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USE OF CHITOSAN AS A POTENTIAL ANTIOXIDANT ALTERNATIVE TO SULFITES IN OENOLOGY

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Acknowledgments

Y finalmente llegó el momento....el momento de cerrar otro capítulo de mi vida, un intenso capítulo que no habría podido vivir sin todas las personas presentes en la siguientes lineas. Quería pedirte perdón, querido lector, por utilizar más de una lengua.

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Abstract

In the present PhD thesis work, the efficacy of chitosan, a natural polysaccharide, as a potential antioxidant alternative to the use of sulphur dioxide (SO_2) in oenology was studied.

For this purpose, a preliminary characterization of the antioxidant properties of chitosan was carried out. Using the Electron Paramagnetic Resonance (EPR) technique, the antiradical capacity of chitosan against hydroxyl (·OH), or 1-hydroxyethyl (1-HER) radicals, was evaluated. On the other hand, by the application of HPLC, the effect of chitosan on compounds related to oxidation, such as glyoxylic acid and acetaldehyde, was studied.

Furthermore, indirect antioxidant mechanisms of chitosan, such as capacity of metal chelation, and hydrogen peroxide scavenging were evaluated by flame atomic absorption spectrometry and fluorimetry respectively.

The antioxidant activity experiments were carried out both in model and in real wines, showing promising results.

Once characterized, winemaking processes in the presence of chitosan were carried out both at micro-scale in the laboratory and at semi-industrial levels, evaluating its effect on the inhibition of browning (abs 420), the total content of polyphenols (abs 280), on organic acids and on the phenolic and volatile profile. For this purpose, chromatography techniques such HPLC-DAD-MS/MS, and GC/MS were used.

In addition, sensory analysis with trained panels have been conducted to study the sensory profile of different wines made in the absence of sulfites and in the presence of chitosan, ascorbic acid and glutathione, in order to study the interaction of other well-known antioxidants with chitosan.

Contents

Premise

Oxidation of wines is one of the main problems to be faced by winemakers as it not only negatively affects the organoleptic but also the healthy properties of the final product. Polyphenols are the most vulnerable compounds to oxidative processes, developing browning phenomena of white wines.

The most widespread control strategy in oenology is the addition of sulfur dioxide, a potent antimicrobial and antioxidant agent that can be applied during all the stages of winemaking process. However, it has been shown that sulfites generated from this additive are related to adverse effects on human health, causing allergies in most cases, and triggering cancerous diseases.

For this reason, there is a growing interest in discovering new physical-chemical alternatives to the use of sulfites in winemaking. In spite of a quite large number of researches dealing with this subject, none of the supposed technological substitutes have been capable to completely replace the use of sulfur dioxide by itself.

The present doctoral thesis will focus mainly on the study of a recently admitted adjuvant in oenology, chitosan. This natural and abundant biopolymer has been certified as a GRAS (Generally Recognised as Safe) by the Food and Drug Administration (FDA) because of its bioavailability, biocompatibility and low toxicity. For this reason, the application of chitosan in food science is booming. In addition, studies in biological matrices have demonstrated an interesting antioxidant behaviour of this polysaccharide.

Therefore, this project will be based on the premise that chitosan could be used for antioxidant purposes in oenology and candidate an alternative to the use of sulfites in winemaking processes.

1. Introduction

1.1. Oxidation processes occurring in wines

1.1. Oxidation processes occurring in wines

Oxidation of wines is one of the main problems to be faced by winemakers as it adversely influences the overall quality of the final product. During oxidation, polyphenols are among the most susceptible species to be oxidized by reactive oxygen species, leading to the browning of wines. In this chapter, the oxidation mechanisms that leads to wine browning will be reviewed:

1.1.1. Reactive oxygen species (ROS)

Free radical species take part in many reactions processes on biological systems and are often related to spoiling phenomena of foods. ROS is a collective term to describe oxygen radical species, superoxide anion ($O_2\cdot$), hydroperoxyl (HOO·), hydroxyl (HO·), peroxyl (ROO·), alkoxyl (RO·) and other non-radical species that can be oxidative precursors by its easy conversion into radicