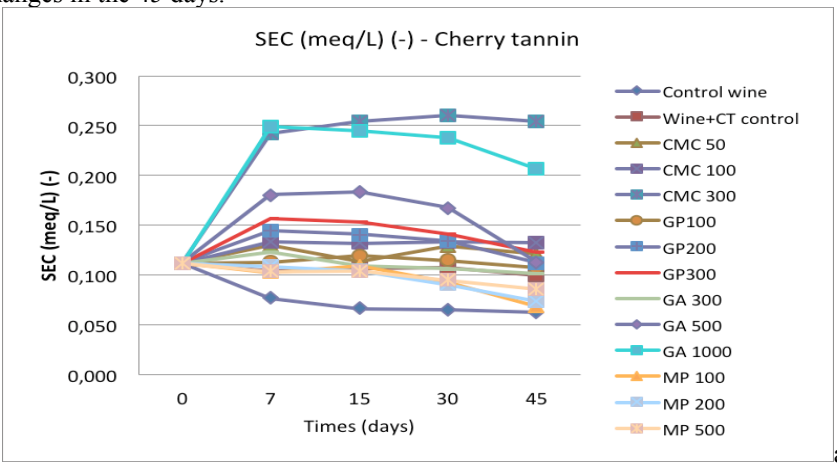
e

Figure 92 a b c d e – POM-test for the samples with Grape skin tannin and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.

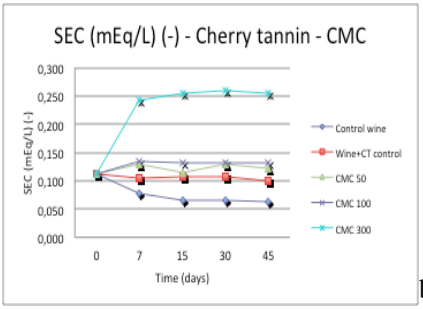
Surface Electrical Charge

The addition of tannin and polysaccharides has led to the increase of the surface electrical charge for all samples compared to control (Figure 93, 94, 95). In particular, the highest values were found for the samples with the addition of gum

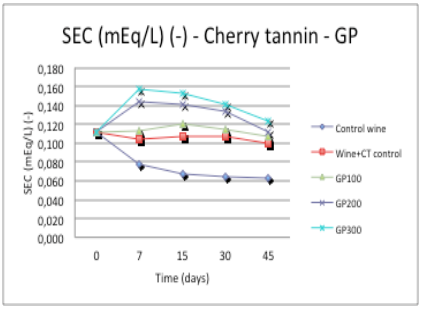
arabic (all the three concentrations) (Figures 93d, 94d, 95d) and carboxymethylcellulose at the highest concentrations (Figures 93b, 94b, 95b). It could be noted, however, how after the initial rise in value, there are no substantial changes in the 45 days.



a



b



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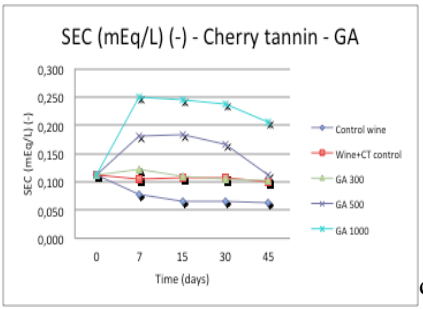
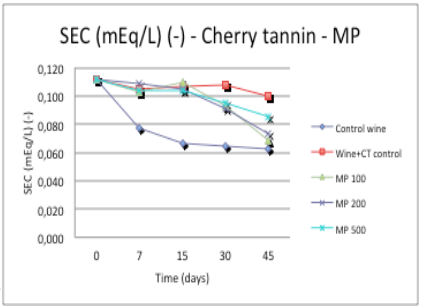
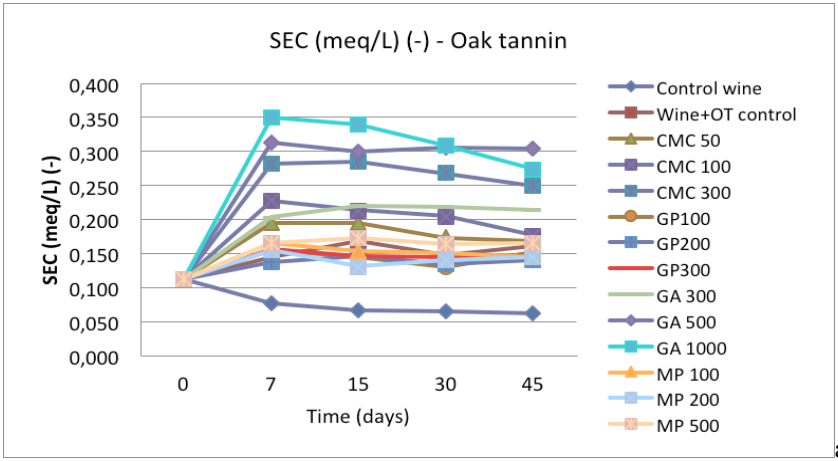


d



e

Figure 93 a b c d e– SEC (meq/L) (-) for the samples with Cherry tannin and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.



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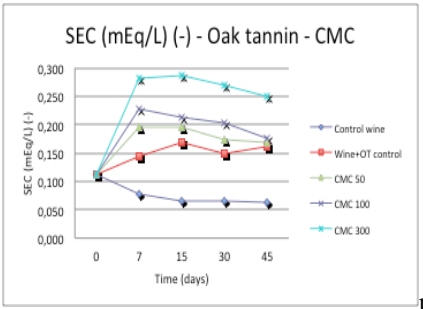

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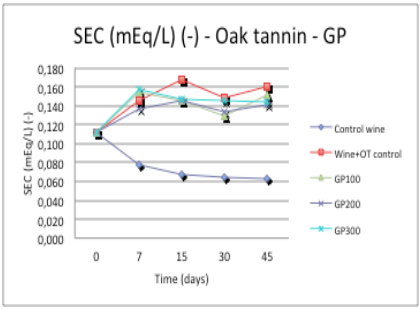

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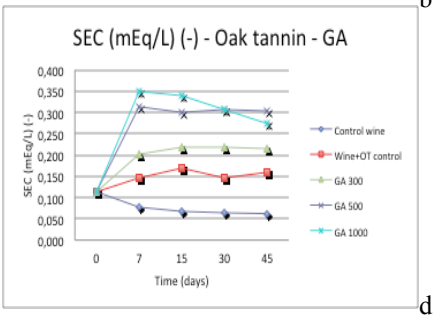
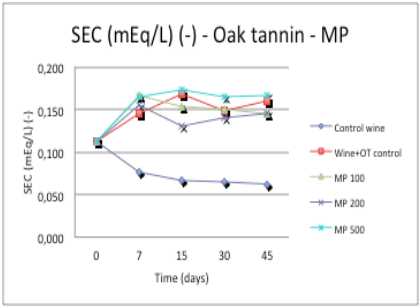
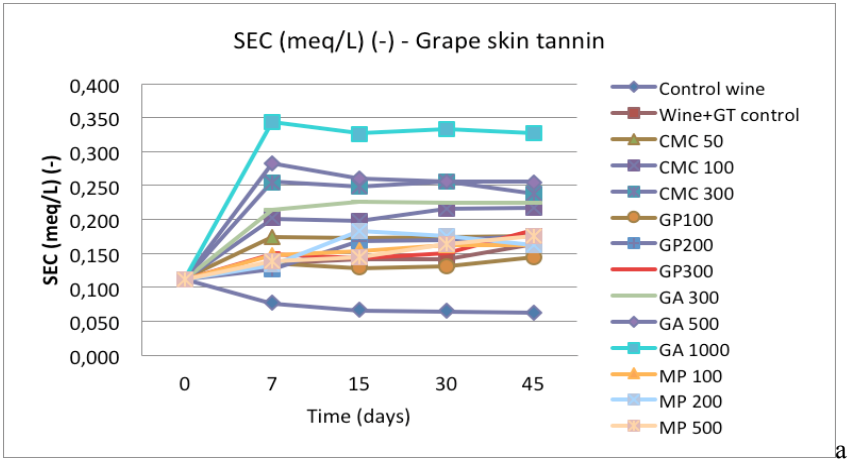


d

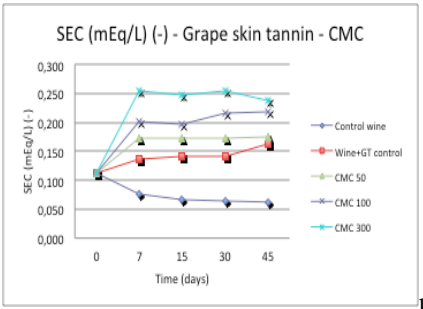


e

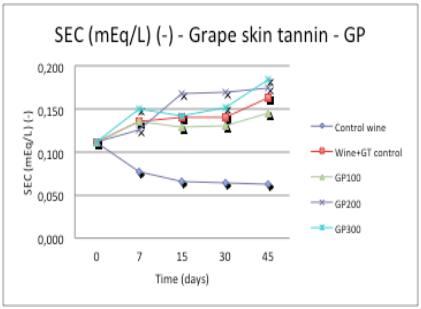
Figure 94 a b c d e– SEC (meq/L) (-) for the samples with Oak tannin and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.



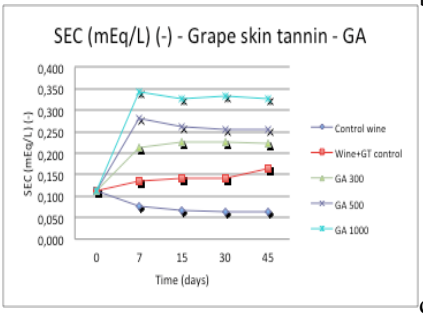
a



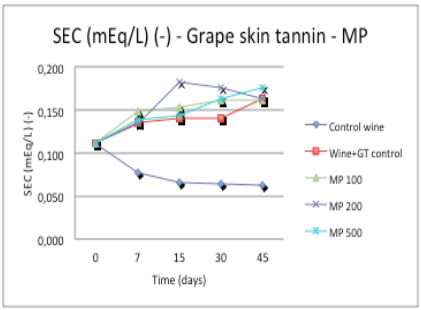
b



c



d



e

Figure 95 a b c d e– SEC (meq/L) (-) for the samples with Grape skin tannin and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.

This parameter seems, then, not correlated with turbidity, confirming that the precipitations, that occur due to the formation of aggregates, are probably only slightly influenced by the surface electrical charge.

Particle Size

Regarding the hydrocolloidal diameter, being difficult a graphical representation of results, related to the measure of more than one distributions of diameters of particles, for convenience it was preferred to report in Table 11 all values of sizes at 7 and 45 days and comment directly on the most interesting data.
The bold numbers are related to outliers values, which are beyond the capacity of the instrument, thus indicating the presence of particles whose size is greater than 5 microns.
All the particle sizes were measured after mixing the samples in order to obtain also the dimensions of particles that could have been present in the sediment.
Evaluating the data obtained it can be noticed that there is no unique trend in the variation of size between samples. The effect of a tannin and a polysaccharide added affects this parameter in different ways. For example, while with the cherry tannin, the addition of carboxymethylcellulose at all three concentrations leads to an increase in particle size, the opposite happens with the same addition but with grape skin tannin added to the wine. In this case, carboxymethylcellulose seems to favor the formation of smaller particles. The same effect is noted for samples containing oak tannin, but to a lesser extent.
In general, the addition of mannoproteins leads to an increase in particle size, while the addition of polysaccharides leads to an increase in size only in samples with cherry tannin, in the other samples, it favors the formation of aggregates whose size is smaller. This suggests, once again, that turbidity and particle size are not correlated, because the grape polysaccharides are the compounds that increase the turbidity the most, but do not favor the formation of larger particles.
The outliers, if confirmed, are evidence of the formation of large aggregates of macromolecules in the sediment.
It seems clear that the measurement of particle size is an important factor to be studied in order to optimize the processes of stabilization.

	Particle Size at 7 days (nm)						Particle Size at 45 days (nm)					
	Peak 1		Peak 2		Peak 3		Peak 1		Peak 2		Peak 3	
	Diameter	%	Diameter	%	Diameter	%	Diameter	%	Diameter	%	Diameter	%
Control wine	55	10,0	**8328**	90,0			2627	100				
Wine+CT control	90	16,0	1029	53,1	9191	30,8	11	93,4	2866	6,6		
CMC 50	11	100					2472	100				
CMC 100	11	98,8	1784	1,2			16	18,0	884	37,2	**7655**	44,8
CMC 300	548	100					37	10,8	1802	89,2		
GP100	18	12,7	800	11,7	**8692**	61,3	200	14,0	2614	86,0		
GP200	11	100					12	39,2	319	60,8		
GP300	696	100					58	4,0	145	9,8	1766	86,3
GA 300	144	3,9	4573	96,1			21	43,0	3040	57,0		
GA 500	119	100					11	97,9	2699	2,1		
GA 1000	11	94,5	340	5,5			82	6,6	519	6,5	3434	86,9
MP 100	175	13,8	2479	86,2			33	8,7	616	17,4	4709	73,9
MP 200	27	16,2	288	18,5	4897	65,3	87	9,7	1490	90,3		
MP 500	133	35,7	1715	64,3			33	16,5	850	24,7	**6283**	58,8
Wine+OT control	52	4,5	458	25,6	2965	70,0	87	5,1	882	39,3	**5883**	55,6
CMC 50	186	6,3	860	93,7			60	18,4	1835	81,6		
CMC 100	583	47,4	**5419**	52,6			492	100				
CMC 300	122	4,5	606	46,0	4528	49,6	71	16,3	1663	83,7		
GP100	252	12,5	642	31,9	3646	55,6	606	57,5	**5238**	42,5		
GP200	279	19,9	1872	80,1			60	4,7	995	95,3		
GP300	731	100					61	9,6	820	90,4		
GA 300	178	12,3	965	87,7			146	10,3	1500	89,7		
GA 500	249	18,8	1162	81,2			954	100				
GA 1000	11	7,0	63	4,1	331	88,9	36	11,7	552	26,4	3267	61,9
MP 100	313	34,6	1913	65,4			111	100				
MP 200	466	100					82	22,1	1000	77,9		
MP 500	399	71,5	**6811**	28,5			12	20,0	1219	80,0		
Wine+GT control	148	24,5	2154	75,5			33	17,1	2536	82,9		
CMC 50	250	23,5	4302	76,5			11	98,3	2669	1,7		
CMC 100	11	79,6	509	4,8	**6298**	15,6	37	10,5	1002	18,8	3502	70,6
CMC 300	127	16,2	1356	83,8			68	3,6	1110	96,4		
GP100	164	21,3	1286	78,7			11	74,4	1587	25,6		
GP200	256	24,9	2733	75,1			918	100				
GP300	338	34,2	2422	65,8			92	11,8	1398	88,2		
GA 300	11	89,0	225	2,1	714	8,9	49	100				
GA 500	51	10,0	686	44,1	**8708**	45,9	11	98,4	1431	1,6		
GA 1000	148	25,4	2757	74,6			34	11,0	1873	89,0		
MP 100	334	35,3	3380	64,7			28	16,3	402	5,0	2109	78,6
MP 200	291	100					68	3,1	644	35,2	2867	61,7
MP 500	154	30,3	311	69,7			55	6,3	698	59,1	**8702**	34,6

Table 11 – Particle size white wine added with tannins and polysaccharides.

6.3 Analysis for model wine added with grape skin tannin and polysaccharides

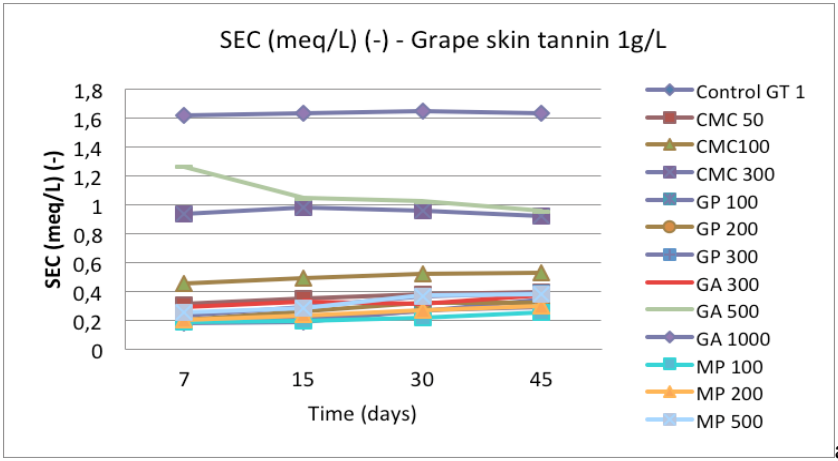
Surface Electrical Charge

With regard to the measurement of surface electrical charge, the increase of the concentration of tannin generally led to an increase of the electrical charge for all samples, to be noted however that in all the three cases the highest values were found in the samples with the addition of GA 500 and 1000 and CMC 100 and 300.

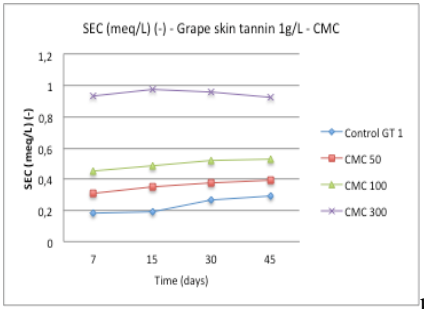
In the trial with grape skin tannin at 1 g/L the highest values were given by the samples added also with gum arabic (Figure 96d) and CMC (96b).

The same trend was found in samples with grape skin tannin at 2 and 4 g/L (Figure 97 d and c, Figures 98 d and c), but clearly the values were higher, due to the higher concentration of the tannin, that causes an increase in the negative charge.

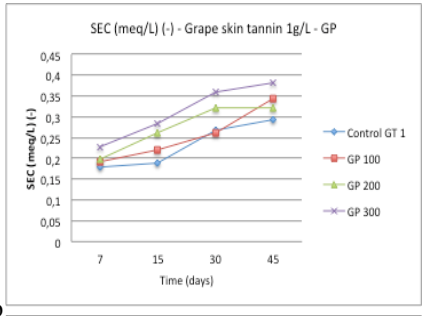
As it can be easily seen in Figures 96, 97 and 98, for all samples the surface electrical charge remains constant over time, it is unaffected, showing values with trends similar to those of control. This confirms what has already been found in tests with the white wine and red wine. Confirming that the interactions between the molecules do not appear to depend only on surface electrical charge.



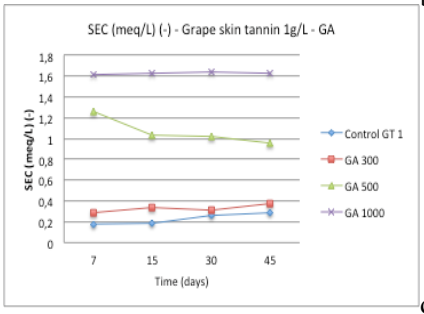
a



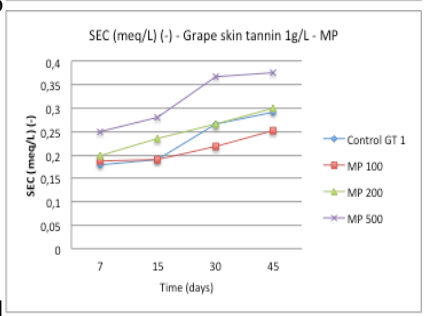
b



c

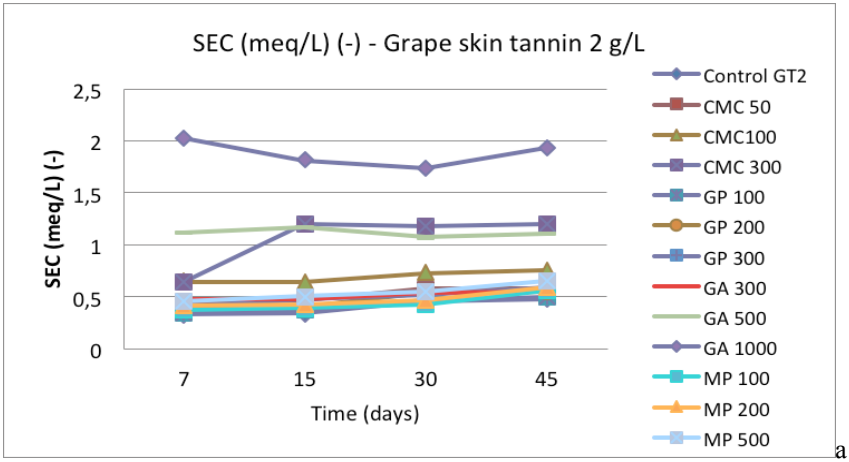


d

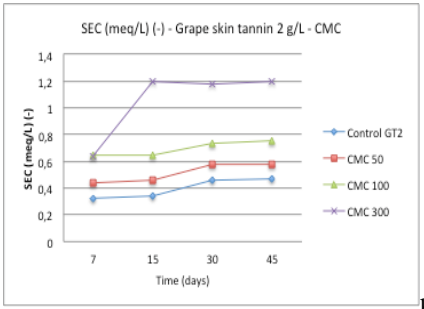


e

Figure 96 a b c d e– SEC (mEq/L) (-) for the samples of model wine with Grape skin tannin at 1 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.



a



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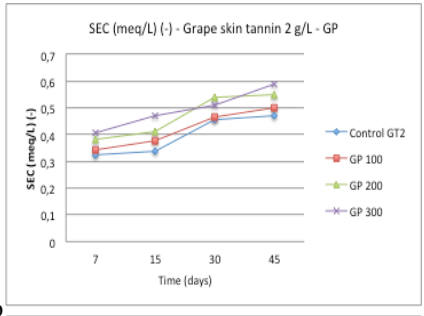
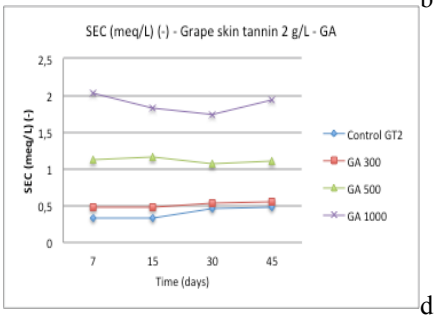
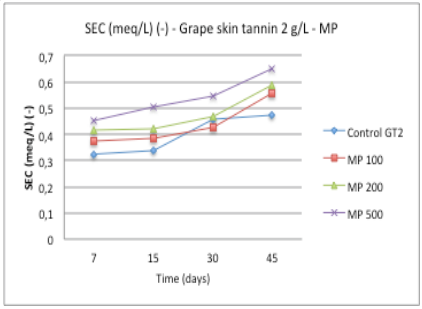


c

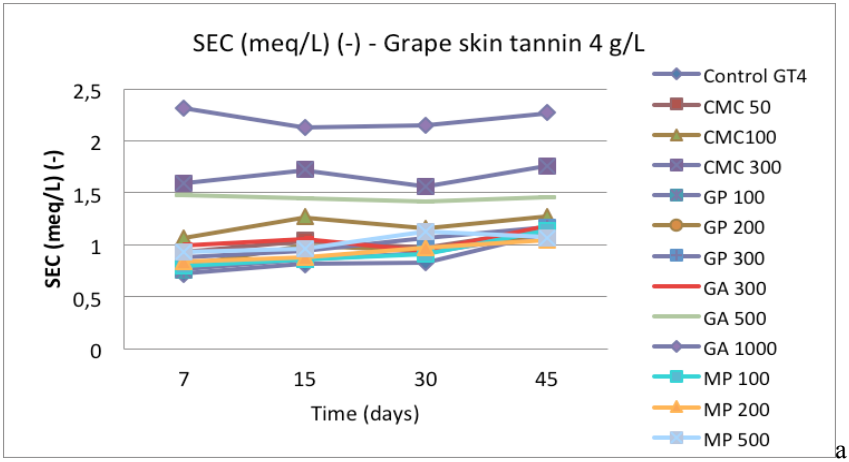


d

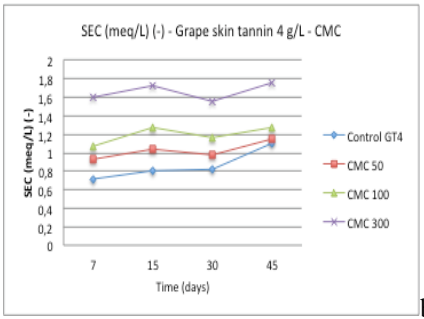


e

Figure 97 a b c d e– SEC (meq/L) (-) for the samples of model wine with Grape skin tannin at 2 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.



a



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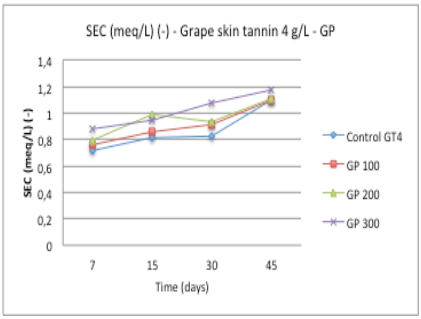

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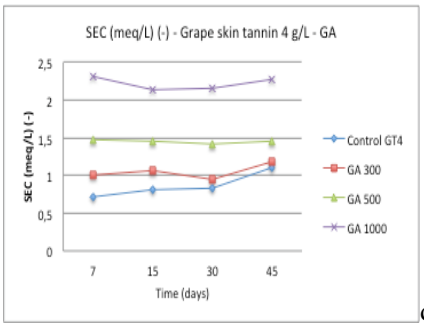
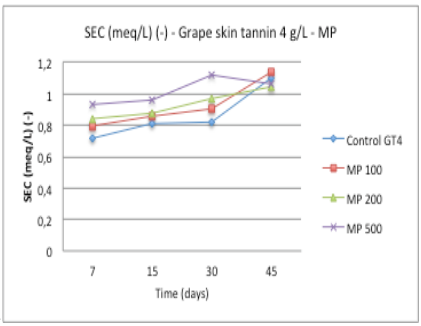


d



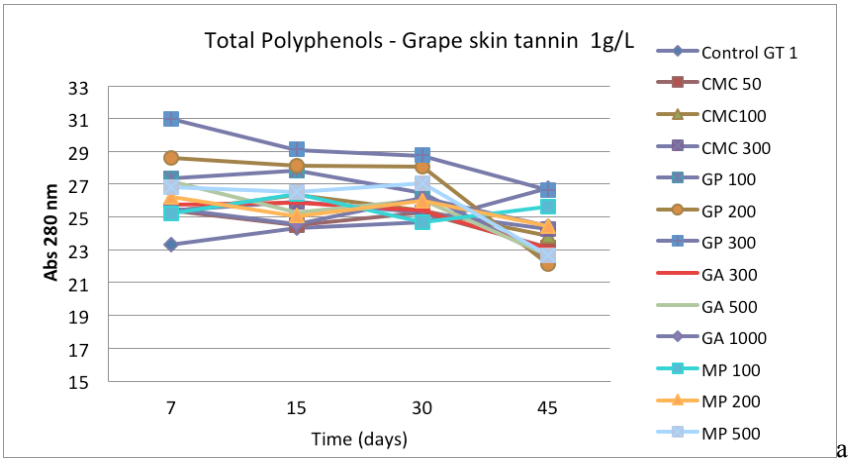
e

Figure 98 a b c d e– SEC (meq/L) (-) for the samples of model wine with Grape skin tannin at 4 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.

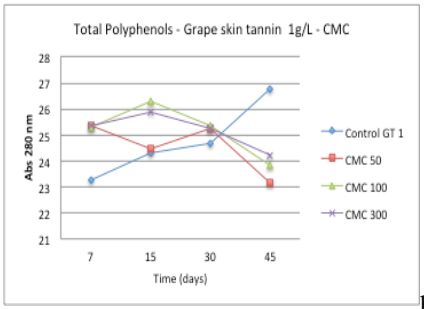
Total Polyphenols

The evaluation of the the total polyphenols through the absorbance measurement

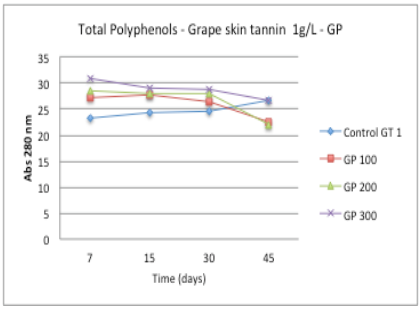
at 280 nm, clearly showed that a higher concentration of tannins implied a higher value of absorbance (Figures 99, 100, 101). But in general, the trends over the 45 days did not show large differences, indicating that the addition of various polysaccharides, does not seem to influence this parameter, as already shown in previous tests. Only at the concentration of grape skin tannin at 2 g/L the addition of grape polysaccharides showed slightly different and higher values (Figure 100c). The values decreased more over time for the parameters with tannin at the concentration of 4 g/L (Figure 101). This trend highlights the stabilizing role of the polysaccharides on polyphenols.



a



b



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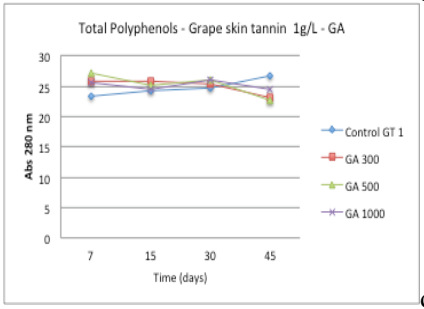

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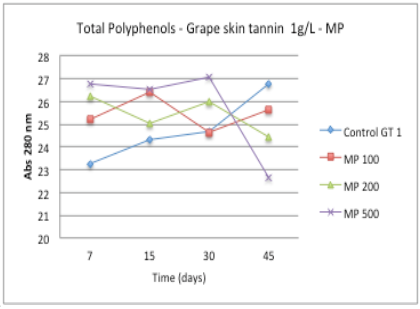
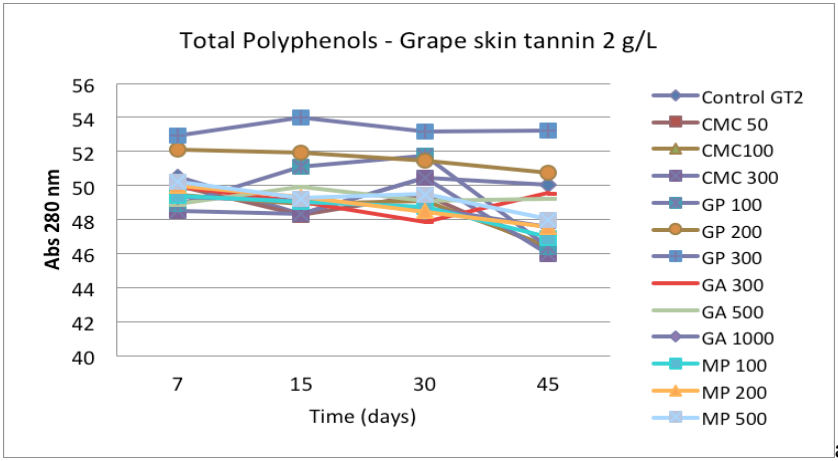


e

Figure 99 a b c d e - Total polyphenols in white wine added with Grape skin tannin at 1 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.



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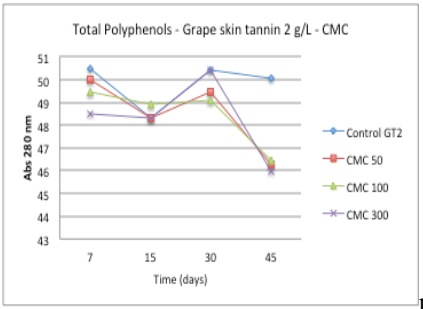
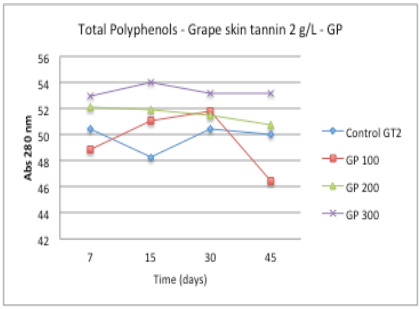


b



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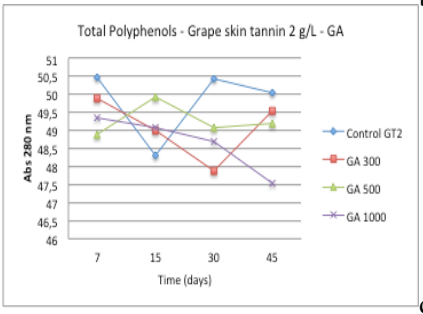

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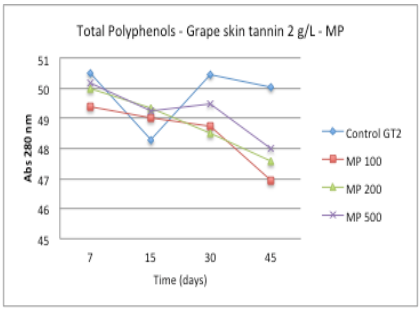
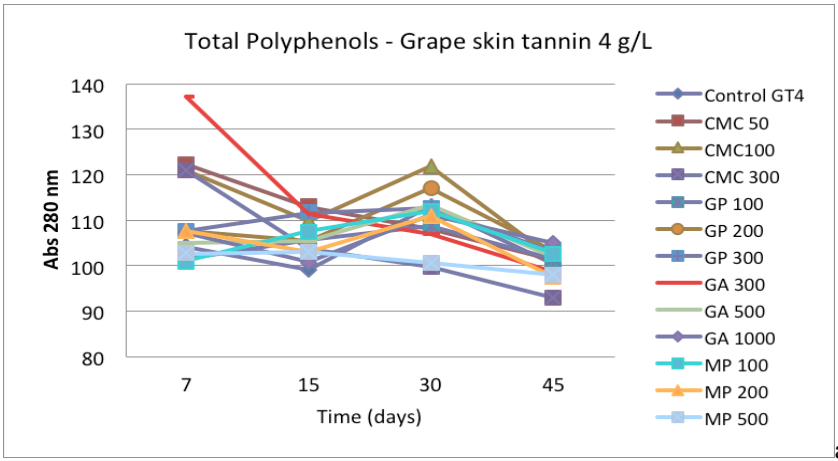
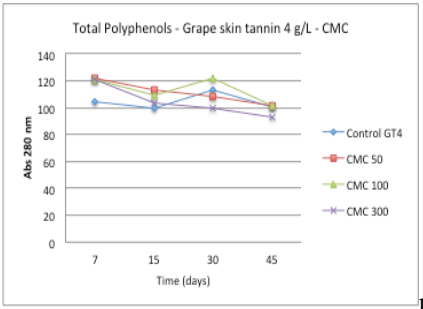


e

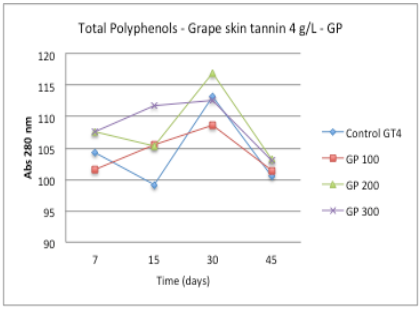
Figure 100 a b c d e - Total polyphenols in white wine added with Grape skin tannin at 2 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.



a



b



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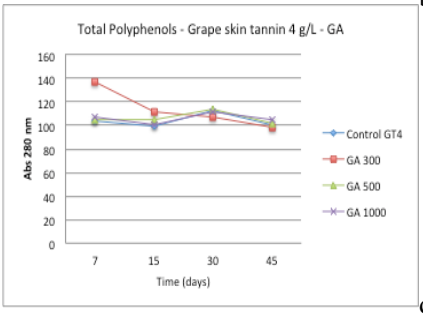

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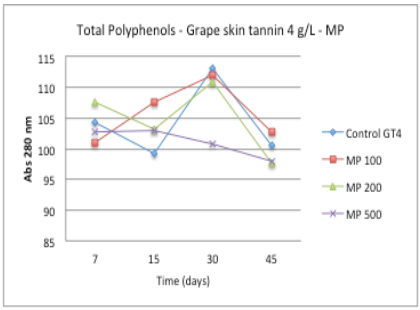
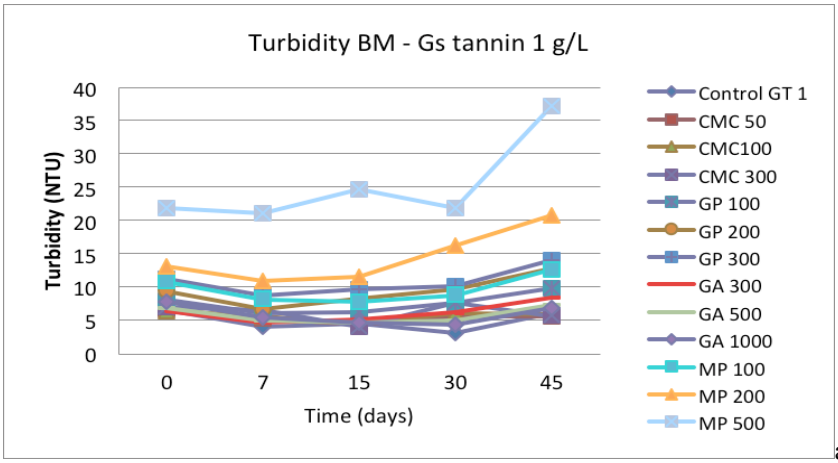


e

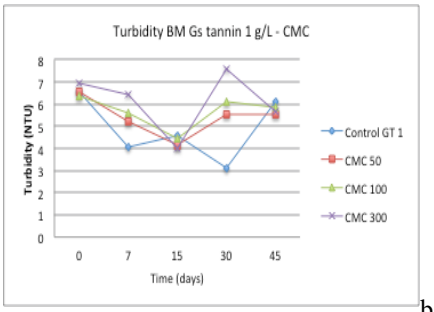
Figure 101 a b c d e - Total polyphenols in white wine added with Grape skin tannin at 4 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.

Turbidity

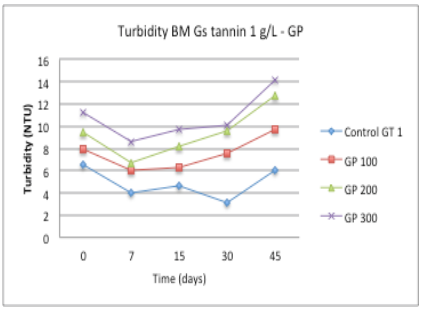
The tests with model wine, have shown that in particular the addition of mannoproteins at all the three concentrations resulted in an increase in the initial turbidity and in the total one during the 45 days (Figures 102e, 103e, 104e, 105e, 106e, 107e). This indicates that in this case, these compounds appear to have favored increased sedimentation, binding the tannin present in solution. The same behavior, but to a lesser extent, was obtained by the addition of grape polysaccharides (Figures 105c, 106c and 107c). The addition of gum arabic and carboxymethylcellulose did not lead to evident changes compared with control (Figures 105d and b, 106d and b, 107d and b).



a



b



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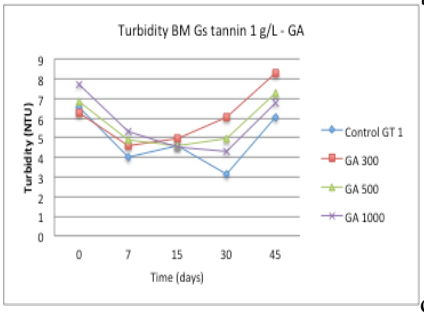
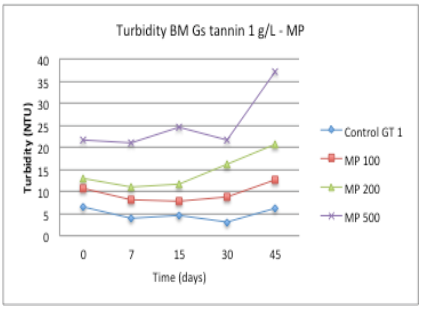
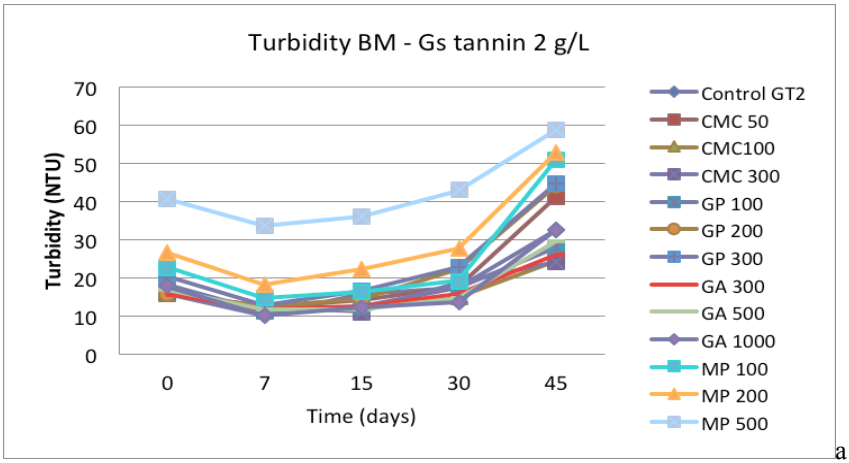


d

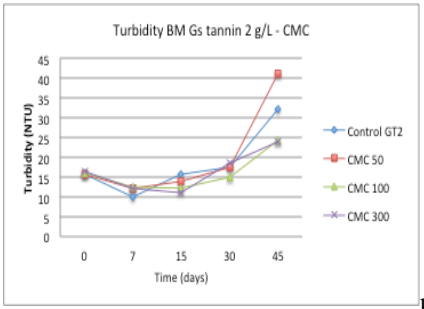


e

Figure 102 a b c d e – Turbidity (NTU) before mixing the samples with Grape skin tannin at 1 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP (BM = before mixing).



a



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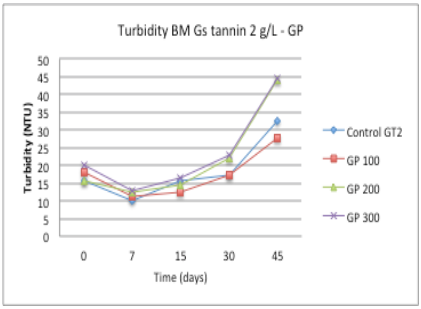

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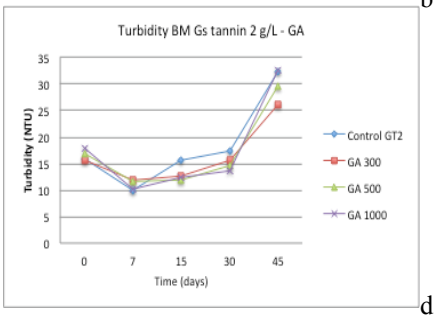

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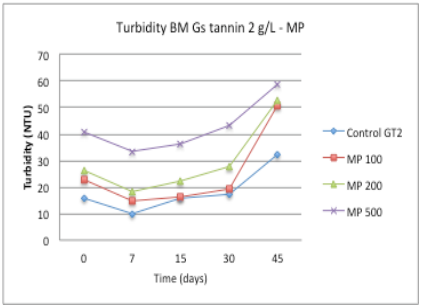
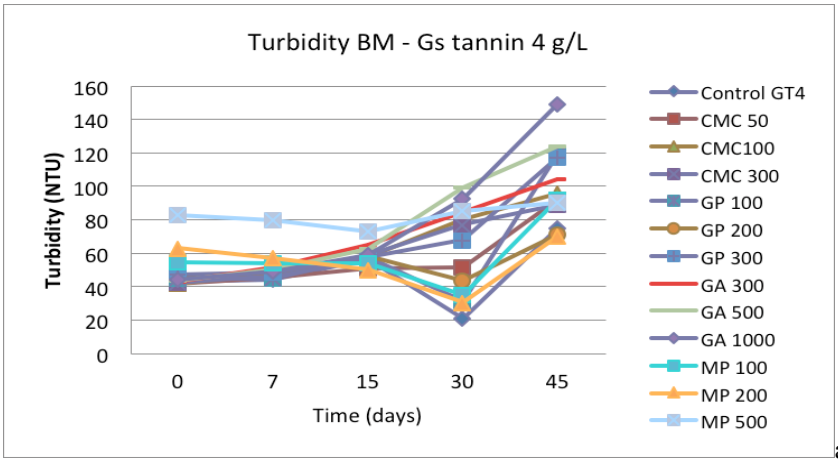


e

Figure 103 a b c d e – Turbidity (NTU) before mixing the samples with Grape skin tannin at 2 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP (BM = before mixing).



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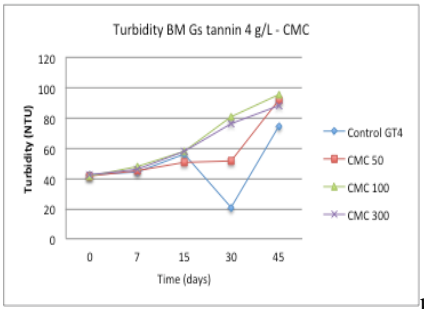

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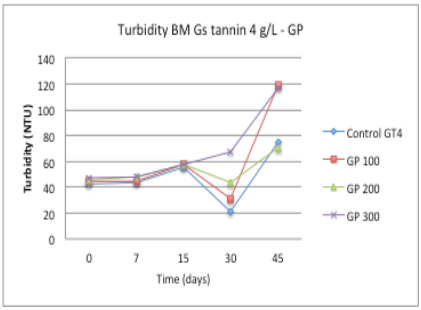

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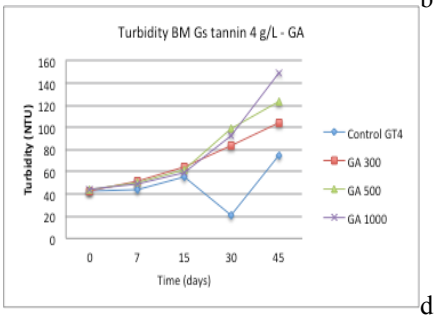

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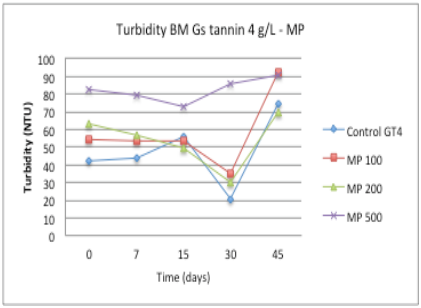
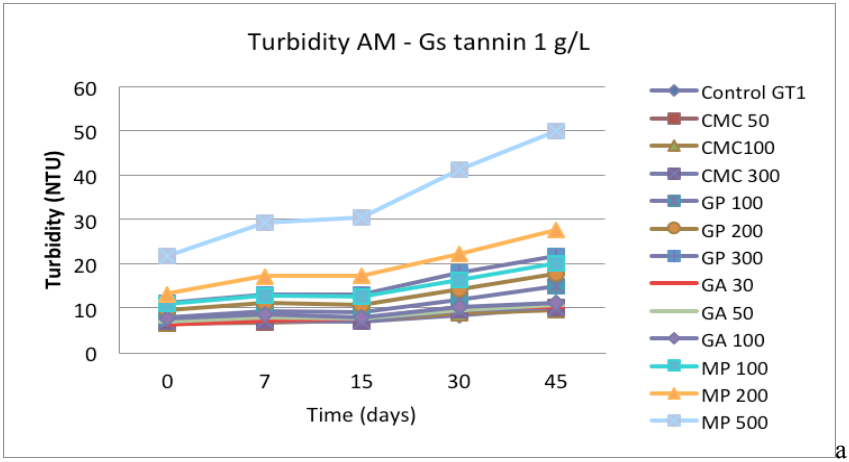


e

Figure 104 a b c d e – Turbidity (NTU) before mixing the samples with Grape skin tannin at 4 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP (BM = before mixing).



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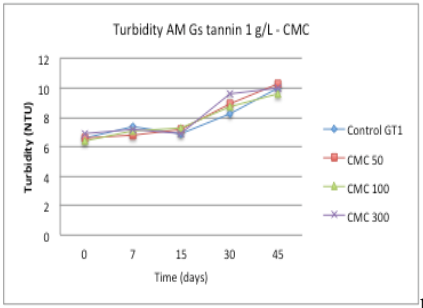

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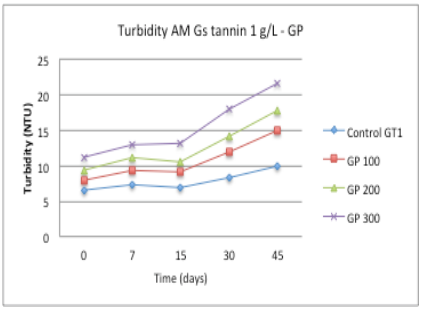

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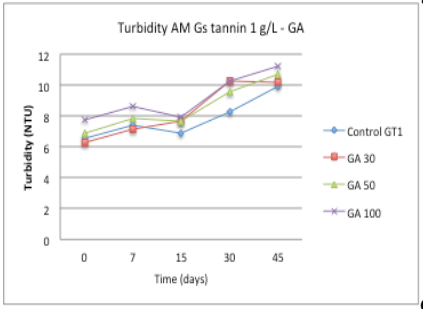

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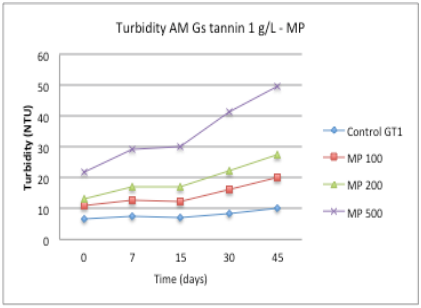
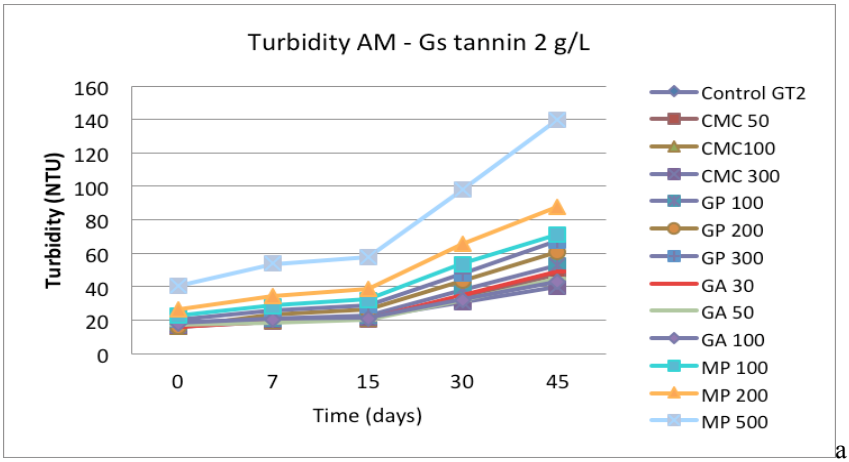


e

Figure 105 a b c d e – Turbidity (NTU) after mixing the samples with Grape skin tannin at 1 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP (AM = before mixing).



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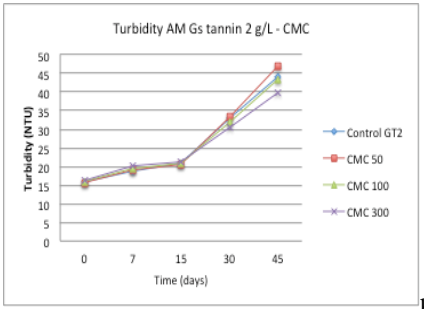

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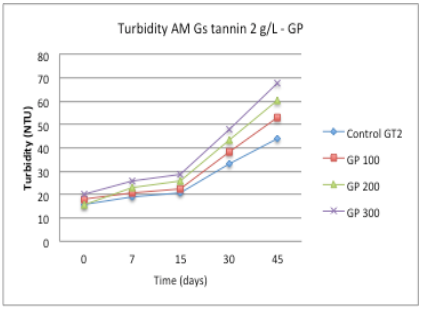

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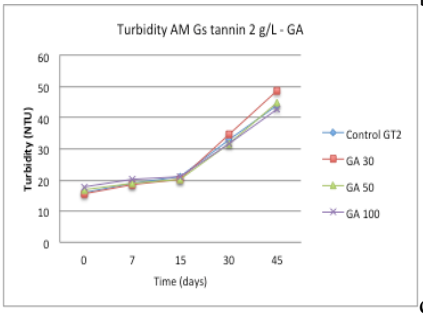

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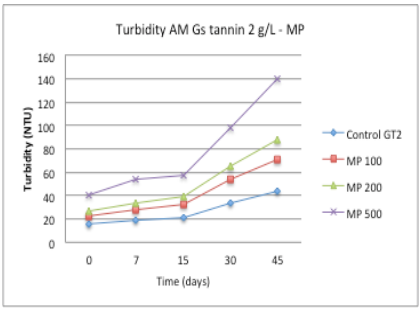
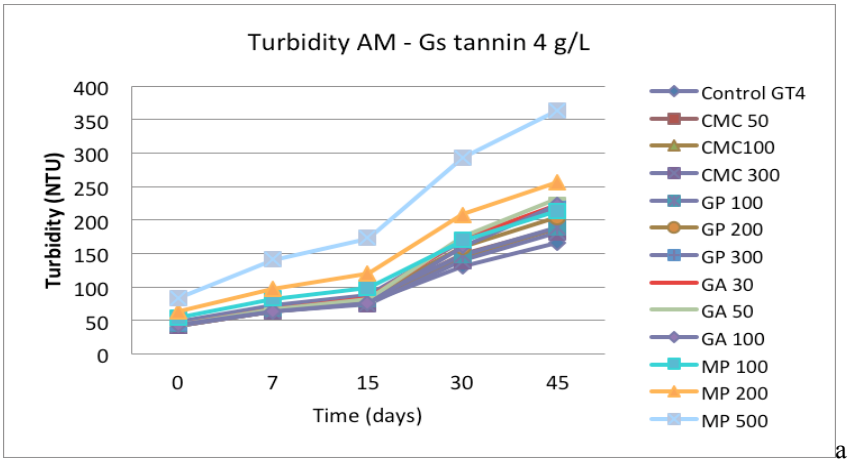


e

Figure 106 a b c d e – Turbidity (NTU) after mixing the samples with Grape skin tannin at 2 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP (AM = before mixing).



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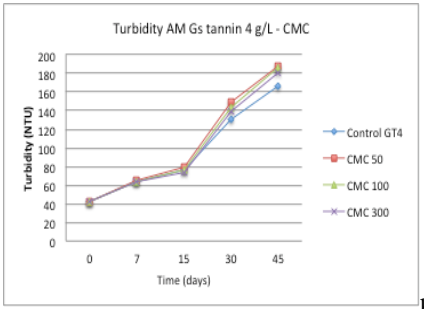

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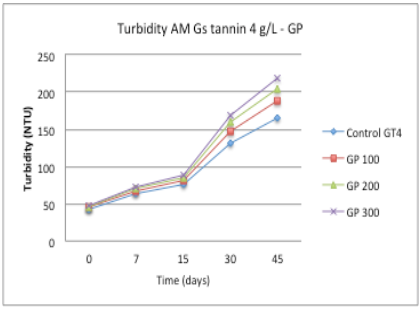

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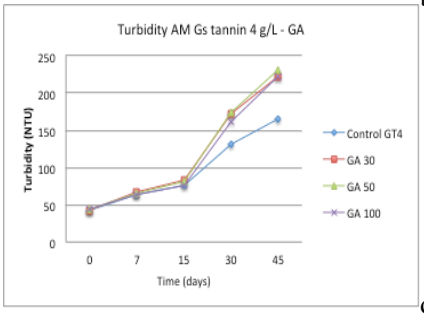

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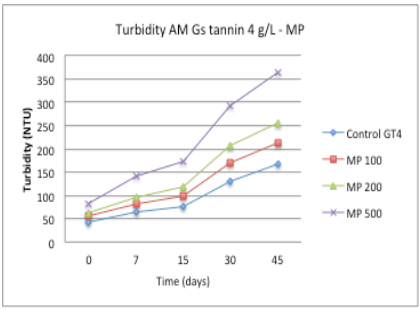


e

Figure 107 a b c d e – Turbidity (NTU) after mixing the samples with Grape skin tannin at 4 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP (AM = before mixing).

Considering the different behavior of the various polysaccharides, turbidity measurements can be useful in defining the limits between colloidal stability and instability.

Particle size

Also in this case, it was decided to consider the values at 7 and 45 days to facilitate the understanding of the values obtained.

Table 12 shows the values of the distributions, and their percentage of presence in solution. Values in bold indicate the outliers that can not be trusted, as out of range compared to the potential of the instrument.

The measurement of particle size showed different trends for all the samples at different concentrations.

For solutions containing grape skin tannin at 1 g/L, the control sample showed outliers, indicating the presence of large particles, which were maintained over time.

The presence of CMC at low concentrations showed initial values of the particles out of scale and then the formation of particles of reduced size at 45 days. At concentrations of 100 and 300 mg/L instead it favored the formation of large particles whose values were out of scale.

CMC added to the model solutions containing 2 g/L of grape skin tannin has facilitated the formation of particles larger than control at 7 days, then increased over time but to a lesser extent than the control for the concentration at 50 mg/L and to a greater extent for the solution with 300 mg/L, while they were reduced for the solution at 100 mg/L.

In the model solution with 4 g/L of tannin added, adding the CMC did not bring great changes in size at 7 days, while at 45 days it favored the formation of large particles, in fact, once again, off scale values were found. Contrary to what it might be expected, the solutions with the highest concentration of this adjuvant favored the formation of small particles compared to control and similar in dimension to those obtained from the same solution at the time 7 days.

Grape polysaccharides added, generally have favored the formation of larger particles during the 45 days. In solutions at 1 g/L of tannin large size particles were already present at 7 days, whose size was then kept constant over time for the concentration of polysaccharides at 100 mg/L. At the other concentrations, the formation of particles larger than those at 7 days, although reduced compared with control, was noted.

The same can be said with regard to solutions with grape skin tannin at 2 g/L, but in this case, the 3 concentrations of grape polysaccharides added led to particle sizes, at 45 days, similar to one another, and smaller than those of the control.

In solutions of 4 g/L of tannin, grape polysaccharides reversed their trend, favoring after 45 days the formation of particles smaller than those at 7 days and smaller than those of control. Only at the highest concentration of polysaccharide added, there was a large increase in size.

The addition of gum arabic in solutions of tannin, and at three different concentrations, as for other polysaccharides added, did not produce a linear trend for all tests. In solutions of tannin at 1 g/L, in fact, it generally provided the formation of large particles, with outliers. In solutions of tannin at 2 g/L it

facilitated the formation of large particles at lower concentrations (300 and 500 mg/L) and led to the formation of particles of reduced size after 45 days.
The addition of this polysaccharide to solutions of tannin at 4 g/L, instead, has encouraged the formation of particles of small size for all concentrations (300-500 and 1000 mg/L).
Mannoproteins added to the 1 g/L solutions of grape skin tannin led to the formation of particles smaller than those of control, but generally larger than those given at 7 days, except for the concentration at 100 mg/L.
In the 2 g/L solution of tannin, at 45 days, it was noticed that these compounds have favored the formation of particles larger than the control, except for the concentration at 100 mg/L as in the previous case, which has favored particles reduced in size.
As for the solutions of 4 g/L of tannin mannoproteins to all three concentrations favored the formation of particles much smaller than the control. These data would seem to be inconsistent with the turbidity data obtained indicating the mannoprotein-containing samples as those that provided the highest values of this parameter. The increased turbidity of these samples after shaking was assumed as a greater precipitation due to aggregation of tannin with these polysaccharides. This behavior could still be explained by an increase in turbidity due to small particles that do not fall, but remain in solution. However, for technological purposes, the turbidity caused by these particles appears to be undesirable.

	Particle Size at 7 days (nm)						Particle Size at 45 days (nm)					
	Peak 1		Peak 2		Peak 3		Peak 1		Peak 2		Peak 3	
	Diameter	%	Diameter	%	Diameter	%	Diameter	%	Diameter	%	Diameter	%
Control GT1	50	20	1428	50	**8257**	30	86	5,3	**7093**	94,7		
CMC 50	31	24	869	12	**7283**	64	343	100				
CMC100	32	33	285	3,7	2804	63	18	13	590	18,4	**8729**	68
CMC 300	87	20	2342	80			363	7,3	**8732**	92,7		
GP 100	77	15	514	31	3136	54	75	8,5	3760	91,5		
GP 200	86	21	1461	79			2763	29	3825	70,8		
GP 300	45	16	487	19	3449	65	94	4,3	609	30,6	**8764**	65
GA 300	44	32	2140	68			448	25	5218	75,2		
GA 500	119	18	2508	82			35	6,7	259	21,1	2070	72
GA 1000	33	29	1594	51	**9013**	21	62	5,4	651	29	**7286**	66
MP 100	80	22	1546	78			58	18	998	81,9		
MP 200	70	4,8	317	29	2774	66	825	34	**6250**	65,7		
MP 500	195	35	1498	65			44	5,5	641	23,8	3984	71
Control GT2	2199	100					271	11	4851	89,5		
CMC 50	47	18	260	7,5	2645	75	103	3,3	329	10,1	3049	87
CMC100	11	90	1028	1,9	**8403**	8	288	19	3083	80,6		
CMC 300	66	14	6076	87			542	15	8720	84,7		
GP 100	42	31	1970	69			274	6,3	3849	93,7		
GP 200	60	8,4	320	13	3338	79	399	14	3026	85,6		
GP 300	42	34	220	66			451	18	3004	81,8		
GA 300	77	10	524	13	4546	76	451	27	**6148**	73,2		
GA 500	56	30	1569	70			277	33	2882	67,1		
GA 1000	11	92	295	1	2998	7	158	15	855	84,7		
MP 100	43	17	468	8,7	3235	74	181	4,2	2518	95,8		
MP 200	126	20	1859	80			53	4,2	828	29,7	4404	66
MP 500	81	100					863	58	**8687**	41,6		
Control GT4	123	11	1539	89			1033	51	**7128**	49,5		
CMC 50	120	15	1792	85			605	25	**5689**	74,8		
CMC100	196	19	2102	81			712	38	**8719**	62		
CMC 300	77	5,7	515	24	1878	70	243	17	1292	83,2		
GP 100	125	13	1356	87			791	100				
GP 200	286	19	1831	81			1375	100				
GP 300	109	9,6	1188	90			851	83	**8358**	16,8		
GA 300	113	16	1101	85			749	100				
GA 500	210	22	1297	78			636	100				
GA 1000	79	13	1011	87			609	100				
MP 100	36	5,2	1311	95			1020	100				
MP 200	114	5,9	1545	94			1264	100				
MP 500	54	3,3	1363	97			1135	100				

Table 12 – Particle sizes at 7 and 45 days (nm).

6.4 Analysis for model wine added with oak tannin and polysaccharides

The assessments carried out on samples of model wine solutions with oak tannin, in three different concentrations (1-2 and 4 g/L), and the four polysaccharides, at three different concentrations, partly confirmed what had already been found in previous tests and provided useful and more significant information. Having replicated the tests, it was possible to evaluate statistical differences between the data obtained.
The following graphs show the mean values of all the data obtained from the three replications. The absence of error bars is due to the fact that with their presence it would have been difficult to understand the graphs, given the amount of data present.

Surface Electrical Charge
As for the surface electrical charge, the addition of polysaccharides has led to an increase in overall negative charge of the solution compared to the control. This increase occurred in particular in samples with gum arabic (Figures 108d, 109d and 110d) and carboxymethylcellulose (Figures 108b, 109b and 110b), as confirmed by the analysis of variance (Figure 112). The addition of grape polysaccharides and mannoproteins did not lead to such evident increases (Figures 108c and e, 109 c and e, 110 c and e). It may be noted that, like in chapters 6.2 and 6.3, in this case after the initial increase due to the addition, the surface electrical charge suffered only minor variations, remaining generally constant over time for each sample.
To confirm this, the one-way analysis of variance showed that there were no significant differences for this parameter with respect to time. This consideration, once again, serves to emphasize the fact that the interactions that lead to the formation of aggregates that precipitate, is not only related to the electrical charge of the samples particles.
The analysis of variance showed significant differences between samples with different concentrations of tannin added. As the tannin itself provides negative charges to the solution, by increasing its concentration, consequently, increases the amount of negative charge provided (Figure 111). The same consideration can be made for the concentration of the polysaccharides added, a higher concentration brings greater charge to the system (Figure 113). The factorial analysis of variance between the three tannin concentrations and the polysaccharides added showed that the values of surface electrical charge are influenced by both treatments, indicating a synergistic effect of the two (Figure 114).
The same analysis performed to evaluate the effect of time (7 and 45 days) and the different polysaccharides on this parameter, showed that there are no statistical differences among all the samples. Only samples with gum arabic gave values that are statistically different, but this difference did not change over time. The values remained constant (Figure 115).

The factorial ANOVA between the variables tannin concentration and time (7 and 45 days) showed that surface electrical charge values were statistically different depending only on the concentration of the tannin, but they were constant over time (Figure 116).

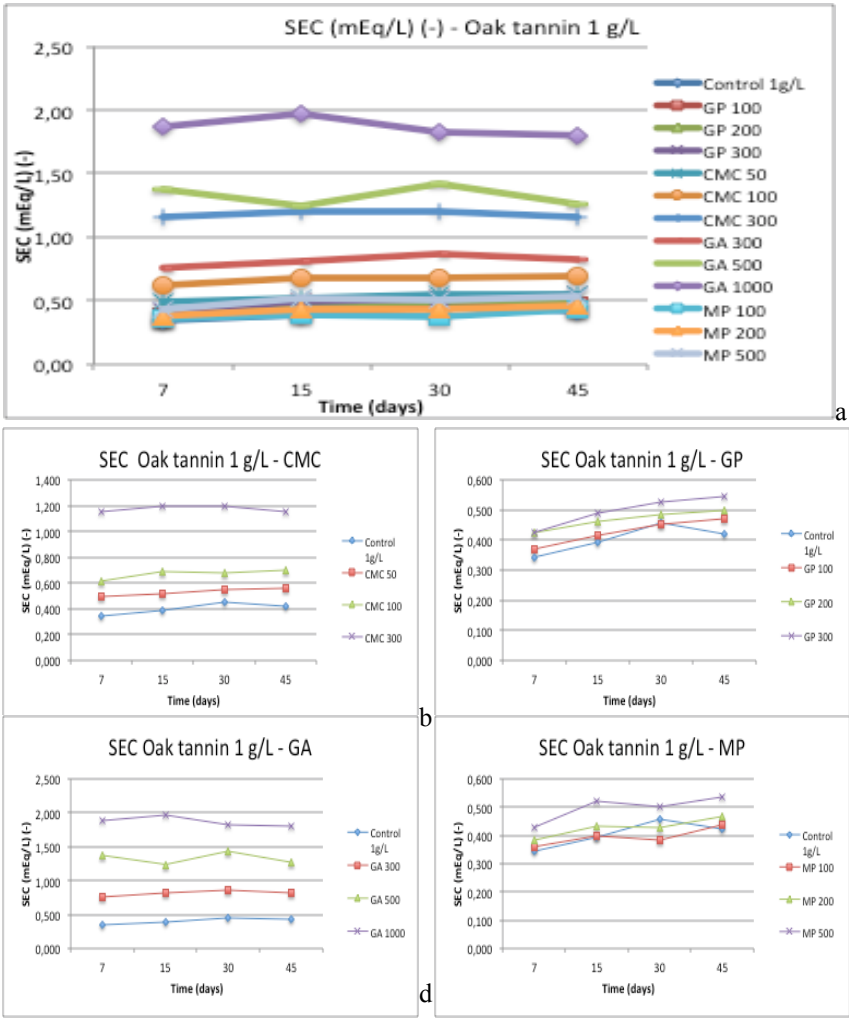
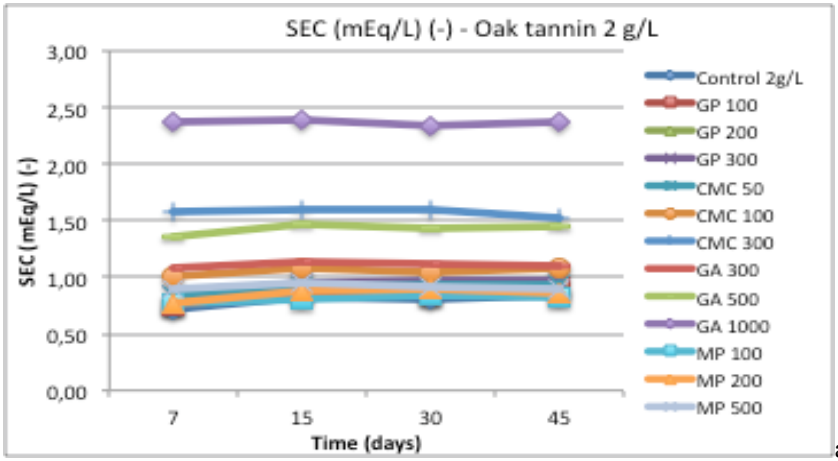


Figure 108 a b c d e – SEC (mEq/L) (-) for the samples of model wine with Oak tannin at 1 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.



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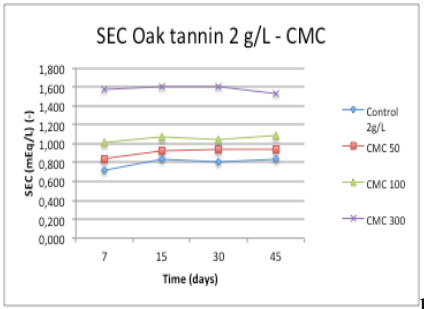

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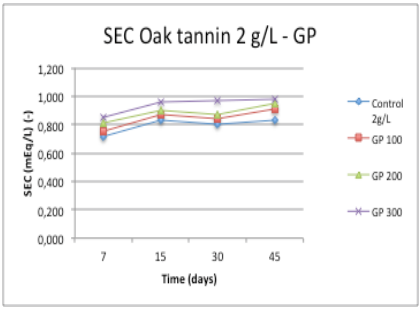

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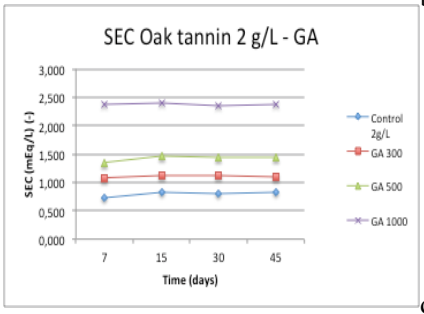

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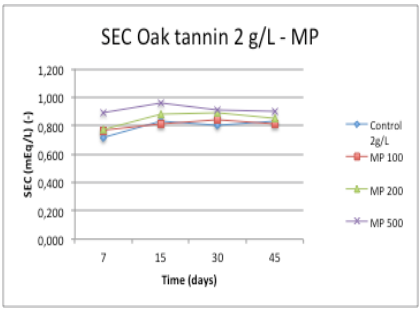
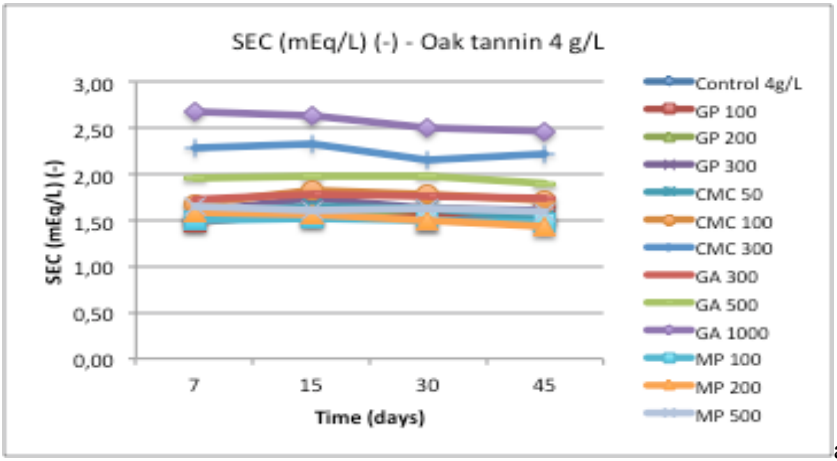


e

Figure 109 a b c d e– SEC (mEq/L) (-) for the samples of model wine with Oak tannin at 2 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.



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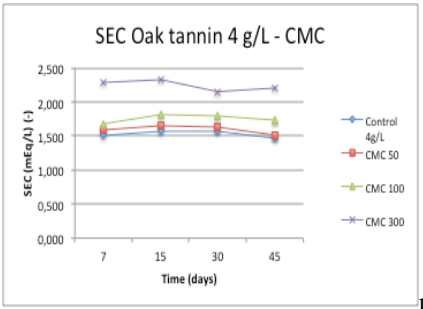
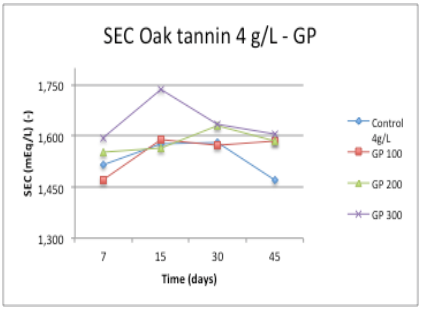
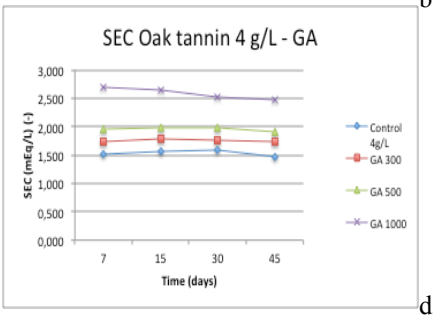


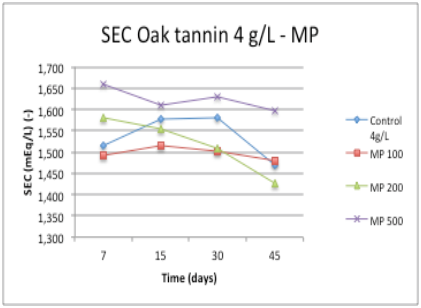
b



c



d



e

Figure 110 a b c d e– SEC (mEq/L) (-) for the samples of model wine with Oak tannin at 4 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.

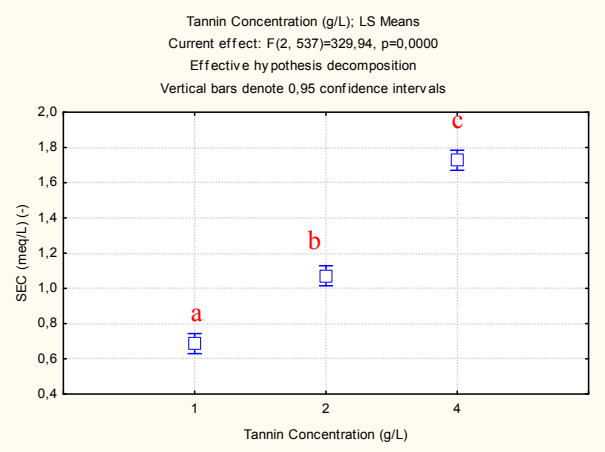


Figure 111 – One way ANOVA for SEC related to tannin concentration.
Different letters identify samples that are significantly different for p <0.05.

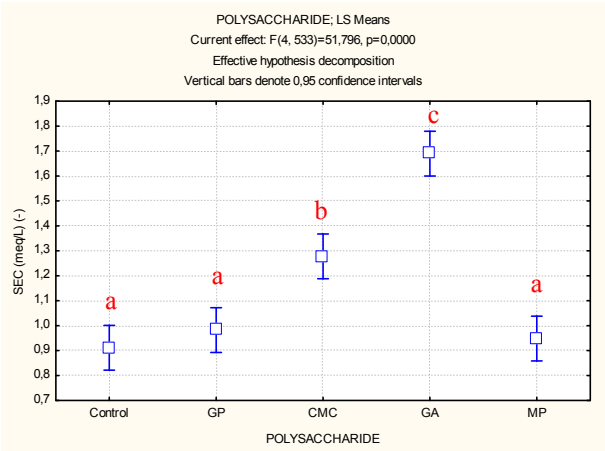


Figure 112 – One way ANOVA for SEC related to the type of polysaccharide added.
Different letters identify samples that are significantly different for p <0.05.

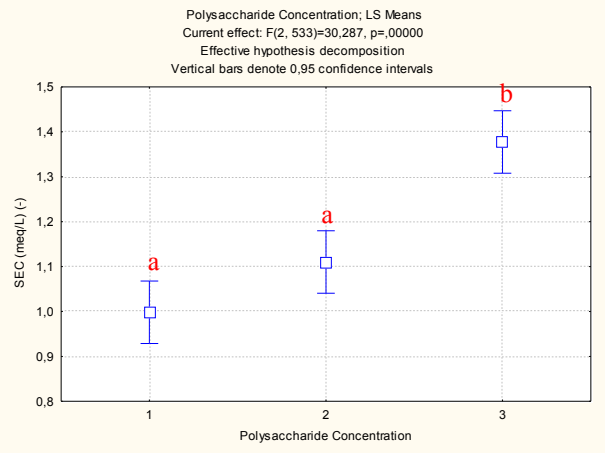
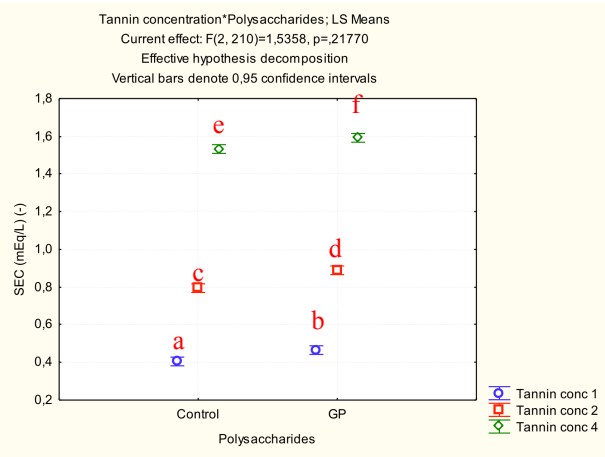
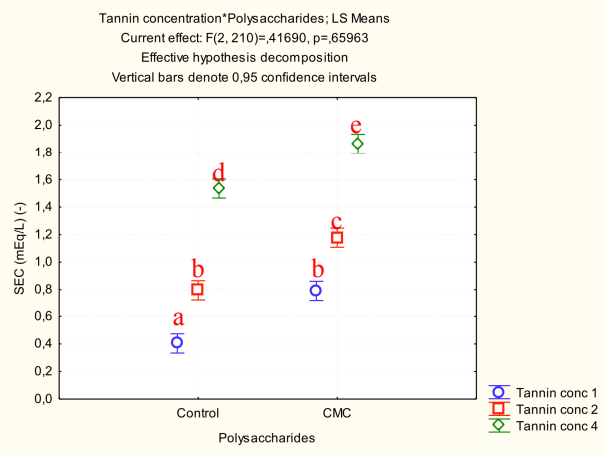


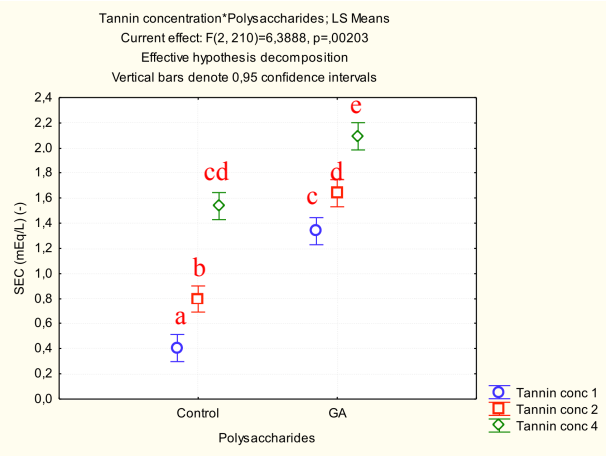
Figure 113 – One way ANOVA for SEC related to polysaccharide concentration. Different letters identify samples that are significantly different for $p < 0.05$.



a



b



c

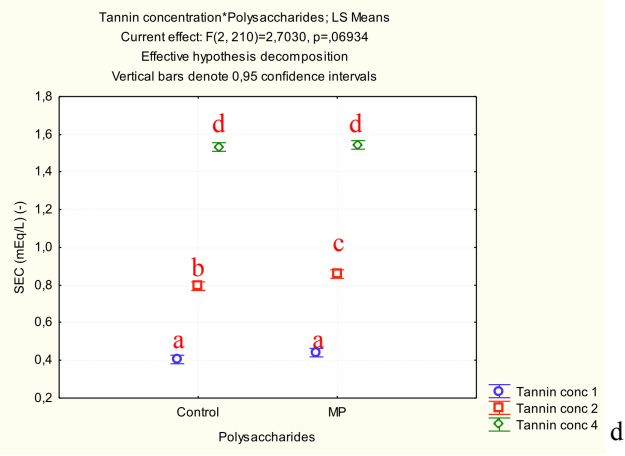


Figure 114 – Factorial ANOVA for Tannin concentration and Polysaccharides a: Control-GP, b: Control-CMC, c: Control-GA, d: Control-MP.
Different letters identify samples that are significantly different for $p < 0.05$.

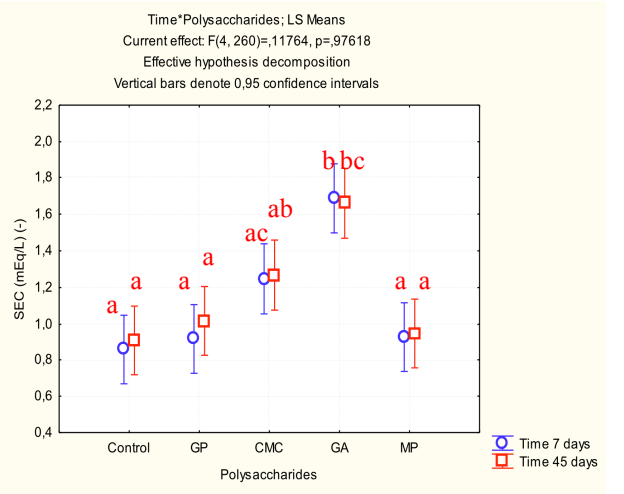


Figure 115 - Factorial ANOVA for Time (7 and 45 days) and Polysaccharides. Different letters identify samples that are significantly different for $p < 0.05$.

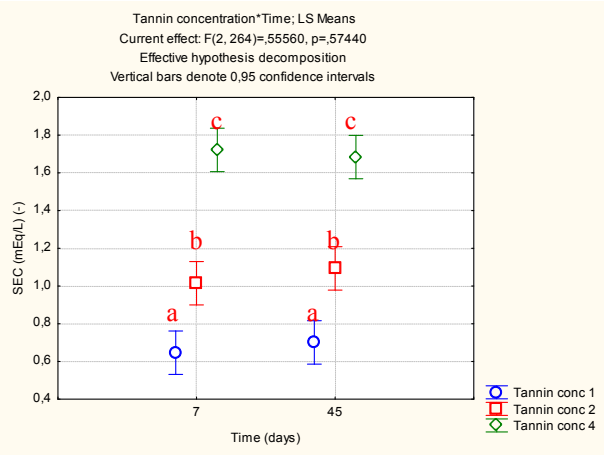
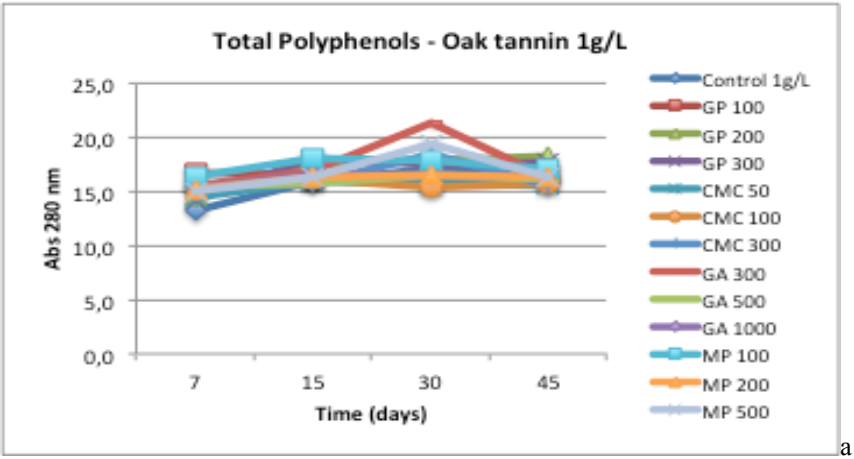


Figure 116 – Factorial ANOVA for Tannin concentration and Time (7 and 45 days).
Different letters identify samples that are significantly different for $p < 0.05$.

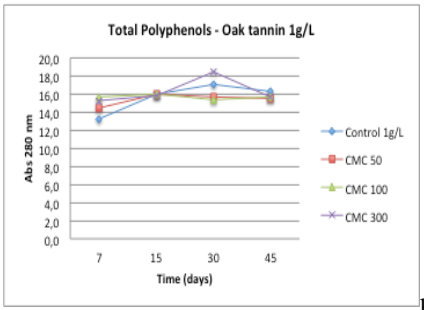
Total Polyphenols

Also in this case, the assessment of total polyphenols by measuring absorbance at 280 nm confirmed the initial increase compared to control, due to the addition of tannin and polysaccharides. Then the value remained constant over the 45 days, confirmed by the analysis of variance (Figures 117, 118, 119, 120 and 121). This seems to confirm the stabilizing role polysaccharides against polyphenols reduction.

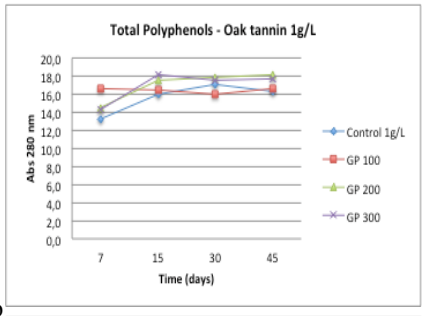
The various types of polysaccharides seem to have affected this parameter in different ways, in particular, the addition of carboxymethylcellulose resulted in absorbance values at 280 nm closer to control than the other samples (Figure 122). In this case, however, the different concentrations of polysaccharides did not lead to differences between the samples.



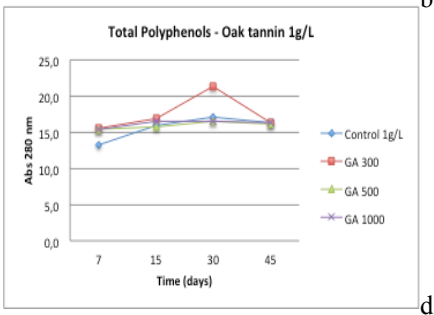
a



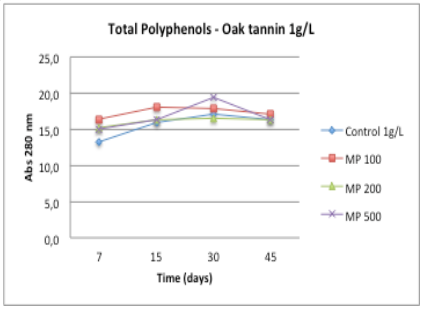
b



c

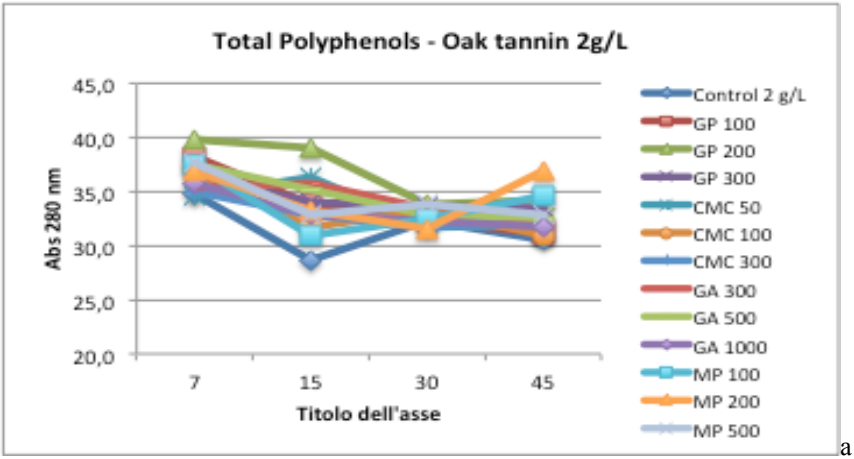


d

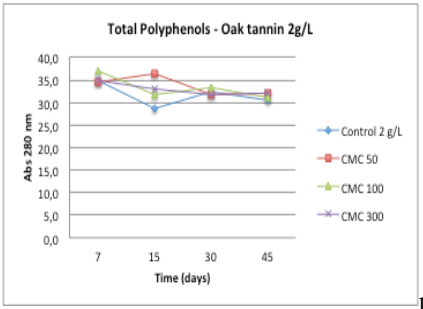


e

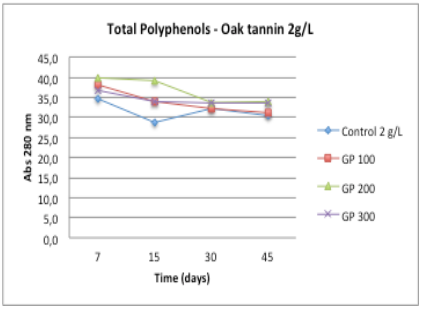
Figure 117 a b c d e - Total polyphenols in white wine added with Oak tannin at 1 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.



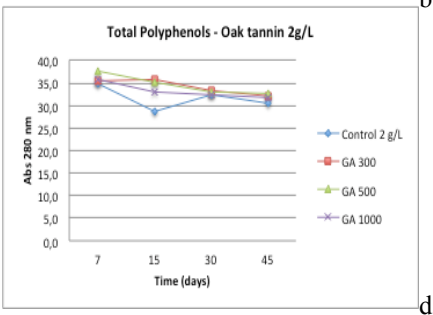
a



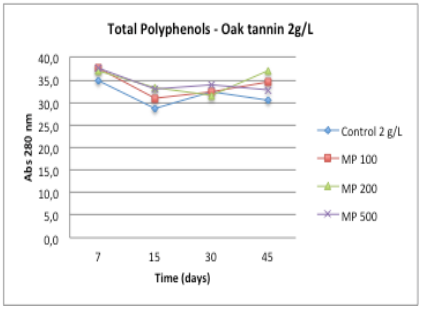
b



c

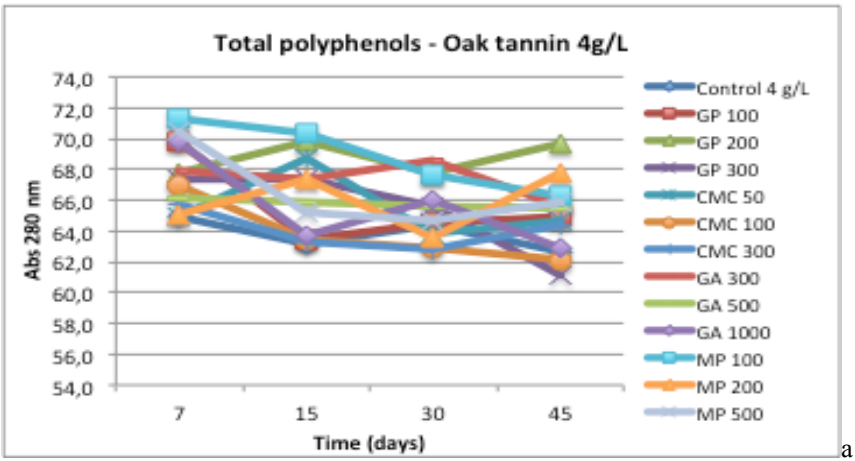


d

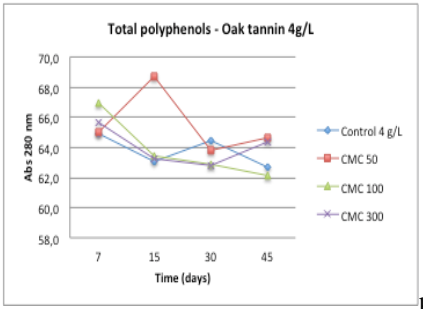


e

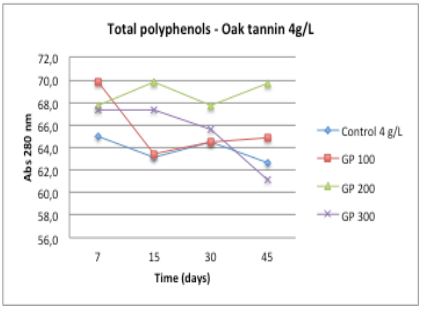
Figure 118 a b c d e - Total polyphenols in white wine added with Oak tannin at 2 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.



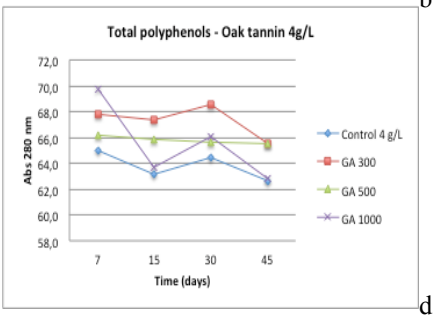
a



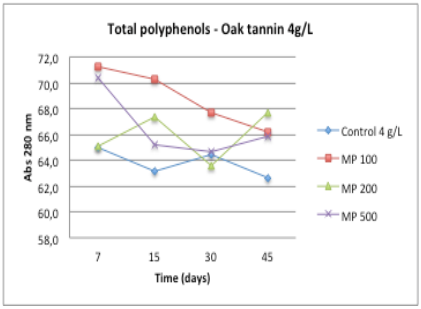
b



c



d



e

Figure 119 a b c d e - Total polyphenols in white wine added with Oak tannin at 4 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP.

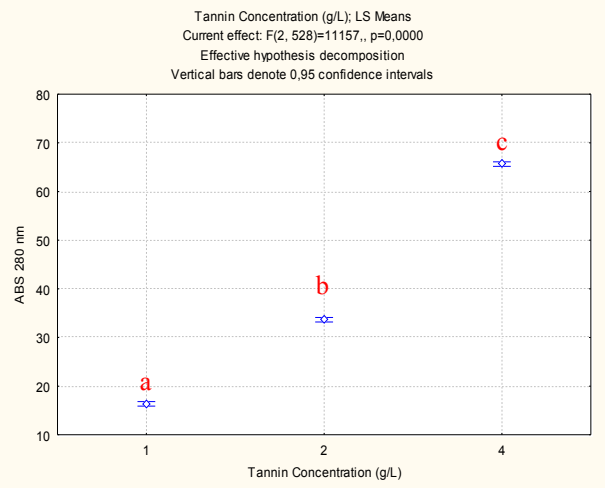


Figure 120 – One way ANOVA for Abs 280 nm related to tannin concentration.
Different letters identify samples that are significantly different for p <0.05.

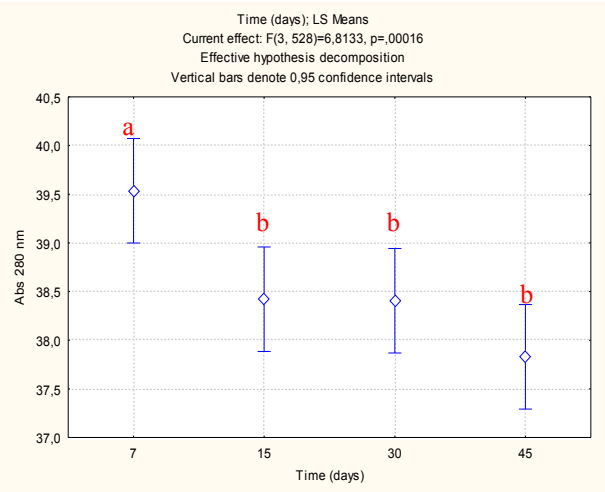


Figure 121 – One way ANOVA for Abs 280 nm related to time.
Different letters identify samples that are significantly different for p <0.05.

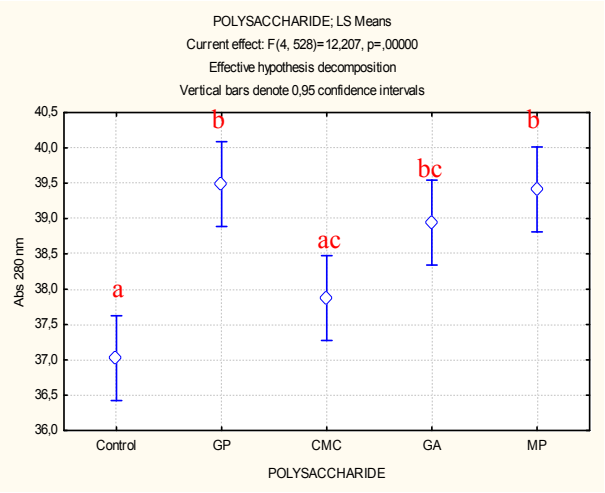
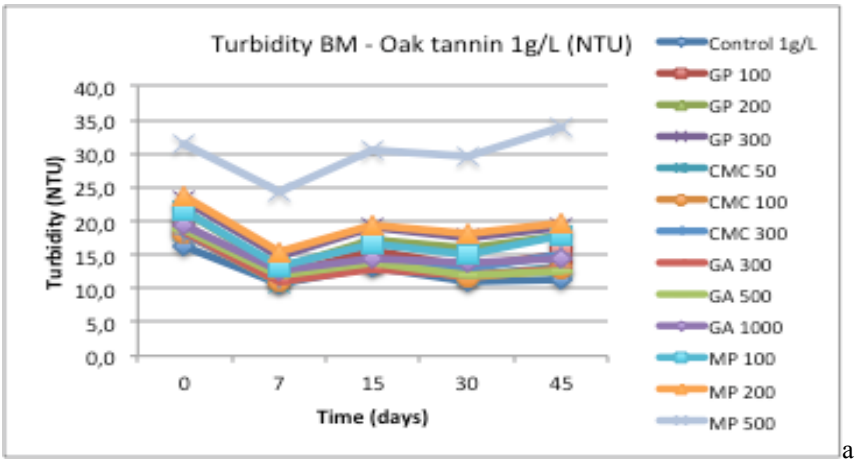


Figure 122 – One way ANOVA for Abs 280 nm related to the type of polysaccharide. Different letters identify samples that are significantly different for $p < 0.05$.

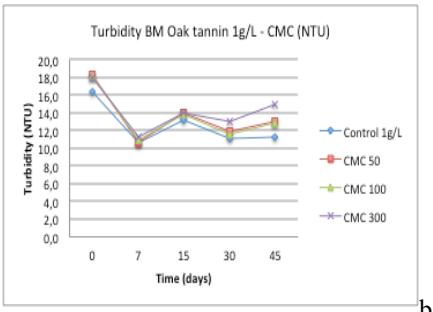
Turbidity

The turbidity before mixing the sample, like in all previous trials, showed differences in values relating to different concentrations of tannin added (the higher the concentration, the higher was the value of turbidity) (Figures 123, 124, 125 and 126). Also among the tests with three different concentrations it can be seen that the samples that presented the highest values of turbidity are always those which have also been added with mannoproteins (Figures 123e, 124e and 125e) and grape polysaccharides (Figures 123c, 124c and 125c), as confirmed by the analysis of variance (Figure 127).

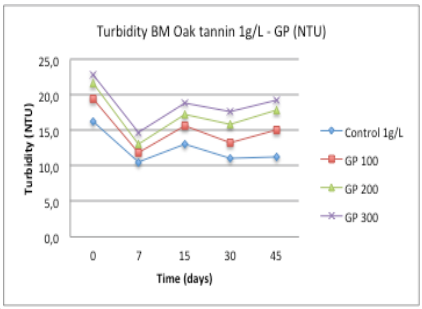
In addition to this, also a higher concentration of polysaccharide added has led to an increase in turbidity, which was confirmed by the analysis of variance (Figure 128).



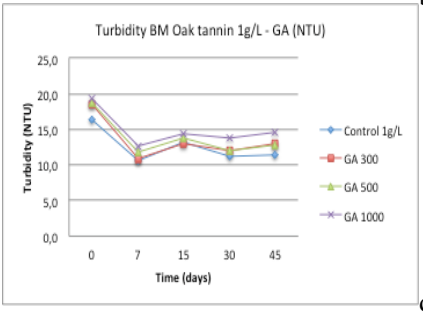
a



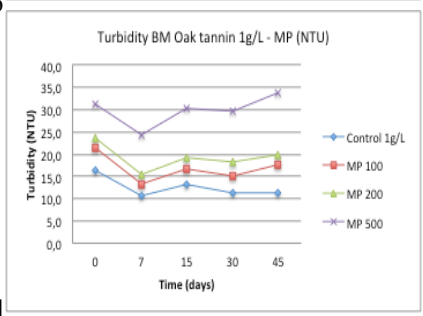
b



c

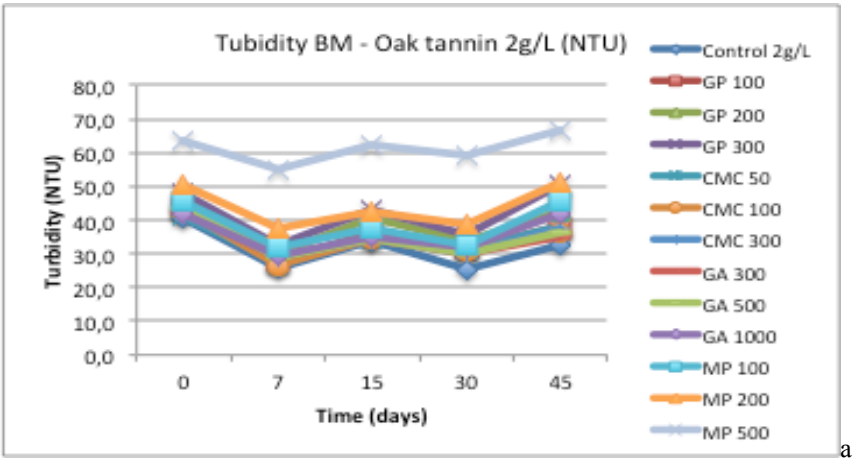


d

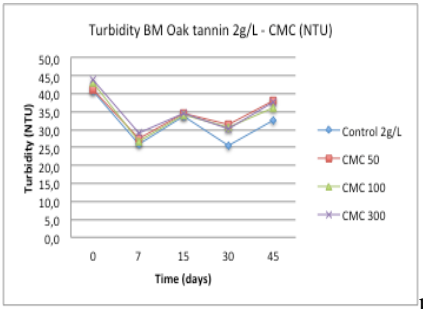


e

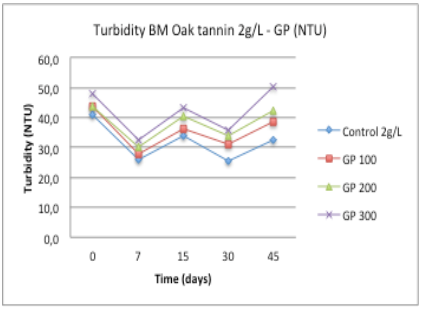
Figure 123 a b c d e – Turbidity (NTU) before mixing the samples with Oak tannin at 1 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP (BM = before mixing).



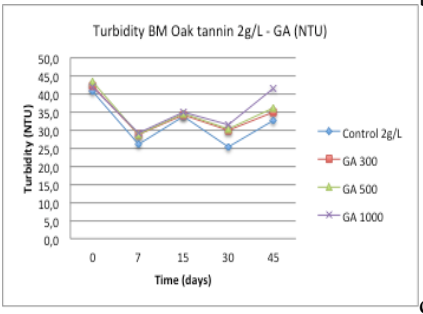
a



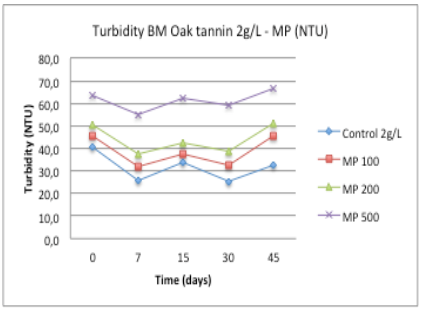
b



c

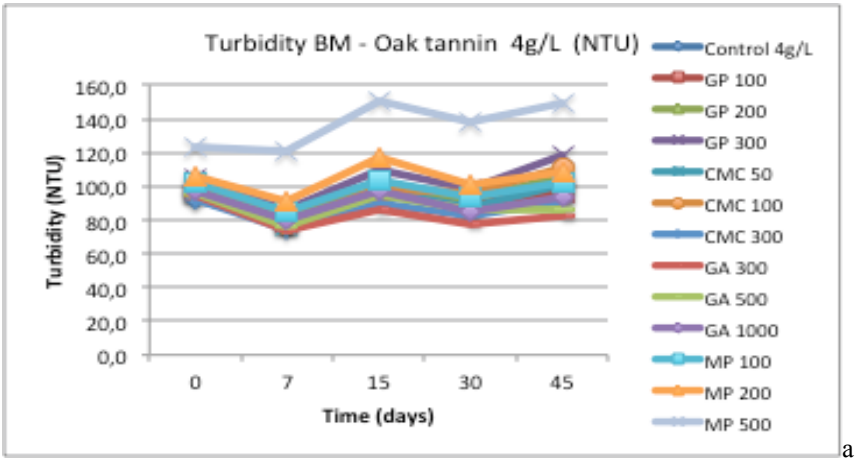


d

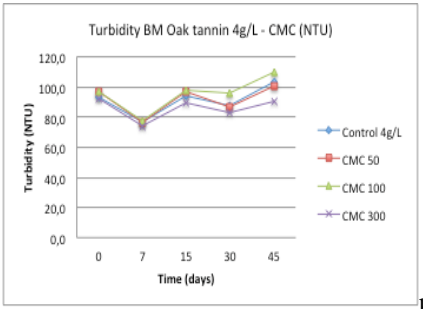


e

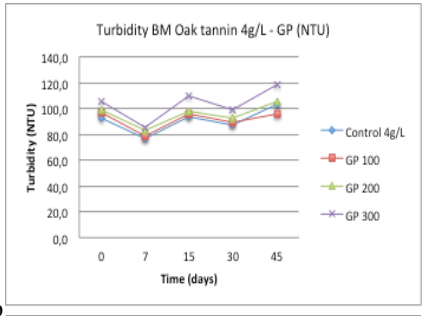
Figure 124 a b c d e – Turbidity (NTU) before mixing the samples with Oak tannin at 2 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP (BM = before mixing).



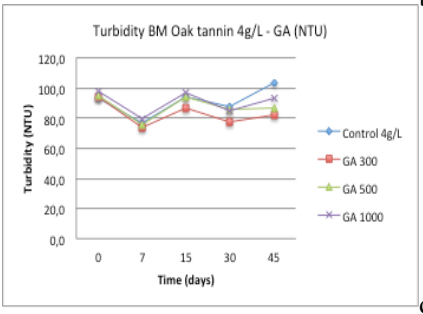
a



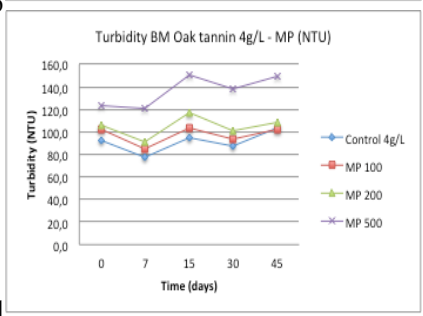
b



c



d



e

Figure 125 a b c d e – Turbidity (NTU) before mixing the samples with Oak tannin at 4 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP (BM = before mixing).

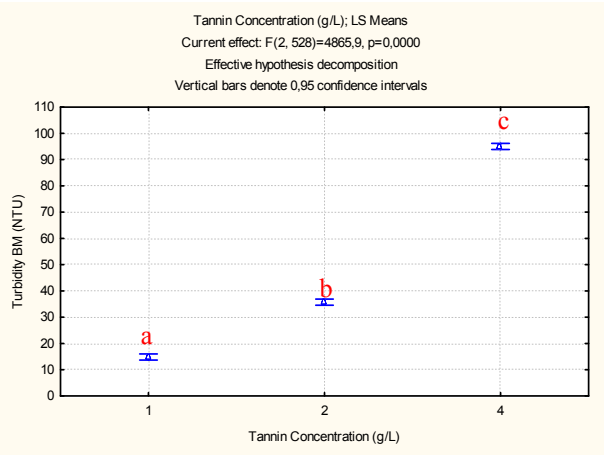


Figure 126 – One way ANOVA for Turbidity BM related to tannin concentration.
Different letters identify samples that are significantly different for p <0.05.

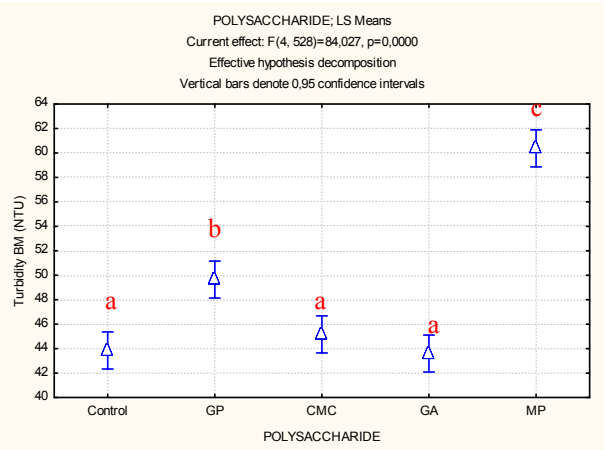


Figure 127 – One way ANOVA for Turbidity BM related to the type of polysaccharide.
Different letters identify samples that are significantly different for p <0.05.

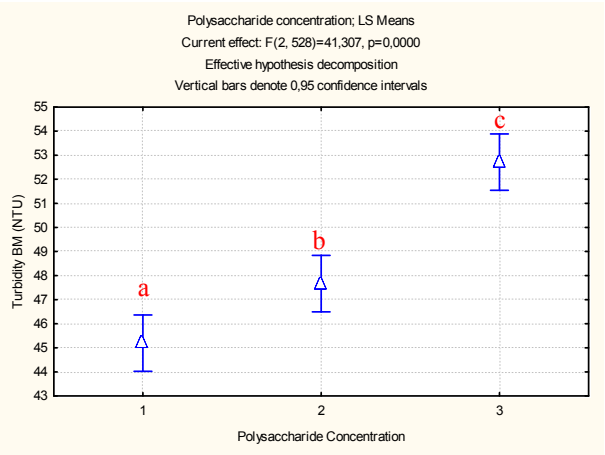
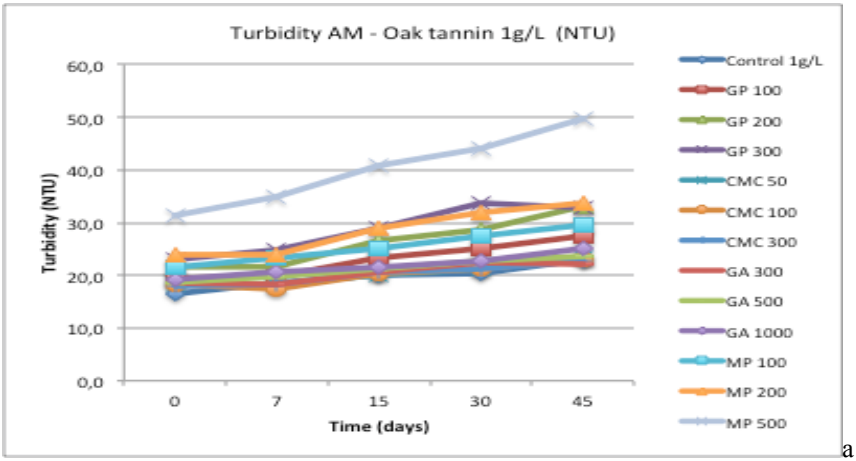
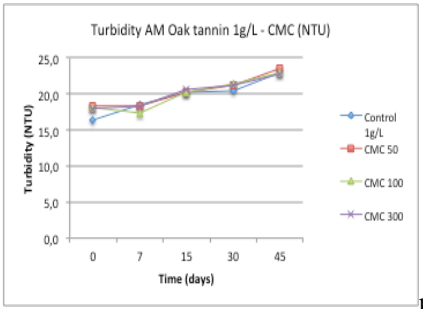


Figure 128 – One way ANOVA for Turbidity BM related to the polysaccharide concentration. Different letters identify samples that are significantly different for $p < 0.05$.

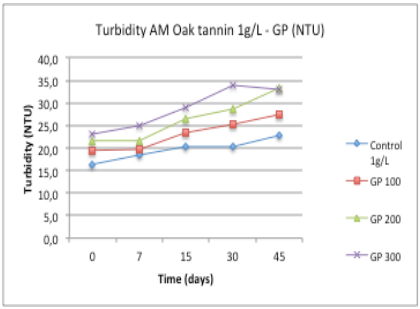
As for the turbidity after mixing the samples, it can be seen that it increased over time more than the one of the control with all the samples with significant differences at each measurement interval (7-15-30-45 days) (Figure 132). Clearly the values of turbidity increased with the increase of the oak tannin concentration.
In this case the samples that showed the highest values of turbidity were those added with mannoproteins and grape polysaccharides (Figures 129 e and c, 130 e and c, 131 e and c, 133) particularly those with higher concentrations of these two polysaccharides (Figure 134).
Since the total polyphenols do not seem to vary over time, only a precipitation of the polysaccharides can be assumed.
The fact that the addition of carboxymethylcellulose did not influence the turbidity parameter, suggests that this adjuvant can be used for traditional purposes without interacting negatively with the other macromolecules, avoiding the instability of the colloidal system. And since this consideration can be made also for the tests with grape skin tannin, it seems that the carboxymethylcellulose effect is independent of the nature of the tannin.



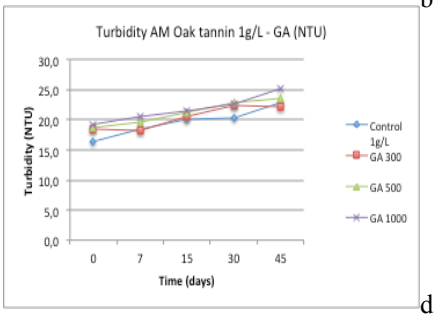
a



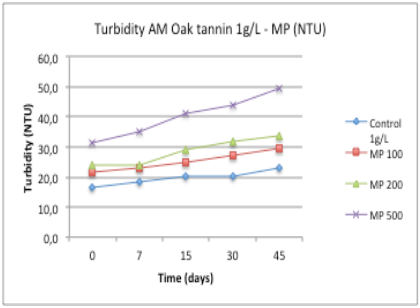
b



c

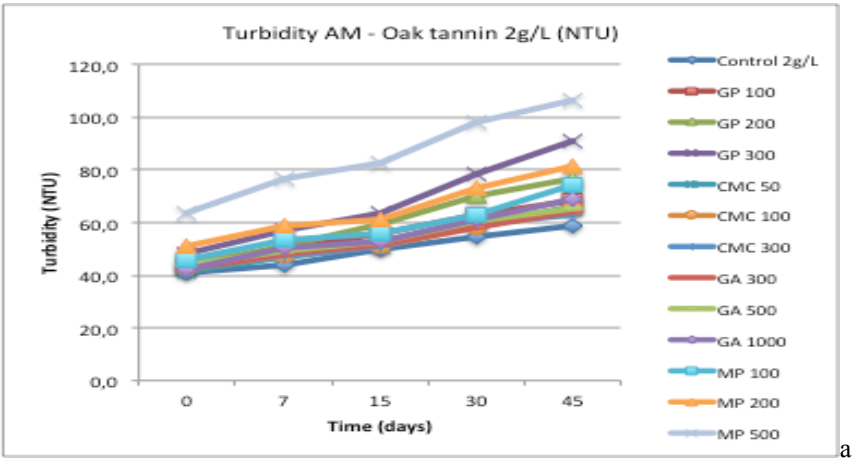


d

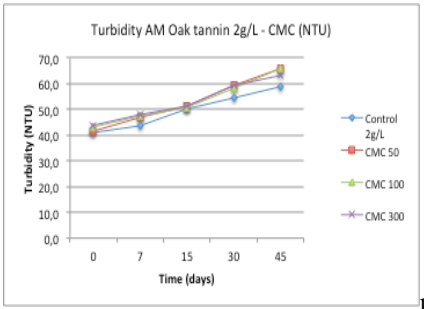


e

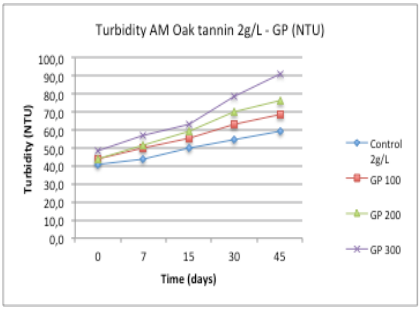
Figure 129 a b c d e – Turbidity (NTU) after mixing the samples with Oak tannin at 1 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP (AM = before mixing).



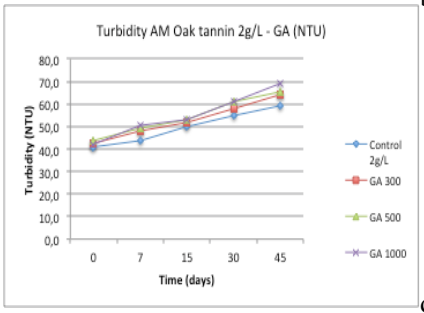
a



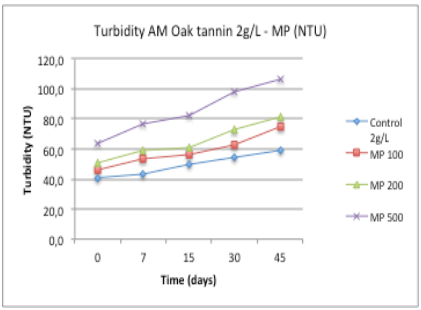
b



c

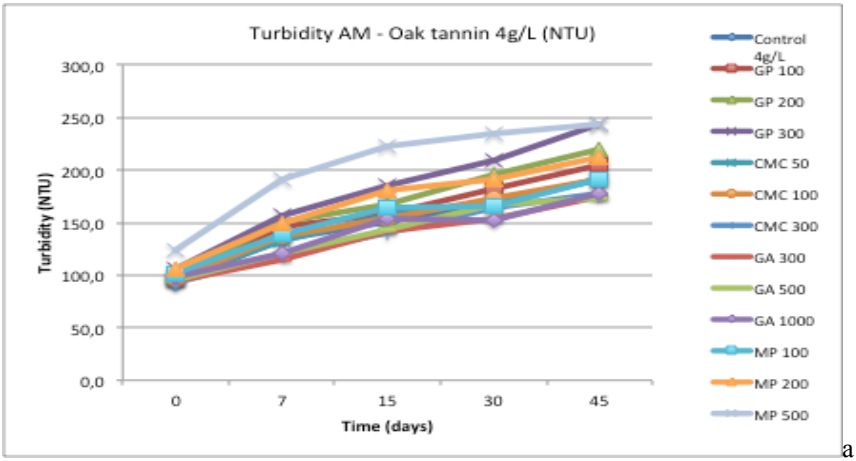


d

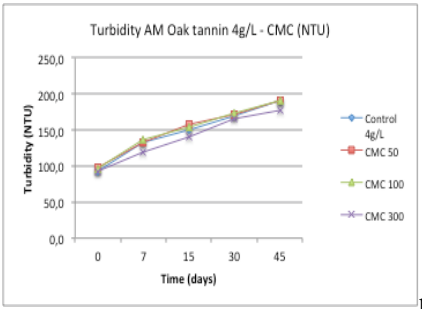


e

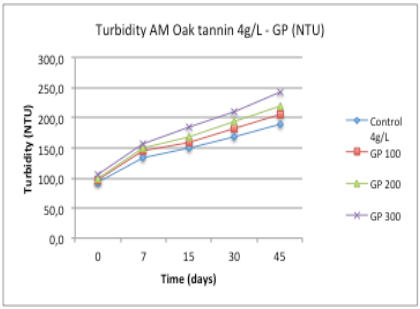
Figure 130 a b c d e – Turbidity (NTU) after mixing the samples with Oak tannin at 2 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP (AM = before mixing).



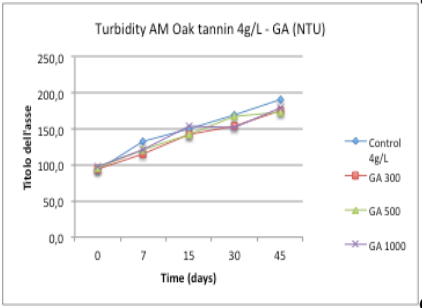
a



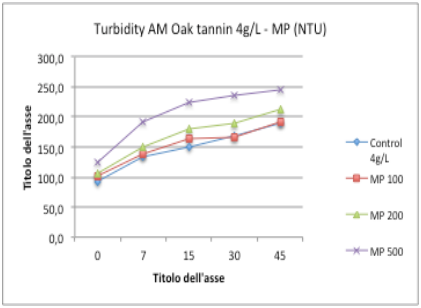
b



c



d



e

Figure 131 a b c d e – Turbidity (NTU) after mixing the samples with Oak tannin at 4 g/L and the different polysaccharides. b: added with CMC; c: added with GP, d: added with GA and e: added with MP (AM = before mixing).

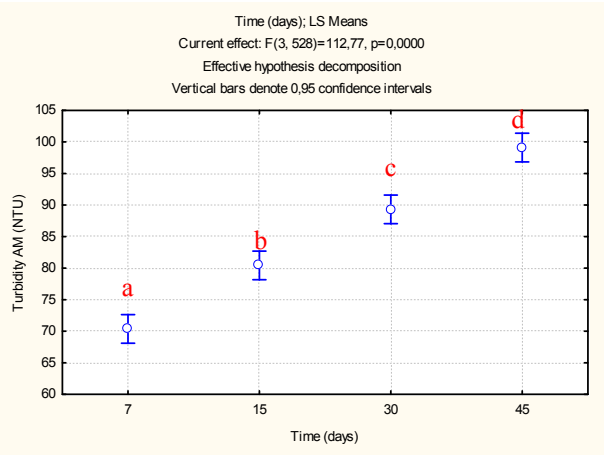


Figure 132 – One way ANOVA for Turbidity AM related to time.
Different letters identify samples that are significantly different for p <0.05.

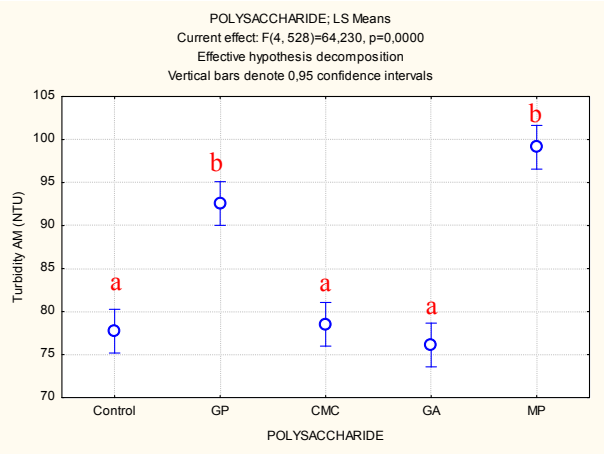


Figure 133 – One way ANOVA for Turbidity AM related to the type of polysaccharide.
Different letters identify samples that are significantly different for p <0.05.

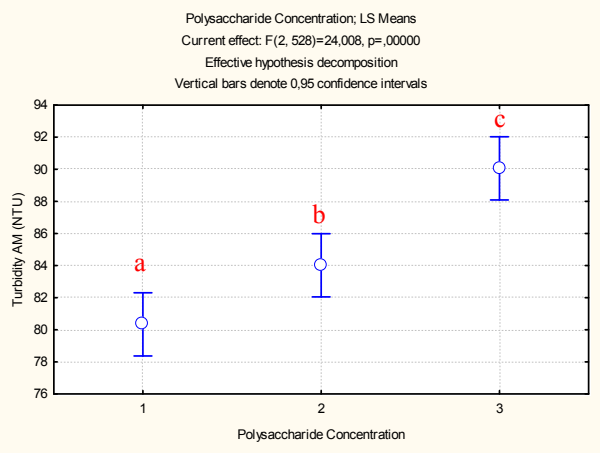


Figure 134 – One way ANOVA for Turbidity AM related to the polysaccharide concentration. Different letters identify samples that are significantly different for $p < 0.05$.

Particle Size

With regard to particle size measures related to the repetitions of the model samples prepared in solution by adding the oak tannin at three different concentrations, it was necessary to report in Table 13 only one of the measurements taken at 7 days and of the measures at 45 days, in order to make the data comparable. The reason for this choice is the impossibility to obtain an average value, as most of the solutions showed the presence of more than one size distribution, with different percentages,.

The 1 g/L tannin solutions showed an increase in size of the control within 45 days. The addition of grape polysaccharides presented particles whose sizes have increased over time, but were smaller compared to the control. In solutions at 2 g/L of tannin, grape polysaccharides at a concentration of 100 mg/L showed an increase in particle size even compared to the control, which instead, maintained particles of about the same size over time. At the concentrations of 200 and 300 mg/L instead, grape polysaccharides showed particles larger at 7 days, then decreased to values lower than the control ones at 45 days.

In the 4 g/L tannin solutions, for which the control presented a particle size constant over time, grape polysaccharides favored the formation of particles larger than the control, especially at 45 days.

The CMC in solutions at 1 g/L of tannin led to an increase in the size of the particles that were even higher than the ones of the control, but only at concentrations of 50 and 100 mg/L, while at 300 mg/L showed values that remained constant over time, and lower than the control.

Added to the solutions at 2 g/L of tannin, the CMC showed the same behavior described above. While for the addition to the solutions at 4 g/L of tannin, it showed higher values at 45 days than those at 7 days, which were higher than the

control.
The addition of gum arabic led to the formation of particles of smaller size compared to the control solution of tannin at 1 g/L. The opposite, instead, occurred in the solutions of 4 g/L of tannin, where, while remaining constant over time, the particles reached a size larger than the control ones. In spite of the size comparable to that of the control at 7 days, the addition of gum arabic formed larger particles compared to the control for the lower concentration of the polysaccharide and smaller at the other concentrations, in solutions at 2 g/L of tannin. In this case it is possible to notice how the same behavior already evident in the tests with the grape skin tannin is repeated.
The addition of mannoproteins resulted in particles smaller than in the control solutions at 1 g/L of tannin. With the tannin in the concentration of 2 g/L, mannoproteins favored the formation of smaller particles than those obtained at 7 days and compared to the control, whereas, in solutions of 4 g/L it favored the formation of particles larger than the control at the concentrations of 100 and 500 mg/L and similar to the control for the 200 mg/L concentration. This behavior differs from what was shown by the tests with grape tannins.
The different answers given by the polysaccharides with the two tannins and in their different concentrations, suggests a different reactivity of these substances towards tannins.
The formation of stable colloids of large size carries the risk of retention in filtration systems. Consequently, these studies are interesting as they provide insight into what stage of the wine production process is more appropriate for the use of the adjuvants tested. Also in light of the fact that grape tannins are already present in the medium, as well as used as adjuvants, oak tannins may be present only as exogenous adjuvants or arise from wines aged in barrels.

	Particle Size at 7 days (nm)						Particle Size at 45 days (nm)					
	Peak 1		Peak 2		Peak 3		Peak 1		Peak 2		Peak 3	
	Diameter	%	Diameter	%	Diameter	%	Diameter	%	Diameter	%	Diameter	%
Control OT 1	62	24	1118	76,4			178	22	2019	78		
GP 100	67	8,9	745	91,1			245	23,1	1047	76,9		
GP 200	43	14	959	85,9			185	20,5	1263	79,5		
GP 300	154	100					119	15,9	1188	84,1		
CMC 50	67	22	1077	77,7			72	15,5	2340	84,5		
CMC 100	240	24	1409	75,8			146	10,5	461	22,4	3028	67
CMC 300	136	15	1460	84,6			278	27	1599	73		
GA 300	141	20	1199	80,5			136	21,4	1165	78,6		
GA 500	96	25	639	75,4			120	16,5	849	83,5		
GA 1000	96	19	1028	80,8			60	9,4	554	90,6		
MP 100	111	17	977	83,5			327	15,3	778	84,7		
MP 200	132	17	830	83,4			84	22	814	78		
MP 500	178	34	1317	66,3			206	33,5	1324	66,5		
Control OT 2	238	21	1561	79,3			181	18,5	1244	81,5		
GP 100	96	17	1271	82,6			309	25,3	2078	74,7		
GP 200	237	20	1603	79,9			57	8,8	866	91,2		
GP 300	78	2,2	497	36,8	2003	61	26	1,1	785	98,9		
CMC 50	227	24	1611	75,9			35	3,7	588	58,2	**8845**	30
CMC 100	135	15	1301	84,8			33	7,3	693	55,7	4484	31
CMC 300	277	22	1562	78,5			179	15,1	1534	84,9		
GA 300	195	14	1316	85,9			375	28,4	2008	71,6		
GA 500	47	3	371	31,2	1998	66	72	6,5	760	93,5		
GA 1000	283	29	1761	71			72	9,3	856	90,7		
MP 100	127	10	984	89,8			85	3,6	765	76	4664	20
MP 200	12	20	617	55	2572	25	90	10,5	863	89,5		
MP 500	156	12	804	87,8			76	6,8	586	93,2		
Control OT 4	66	7,6	631	92,4			442	100				
GP 100	216	20	1173	79,7			505	64,4	3217	17	**5470**	19
GP 200	895	21	1163	79,3			404	37,6	1288	62,4		
GP 300	506	100					274	16,8	1275	83,2		
CMC 50	67	22	1077	77,7			637	100				
CMC 100	135	7,6	681	92,4			189	12,1	833	87,9		
CMC 300	206	6,2	705	93,8			580	100				
GA 300	122	6,5	607	93,5			266	21,7	996	78,3		
GA 500	201	16	1113	84			220	13,6	973	86,4		
GA 1000	175	13	1001	87,2			142	8,9	903	91,1		
MP 100	205	17	972	82,7			186	15,8	1301	84,2		
MP 200	293	28	1128	72,2			499	100				
MP 500	332	35	1116	64,9			574	74,9	1865	14,2	4243	11

Table 13 – Particle size of model wine solutions with oak tannin and polysaccharides (nm).

7.Conclusions

7.1 Part 1 - Characterization of tannins - Technological trials in cellar

The objectives of this part of the research have been achieved satisfactorily, by increasing the level of knowledge on tannins and their use. The traditional evaluation parameters tested on the tannins formulations are applicable for their characterization and for the discrimination between condensed tannins and hydrolysable ones.

Among the non-traditional parameters used on tannin preparations, measuring the surface electrical charge (SEC) has allowed to know ahead the responsiveness towards proteins. In fact the tannins have negative electrical charge at the pH of wine, so the evaluation of this important parameter has allowed to know better the oenological quality of these compounds. In addition to this technique, a very interesting analytical tool and complementary to the measure of SEC, has been used, the measure of the colloidal diameter by dynamic diffusion of laser light by DLS (Dynamic Light Scattering). Through this measurement, information on the distribution of tannin particle sizes, useful for predicting its future colloidal stability, was obtained. These alternative evaluation methods could become innovative tools in optimizing the use of adjuvants and the winemaking and management processes stabilization (i.e., predict the boundary between system stability and instability, etc.).

With reference to technological tests, differences in behavior have been highlighted between the different preparations of botanical origin, and between preparations of the same botanical origins extracted with different solvents. It has been confirmed that condensed tannins have a positive role on the stabilization of colour, especially if combined with operations of macro-oxygenation. The diethyl ether solvent provided the most positive feedback, while the hydrolysable tannin (Tara tannin) was effective in providing a strong synergistic action in protecting from oxidation.

In light of the encouraging results obtained. The importance of quality control of tannin and adjuvants for the optimization of techniques of vinification is reiterated. Further studies, however, are needed, in order to better assess the technological potential of individual tannins.

7.2 Part 2 - Study on the interactions between tannins and polysaccharides

The study of the interactions between tannins and polysaccharides, carried out by exploiting unconventional techniques has been useful in defining some of the behaviors of these substances in model solutions and wine, where the interactions and effects related to their presence have been influenced also by all the other wine matrix constituents.
The evaluation of their effects in model solutions showed that the polysaccharides added had different reactivity towards the various tannins and behave differently depending on their concentration and on the concentration of the tannin added in solution.
As for the colour component and phenolic substances, it has been noted that the polysaccharides added did not seem to have substantial negative effects, generally maintaining the polyphenolic content and colour constant over time. In tests with the red wines added with polysaccharides, the colour was even slightly improved compared to the control.
The observed reduction of polyphenols is more likely due to their oxidation and subsequent precipitation, natural in wine, rather than to the presence of polysaccharides.
Also the assessments of the antioxidant activity of tannins and polysaccharides have confirmed the higher (oak and grape skin) or lower antioxidant power (cherry) of tannins in white wine. They also have shown that the addition of polysaccharides did not negatively affect their capacity, but has not improved it either, since it showed no differences both between the various polysaccharides added and their different concentrations.
As for the surface electrical charge, all the tests in model wine and wine, have shown that the addition of polysaccharides, in particular gum arabic and carboxymethylcellulose increased the negative charge of the system compared to the control. The most interesting consideration, however, is the fact that after the initial rise, the value of electrical charge for all the tests has not changed over time, confirming that the phenomena related to the interactions between the compounds tested do not only depend on their electrical charge.
The turbidity measurements showed an increase in values over time, particularly for samples containing mannoproteins and grape polysaccharides, both before mixing the samples, as an index of stable colloids present in solution, and after agitation, indicating the pronounced sediment formation.
The addition of CMC did not seem to influence the turbidity parameter, suggesting that this adjuvant can be used for traditional purposes without interacting with the other macromolecules, avoiding the instability of the colloidal system.
The measures of particle size were used to evaluate the dimension of sediment particles that were formed within 45 days, as well as that of the colloids remained stable in solution. The combined effect of tannins and polysaccharides has shown,

however, to vary greatly depending both on the tannin and the polysaccharide used as well as their respective concentrations.
It is important to evaluate the turbidity parameter in relation to the measurement of particle size, because not always a close correlation was noted between the two measures. In fact, high turbidity not always matched larger particles. In this case the increase in turbidity could be interpreted by the presence of a greater number of smaller particles. The risk in this case could be the permanence of some of these particles in solution, causing turbidity of the product.
The turbidity measurement has provided useful information regarding what might be the problems before and after bottling of a wine. Colloidal instability after bottling could disturb the limpidity of the wine itself, causing a decline in the quality of the product.
Tests were also carried out using rather large quantities of these products in order to assess what might be the limit between colloidal stability and instability.
Sedimentation appears to be the most important issue related to the use of these products as adjuvants, as well as the management of those naturally present in wine (grape polysaccharides, tannins, and mannoproteins).
The risk of precipitation may also mean that the formed colloidal aggregates can drag, in their precipitation, a part of the aromatic component, thus affecting the final quality of the wine also from this point of view.
The time factor is crucial for these phenomena both in the management of the product that is naturally present in wine and in that of adjuvants added.

Part 3

Interactions of Flavours with Macromolecules: Tannins and Proteins

1. Introduction

1.1 Aroma in wine

Wine aroma is very important for its responsibility in the character of the final product.

The aroma of a wine starts in the vineyard, where the variety occupies the central place, to which converge the interference of soil, climate, as well as techniques, fertilization, irrigation.

Wine aromas are made up of several hundreds of volatile compounds, at concentrations ranging from several mg/L to a few ng/L, or even less.

In the classification of the aromatic compounds of wine different classes are distinguished:

- Varietal aromas
- Pre-fermentation aromas
- Fermentation aromas
- Post-fermentation aromas

The complexity of wine aromas, which makes them particularly difficult to study, is due to the diversity of the mechanisms involved in their development:

1. Grape metabolism, depending on the variety, as well as soil, climate and vineyard management techniques.
2. Biochemical phenomena (oxidation and hydrolysis) occurring prior to fermentation, triggered during extraction of the juice and maceration.
3.The fermentation metabolisms of the microorganisms responsible for alcoholic and malolactic fermentations.
4. Chemical or enzymic reactions occurring after fermentation, during aging of the wine in vat, barrel and bottle.

Besides the mechanisms of development, their interactions with the other molecules and macro-molecules present in wines should be considered, in fact these phenomena influence aroma volatility and solubility, and thus its release from wine.

1.2 Interactions Between Aroma Compounds and wine macromolecules

The interactions that occur at the molecular scale, between the volatile compounds, and other components of food matrices, are related to the general pattern reported by Lubbers (1993). The links that can be established between molecules are of two types: valence bonds (ionic and covalent bonds) and physical and chemical interactions (Van der Waals forces, hydrogen bonds, hydrophobic interactions).

In the first case they are actual chemical bonds, involving electronic exchanges, while in the latter, interactions are meant as forces of attraction or repulsion between molecules whose normal valency is met.

In general, according to what Lubbers (1993) reports, the bonds that are established between aromatic compounds and other molecules, are weak and characterized by reversibility, but they are an important factor for the perceived aroma. The sensory characteristics of a product thus, directly depend on interactions between the non-volatile constituents and aromatic substances, a change in the conditions of the medium (i.e. temperature), can heavily modify these characteristics, through the variation of the volatility of aromas.

The Odour Activity Value (OAV) (ratio of concentration to threshold) is widely used in the field to predict the potential contribution of an aroma compound to overall product flavour. Pineau *et al.,* (2007) have pointed out that wine matrix components have not been taken into account in the calculation of OAV of wine odorants. Thus, he suggested that aroma impact data for wines based on OAV calculated using sensory threshold values obtained in water or hydroalcoholic solutions may not be accurate, and could be highly overestimated.

Ethanol is, after water, the major component of wines. It directly contributes to wine aroma and overall flavour, since it is substantially above its perception threshold (from 0.1 to 100 ppm) (Bayonove *et al.,* 2000). Because it can influence viscosity of the beverages, it could modify aroma release and thus, aroma perception (Nurgel and Pickering, 2005).

The most studied ethanol effect is related to its capacity to modify solution polarity, thus altering the gas-liquid partition coefficient. An increase in ethanol content has been shown to decrease the activity coefficients of many volatile compounds in wine because of an increase in solubility (Voilley *et al.,* 1991). Many other researches showed that generally the increase of alcohol content reduces the release of volatile compounds from wines (Hartmann *et al.,* 2002, Whiton and Zoecklein, 2000, Pet'ka *et al.,* 2003, Grosch, 2001, Robinson *et al.,* 2009).

Polyphenols, are one of the major non-volatile components in wine, they can interact non-covalently with aroma compounds in solution (King and Solms, 1982, Dufour and Bayonove, 1999a, Dufour and Suvaitre, 2000, Jung *et al.,* 2000), affecting the release of wine aroma compounds.

documented Hydrophobic interactions between phenolic compounds and aroma compounds in water systems showed that they can increase the solubility of aroma

compounds, thereby decreasing the activity coefficient of the aroma compounds (γ_i) (King and Solms, 1982).

$$\gamma_i = (y_i / x_i) \times (p_t / p_i^S)$$

where:
γ_i = activity coefficient of component "i"
y_i = molar fraction of component "i" in the vapor phase
x_i = molar fraction of component "i" in liquid phase
p_t = vapor pressure of the solution
p_i^S = saturating vapor pressure of component "i"
y_i/x_i = Ki liquid-vapor distribution coefficient of component "i"

Dufour and Bayonove (1999b) Catechins and aroma compounds in model wine systems showed weak interactions that are different depending on the type of polyphenols (catechin or tannin), and on the nature of the aroma compound and are related to their hydrophobicity
Polyphenols (flavanols and anthocyanidins) can interact with aldehydes creating condensation products that are directly related to the development of colour and astringency of a wine (Fulcrand *et al.,* 1996, Dallas *et al.,* 1996, Escribano-Bailón *et al.,* 1996, Atanasova *et al.,* 2002, Mateus *et al.,* 2002).
Anthocyanins can form hydrogen bonds with some aroma compounds when they are present at high concentrations (Voilley *et al.,* 1991). Also some phenolic compounds extracted from the barrels wood (ellagic tannins) can participate in polymerization reactions with aroma compounds (Escalona *et al.,* 2002)
Some NMR studies (Jung *et al.,* 2000) have confirmed that interactions between some polyphenols (gallic acid and naringin) and some aroma compounds (i.e. ethyl hexanoate and 2-methylpyrazine) are due to π-π stacking of the gallic acid ring with the aromatic ring of a flavour compound.
Polyphenols can have different effects depending on the type of aroma compound they interact with (Hartmann *et al.,* 2002).
Aronson and Ebeler (2004) showed with their sensoty study that gallic acid (in 1% ethanol solution) significantly decreased the volatility of 2-methylpyrazine, while naringin at the same level had little effect. They also found that ethyl benzoate had little interaction with either polyphenol and that the analytical and sensory data showed the same effect of gallic acid and naringin interactions with 2-methylpyrazine.
The studies on the interactions aroma-polysaccharides have shown that they are due to relatively weak bonds, which depend on many factors such as the nature and concentration of the molecules involved (Nawar, 1966, Solms, 1986). These interactions are due to different mechanisms such as adsorption, formation of inclusion complexes, and modification of the diffusivity of the aroma in the medium.

The effects of simple sugars on the volatility of aromatic substances were subject to different works. Reineccius *et al.,* (1997) found that the presence of polysaccharides, including sucrose, significantly decreases the release of volatile components

According to Voilley (1991), in the presence of polysaccharides, there is a variation of activity coefficients (γi) of aromatic compounds.

Neutral peptic substances (type II arabinogalactans and arabinogalactans-proteins) represent 40% of the polysaccharides in wine and acidic pectic polysaccharides, (i.e. homogalacturonans and rhamnogalacturonans) account for 20% of them.

Different wine polysaccharides isolated from wine, specifically arabinogalactan proteins, monomeric and dimeric rhamnogalacturonans II and mannoproteins, had different effects on the activity coefficients of some volatile compounds (isoamyl acetate, ethyl hexanoate, 1-hexanol, diacetyl), depending on the type of polysaccharide and the nature of the aroma compound (Dufour and Bayonove, 1999b).

Mannoproteins which represent about the 35% of total wine polysaccharides, can be divided into two main groups, the ones that are secreted into the wine by yeast during alcoholic fermentation and those released into wine due to yeast autolysis during aging on lees (Chalier *et al.,* 2007). They can interact with aroma compounds, and their interactions are, also in this case, mainly hydrophobic and dependent on the type of aroma compound and nature of the substrate (Lubbers *et al.,* 1994a, b)

Mannoprotein interacting with aroma compounds, can reduce their volatility due to the fact that probably both the glycosidic and the peptidic parts of these macromolecules may be responsible for the interaction. Moreover, these interactions can be different depending on the conformational and compositional structure of mannoproteins (Chalier *et al.,* 2007).

It was also found that these macromolecules can strongly modify wine aroma composition by either affecting the volatility of indigenous wine aroma compounds or by adding new aroma compounds, and so modifying the original wine aroma profile (Comuzzo *et al.,* 2006).

Interactions between polysaccharides produced by wine lactic bacteria *(Oenoccocus oeni)* during malolactic fermentation have been shown to be responsible for the reduced volatility of some aroma compounds in wines (Boido *et al.,* 2002).

Other than these studies on the role of proteins released by yeast during autolysis (mannoproteins) on wine aroma, little work has been reported on interactions of other proteins with aroma compounds. One study investigating such interactions was published by Druaux *et al.,* (1995). They used model wine solutions and bovine serum albumin (BSA) as a model protein.

1.2.1 Factors influencing the interaction

The increase in temperature increases the concentration of volatile substances in the vapor phase (Lubbers, 1993). This varies depending on the type of interactions that exist between non-volatile aromas and matrix, the intensity of the hydrogen bonds, in fact, decreases at higher temperatures, while in contrast, hydrophobic interactions, increase with the temperature of the system (Lubbers, 1993) .

The presence of salts in the medium increases the volatility of the flavour of whatever nature (Lubbers, 1993), the addition of salts causes changes in ionic strength, greatly influencing the solubility and conformational state of proteins , and hence their retention capacity.

The presence of salts in the wine causes the volatile components to move towards the vapor phase (De La Ossa and Galán, 1986). Even the pH, like temperature and ionic strength, can determine the conformational change and denaturation of the protein matrix, thereby changing the retention of aroma from the substrate (Lubbers, 1993).

1.3 SPME technique

SPME (Solid-phase microextraction) is a solvent-free technique designed for rapid sampling and sample preparation. It was developed by Pawliszyn in the early 1990s in an attempt to redress limitations inherent in SPE and LLE. SPME integrates sampling, extraction, concentration and sample introduction into a single solvent-free step. Analytes in the sample are directly extracted and concentrated to the extraction fibre. The method saves preparation time and disposal costs and can improve detection limits.

SPME may be used to determine partition coefficients if short sampling times are applied: the process must only sample the headspace and not disrupt the equilibrium (Jung and Ebeler 2003). This method has become very popular to study the effect of wine macromolecules on the liquid-vapor equilibrium, (Whiton and Zoecklein 2000, Escalona *et al.,* 200, Hartmann *et al.,* 2002, Aronson and Ebeler 2004).

It has been routinely used in combination with gas chromatography (GC) and GC/mass spectrometry (GC/MS) and successfully applied to a wide variety of compounds, especially for the extraction of volatile and semi-volatile organic compounds from environmental, biological and food samples, such as beer, vodkas, coffee, colas ad wines (Scarlata *et al.,* 1999, Ng *et al.,* 1996, Yang and Peppard, 1994, Bicchi *et al.,* 1997, Elmore *et al.,* 1997, Carlin, 1998).

It is based on the sorption characeristics (adsorption or absorption) of fiber coating materials (Rocha *et al.,* 2001). The analytes (volatiles or semivolatiles) from gaseous, liquid, or solid matrices are first released from the matrices and sorbed onto a fiber coated with an ad(ab)sorbent polymer introduced into the headspace. Following sorption, analytes are either thermally desorbed onto a gas chromatographic (GC) inlet or solvent desorbed into a high-performance liquid chromatographic (HPLC) inlet (Arthur and Pawliszyn, 1990, Chen and Pawliszyn, 1995).

In recent years many studies have optimized the HS-SPME sampling conditions required to sample grape and wine matrices for targeted analytes.

Thus, because of its simplicity, sensitivity, selectivity, and ease of automation, **i**n this research work it was decided to use HS-SPME (Headspace solid-phase microextraction) (Figure 135) to investigate odorants interactions with tannin, proteins and tannin-protein aggregates.

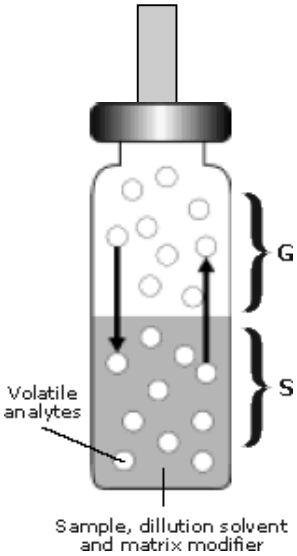


Figure 135 - SMPE Headspace Sampling
G = the gas phase (headspace): The gas phase is commonly referred to as the headspace and lies above the condensed sample phase.
S =the sample phase: The sample phase contains the compound(s) of interest.
Jung *et al.,* 2003.

2. Aim of the research

The aroma is a key feature for the good character of a wine. For this reason it has been extensively studied and it continues to be so. The works presented in the literature have shown that volatile compounds that contribute to the aroma, frequently interact with the molecules and macromolecules of the wine matrix (Saenz-Navajas *et al.,* 2010).

All these macromolecules stay in the colloidal state of the wine. Thus, changes in the colloidal state due to interactions between colloidal substances can influence the odorant component of wine by increasing or decreasing volatility and the perception of some flavour compounds. In fact, besides their direct interactions with macromolecules, volatile compounds can be trapped in the aggregates formed, for example by polyphenols and proteins, no longer contributing to the aromatic fraction of wines.

It is highly desirable, thus, to understand how flavour interactions with the major components of wines can influence their perception.

The studies conducted so far have focused on the investigation of the interaction of individual types of macromolecules with odourous substances in model wine solutions.

The objective of this work is to observe the influence of macromolecules such as polyphenols (tannins) and proteins (BSA), added separately or mixed together to form aggregates (haze) in model wine solutions, on the volatility of some volatile compounds typical of the wine matrix.

3. Matherials and methods

This part of the PhD research has been developed in the Prof. Susan Ebeler's Laboratory in the Viticulture and Enology Department of the University of California-Davis, CA, USA.

3.1 Materials

For experimental purposes, nine volatile compounds, representative of many classes of aromas found in wine, have been taken into account, the considered compounds are presented in Table 14

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Volatile compound	Chemical class
Linalool	terpenes
β-ionone	nor-isoprenoids
Benzaldehyde	phenols
Hexyl acetate	esters
Phenethyl acetate	esters
Ethyl octanoate	esters
1-octanol	primary alcohol
3-octanol	secondary alcohol
3-octanone	ketons

Table 14 - Volatile compounds evaluated in this research

The flavour compounds used in this work (purity > 98%) β-ionone, 1-octanol, 3-octanol, 3-octanone, ethyl octanoate, benzaldehyde, hexyl aldehyde, phenethyl acetate and linalool, were purchased from Aldrich (Milwaukee, Wisconsin, USA). Tannic acid and Bovine Serum Albumin (BSA, purity 96%) were purchased from Sigma (St. Luis, Missouri, USA).
Each flavour compound was solubilized in ethanol, 200 proof (Gold Shield, Hayward, California, USA) to give a concentration of 200 ppm and 100 ppm.
The 20 mm lenght Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber, 50 μm coating thickness, 23 ga needle size, was purchased from Supelco (Bellefonte, Pennsylvania, USA). The fiber was conditioned at 230 °C for 1 hour before the use.
The experimental plan provided the preaparation of model wine solutions (14% EtOH, 5 g/L tartaric acid, buffered to pH 3,5 with NaOH 4N) added with 9 different flavour compounds in two different concentrations, tannic acid at 2,5 g/L concentration and bovine serum albumin (BSA) organized as follows (Figure 136):

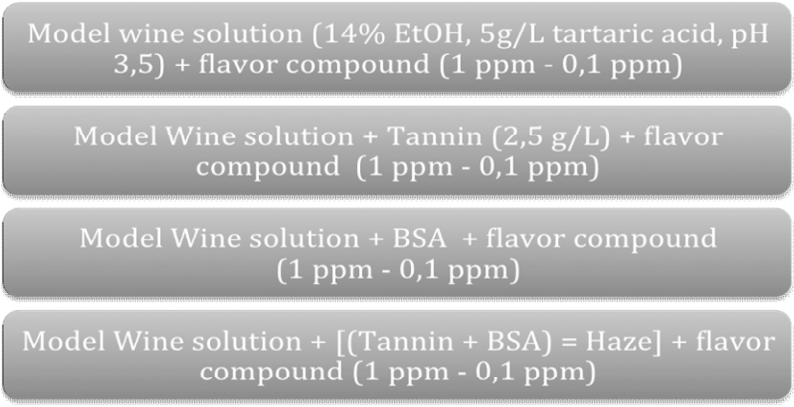

Figure 136 – Experimental plan.

Characterization of matrix interactions were performed preparing three replicates for each solution, placing them in 10 mL amber glass crimp top vials with PTFE/silicone septa, purchased from Agilent (Santa Clara, California, USA) and equilibrating the samples at room temperature for 12 hours before HS-SPME sampling.

3.2 Instruments used

After the equilibration all experimentation was conducted with a Gerstel MPS2 autosampler (Linthicum, Maryland, USA) coupled to an Agilent 6890N gas chromatograph with an Agilent 5975 inert mass selective detector (Santa Clara, California, USA).
The GC oven was equipped with a 30 m DB-WAX capillary column with an inner diameter of 0.25 mm and a film thickness of 0,25 μm (Santa Clara, CA, USA).

3.3 Chromatographic conditions

The injector was held at 240 °C in the splitless mode (when flavour compounds were added in 1 ppm concentration) with a purge-off time of 1.20 min, a 30 mL min^{-1} spit vent flow. The split mode was used when the concentration of the flavour compounds was 0.1 ppm. Helium was used as the carrier gas at a constant flow of 1mL min^{-1}. The temperature program was 40 °C (initial isotherm) for 4 minutes, 6 °C min^{-1} to 100 °C, then 15 °C min^{-1} to 240 °C, held for 10 minutes (last isotherm), with a total run time of 33.33 minutes. The transfer line and ion source were maintained at 240 °C and 230 °C, respectively. The detector collected masses between 50 and 250 amu.

3.4 HS-SPME extraction

The headspace was sampled for 1 minute with the vial at ambient temperature then the fiber was desorbed at 240 °C for 600 seconds.

3.5 GC-MS analysis software

GC-MS interrogation and compound mass spectral data were compared against the NIST 2005 Mass Spectral Library. Peak area integration of unique masses was conducted using MSD Chemstation.

3.6 Statistical Analysis

Regarding the analysis of the headspace using HS-SPME-GC, areas obtained for the absolute peak of the same compounds were evaluated.

Within each comparison, means and standard deviations were calculated, and significant differences (measured by $p < 0.05$) were determined by the 'Honest Significant Difference Test (HSD test) Tukey, using the form basic STATISTICA/W, version 7.0.

4. Results and discussion

The area of the peaks obtained from gas-chromatography of headspace samples is the parameter that has been taken into account to assess and discuss the results of this test.
As for the samples containing benzaldehyde (Figure 137), it was possible to see that, at the concentration of 1 ppm, the addition of tannin, negatively affected the volatility of the aromatic compound significantly reducing the content by 27% in the headspace. This confirms what is present in literature about the ability of polyphenols to bind aromatic compounds (Dufour and Suvaitre, 2000, Jung *et al.,* 2000).
The addition of BSA resulted in a decrease in volatility (12%) but it is not statistically different compared to control.
The aggregate tannin+BSA (haze), seems to have been most influenced by the characteristics of tannin than those of the protein. Its presence in solution, in fact, reduced the volatility of the compound by 23%.
The situation changes if we consider the same aromatic compound at a concentration of 0.1 ppm. At this concentration, the addition of BSA was the only one to have a noticeable effect compared to control by increasing the volatility of the compound by 25%. It can be assumed, therefore, a salting out effect of this protein at low concentrations of benzaldehyde.

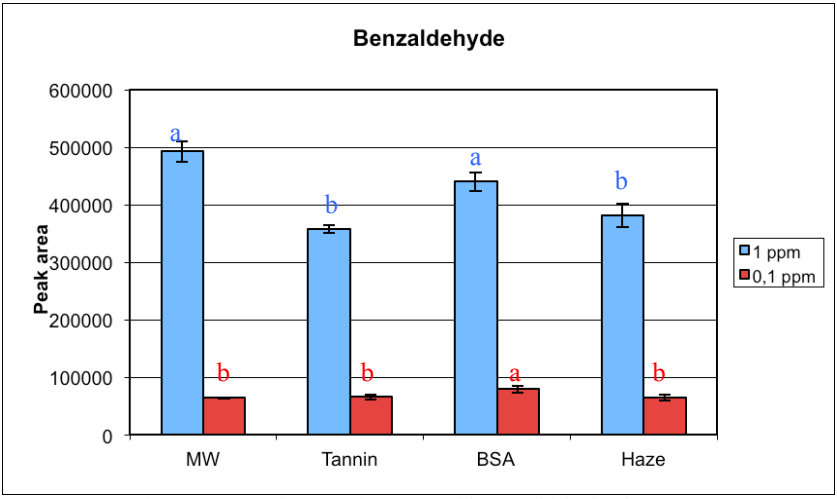

Figure 137 – Peak areas of samples with Benzaldehyde 1/0,1 ppm.
Different letters identify samples that are significantly different for $p < 0.05$.

For the 1-octanol was noted an opposite effect compared to benzaldehyde (Figure 138). At a concentration of 1 ppm, in fact, the only noticeable effect was that due to the addition of BSA, which increased the content in the headspace by 18% compared to control. This effect was lost when the BSA was dissolved together with tannin, as the haze formed there was no salting out effect, it actually decreased by 6% the volatility of the compound. This decrease is however not significant in statistical terms. At a concentration of 0.1 ppm due to the increase in BSA, stood at 19%, remaining the most significant effect, while the formation of haze (tannin + BSA) decreased the volatility by 15% compared to control, differing significantly from the latter.

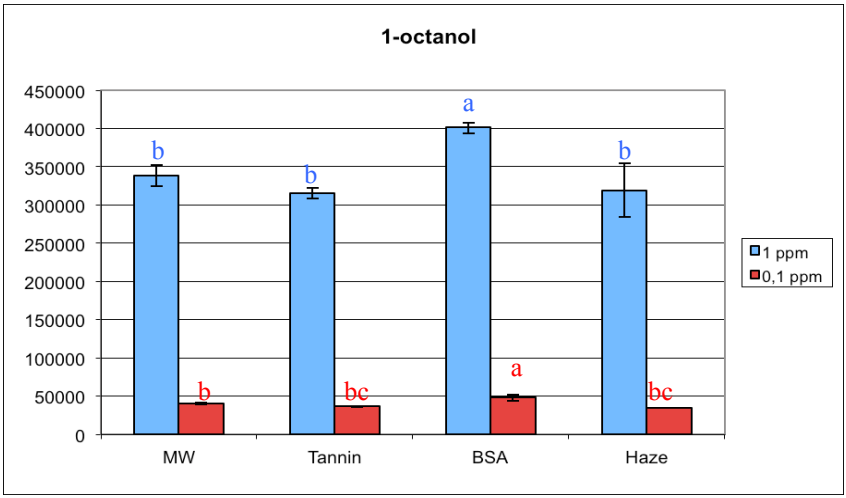

Figure 138 – Peak area for samples with 1-octanol 1/0,1 ppm.
Different letters identify samples that are significantly different for $p < 0.05$.

The β-ionone presented similar results at both 1 ppm and 0.1 ppm concentration, in both cases, in fact, the strongest effects were those due to the tannin, but especially to the haze, their presence reduced the volatility of the compound respectively by 25 and 30% compared to control, in the first case and 34 and 41% in the second case (Figure 139). At a concentration of 0.1 ppm BSA also showed a slight salting out effect, increasing the content of the aroma in the headspace by 7%.

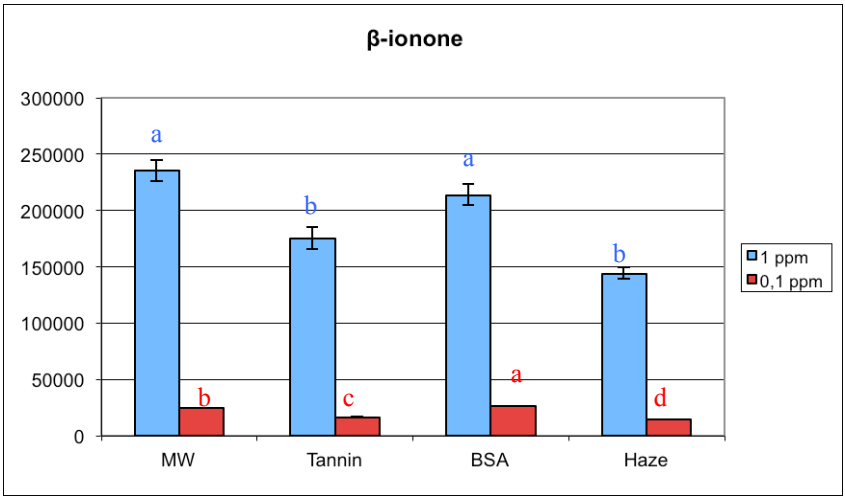

Figure 139 – Peak area for samples with β-ionone 1/0,1 ppm.
Different letters identify samples that are significantly different for p <0.05.

For linalool at a concentration of 1 ppm, it was pointed out the effect of BSA, the addition of which has fostered a percentage increase of 19% (Figure 140). This trend did not occur at a concentration of 0.1 ppm of the aromatic compound, since the presence of BSA resulted in a minimum increase of 1%. A significant effect of tannin added was detected, in fact, it led to a reduction in the volatility by 19%. The simultaneous addition of tannin and BSA, in the case of 1 ppm fostered a behavior more similar to that of tannin, so it is assumed that, at this concentration of flavour, tannin has more influence on the effect of haze compared to the protein. An opposite result was obtained in the case of 0.1 ppm, in which the presence of haze did not affect the volatility, just like the presence of BSA. Therefore, it seems that in this case, the haze is most influenced by the component with the least pronounced effect.

The 3-octanone at 1 ppm did not present significant differences except for the haze that has reduced the volatility by 14% compared to control (Figure 141). At 0.1 ppm, however, in addition to the reduction due to the haze (18%) the reduction due to the presence of tannin (16%) was also significant. It is interesting to note how, for this compound, failed to appear the salting out effect due to the BSA, confirming the findings in previous studies on the BSA in relation to ketones (Jung and Ebeler, 2003).

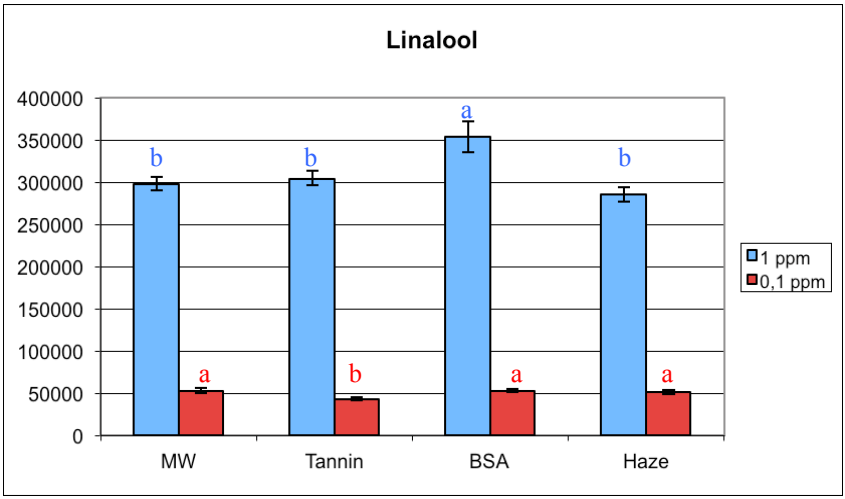

Figure 140 – Peak area for samples with linalool 1/0,1 ppm.
Different letters identify samples that are significantly different for p <0.05.

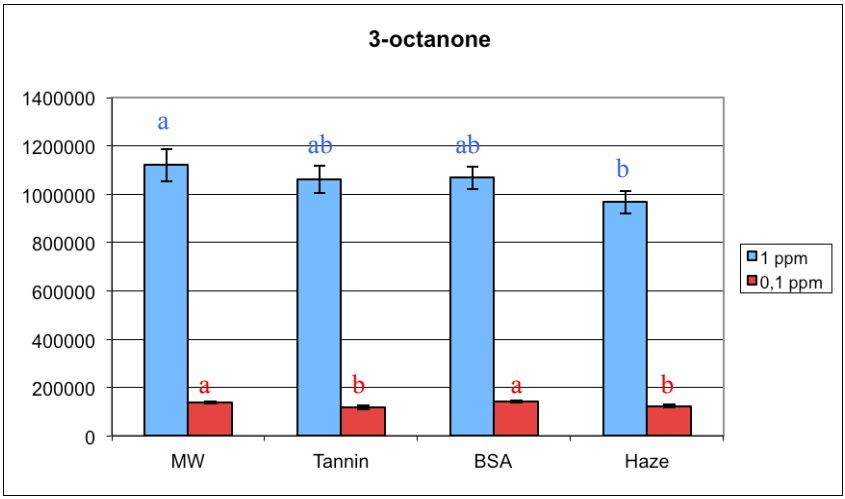

Figure 141 – Peak area for samples with 3-octanone 1/0,1 ppm.
Different letters identify samples that are significantly different for p <0.05.

As for the phenethyl acetate at a concentration of 1 ppm, both the presence of tannin and the presence of BSA added individually resulted in a reduction by 6 and 18% compared to the control (Figure 142). But the most interesting data is that related to the haze, since the synergistic effect of the action of tannin and protein in the aggregate resulted in a 46% reduction in the volatility of the compound.

At 0.1 ppm concentration, however, this behavior was not repeated, only a slight salting out effect related to BSA, which has increased the volatility of 8%, was noticed.

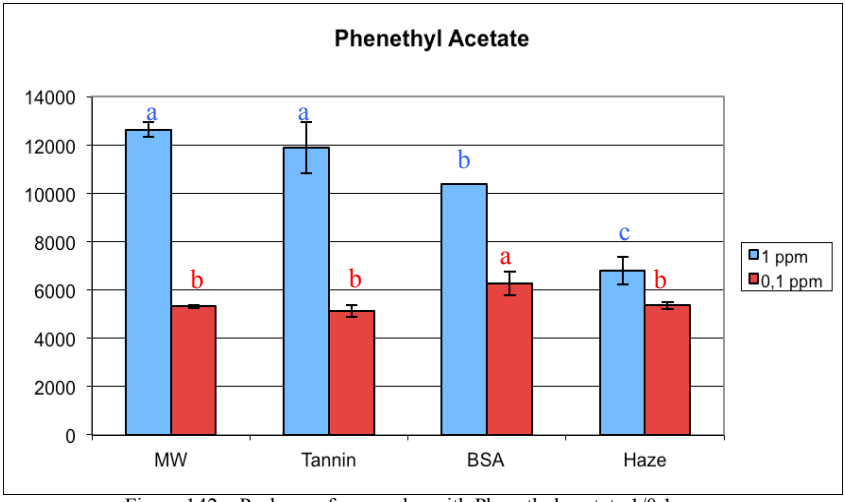

Figure 142 – Peak area for samples with Phenethyl acetate 1/0,1 ppm.
Different letters identify samples that are significantly different for $p < 0.05$.

Samples with hexyl acetate showed similar patterns for both concentrations (Figure 143). The presence of tannin led to a decrease by 15% of the content of the aroma in the headspace of both the solutions at the concentrations of 1 ppm and 0.1 ppm.

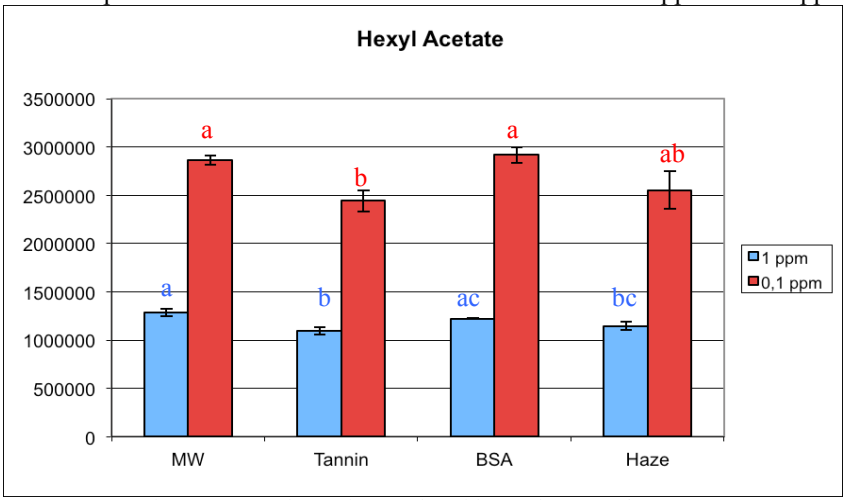

Figure 143 – Peak area for samples with Hexyl acetate 1/0,1 ppm.
Different letters identify samples that are significantly different for $p < 0.05$.

For the ethyl octanoate at 1 ppm both salting-out effect, due to the addition of BSA (10% increase) and negative influence of tannin (15% reduction) and in particular haze (reduction of 18 %) were observed (Figure 144). This effect has been noted markedly for the lower concentration of the aromatic compound (0.1 ppm) where the reduction due to the tannin was by around 16% and by 24% due to the haze.

A separate comment is reserved to 3-octanol, for which only data for the concentration at 1 ppm are present (Figure 145). From which, however, a remarkably similar pattern to that of the ethyl octanoate at the same concentration was deducted: percentage increase of 7% due to the presence of BSA and reduction respectively by 9 and 15% due to tannin and haze.

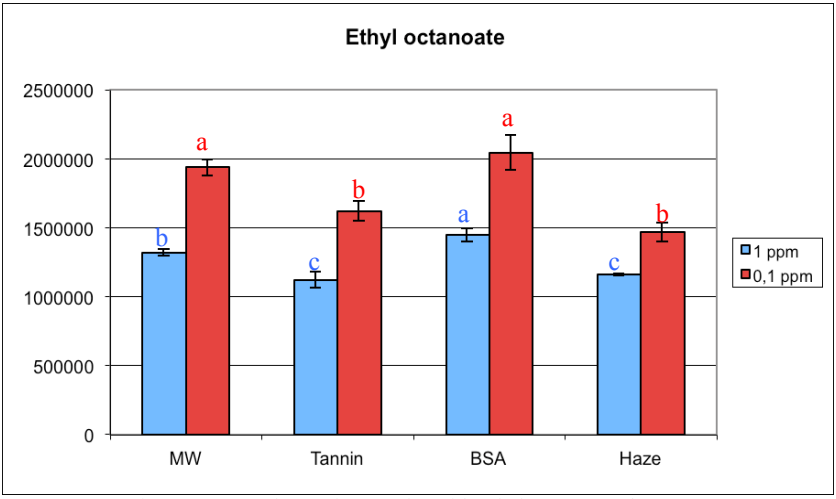

Figure 144 – Peak area for samples with Ethyl octanoate 1/0,1 ppm.
Different letters identify samples that are significantly different for $p < 0.05$.

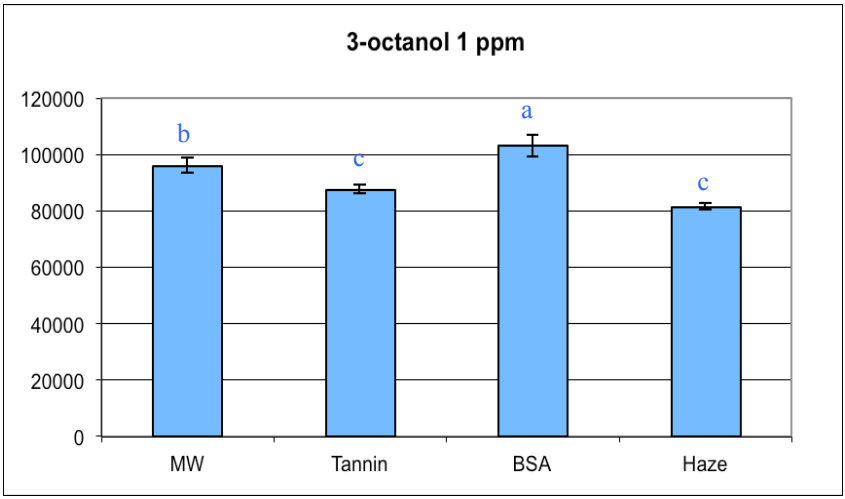

Figure 145 – Peak area for samples with 3-octanol 1 ppm.
Different letters identify samples that are significantly different for $p < 0.05$.

5. Conclusions

The tests have confirmed that the macromolecules present in solution may affect the volatility of aromatic compounds.
In most cases, the presence of tannin led to a reduction in volatility.
In contrast, the addition of protein showed in almost all samples a salting-out effect, increasing the volatility of the same flavours.
The haze formed by the simultaneous presence of tannin and protein in most cases has reduced the volatility of the volatile compound added. This suggests that the aggregates formed, generally, are more influenced by the tannin characteristics than by the ones of the protein.
However, it is possible to affirm that the noted effects depend on the type of aroma present and its concentration, confirming what Hartmann *et al.,* (2002) found. Even the same flavour, at various concentrations, shows different reactivity toward these macromolecules.
It is very likely that in the presence of haze aroma is trapped in the aggregates formed, as well as interacting directly with the macromolecules.
These substances are part of the colloidal system, so, variations in the system can lead to variations of the aromatic components. The formation of stable colloids over time generally promotes sensory stability, as the flavours remain in solution and they are not lost due to colloidal precipitation. Identifying the boundary between stability and instability of soluble colloids is useful to maintain the good sensory quality of a wine.
The results obtained from the study of individual flavours added require integration with the analysis of samples containing a mixture of volatile compounds (belonging to the classes present in the wine) in order to evaluate the synergistic effect of aromatic substances and the effect of macromolecules in presence of different flavours, which ultimately is the situation more similar to that of the wine system.
Sensory stability can not be separated from the optimization of colloidal stability. Studies on this topic may have positive aspects and applications to maintain the good characteristics of a wine.

General Conclusions

This study showed that, despite the obvious advantage of using macromolecules as oenological adjuvants, it is essential to assess the risks associated with their use.

First, by improving the analytical techniques used for the characterization of these substances. The first part of this work has, in fact, permitted to use unconventional techniques in the wine industry for the characterization of commercial preparations of tannins, obtaining useful information that have been exploited in the choice of the tannins to be used in the subsequent technological tests carried out. These technological tests, given the results obtained, confirmed the value and importance of quality controls for adjuvants.

Secondly, by studying the interactions of these molecules and colloidal phenomena related to these interactions. Measurements of the diameter of colloidal particles by DLS, surface electrical charge and turbidity provided interesting information on the limits between colloidal stability and instability, proving to be useful and valid techniques for the study of the colloidal system of wines.

Third, evaluating the effect of macromolecules of the colloidal system on the aromatic component, a key factor in the quality of a wine. The tests have confirmed previous studies on the interactions between aromas and polyphenols. Moreover they provided new information on the effect of aggregates formed from the interactions between tannins and proteins (haze).

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Acknowledgments

At the end of this journey full of exciting challenges I would like to remember and thank all the people who have been around and supported me in times of difficulty and in moments of satisfaction.

First of all I would like to thank my supervisor, Prof. Celotti for the availability, great humanity, ideas, advices, reassurance and for having faith in me.

To Paola, my "travel companion" of these 3 years, goes a huge thank you for having always been close to me, for all the things we shared together and I hope that we will continue to share, the joys and sorrows, the good days and those difficult.

I sincerely thank all the Department of Food Science of the University of Udine, in particular the section “Industrie Agrarie” for the availability and especially thanks to Piergiorgio for his helpful advices. I would also like to thank all the people in the "call center" office for companionship and laughter made together.

I would also like to thank the companies EVER-INTEC and Enartis and in particular Dr. Gianni Branca and Dr. Gianni Triulzi for their helpfulness, kindness and for the products supplied.

I thank the department of Viticulture and Enology of the University of California – Davis, and in particular Prof. Ebeler and the peolpe working in her laboratory, Anna, Arielle and Shaun. I spent six amazing months and I got the opportunity to learn a lot and meet great people, of all Giusy, Alberto, George, Giulia and Silvia, and I thank myself for having chosen to go through this experience.

Thanks to all my dearest friends, with whom I could share this journey and have always had an encouraging word and a joke ready to laugh together.

One of my most sincere thank you goes to Silvia, one of the nicest people I have ever crossed in my path, even though we have known each other for a year it is just as if we had known each other for forever!

Thanks to Mariange a wonderful woman, gentle, kind, just great!

I would like to thank from the bottom of my heart, my FAMILY, the cornerstone of my life, my reference point. Thanks, Mom! Thanks Dad! Thanks Ro!

My last special thought is for Dani, my biggest support over the years, my source of optimism and joy even when it seemed that nothing was going the right way. To you who believe in me even when I do not believe in myself, THANK YOU!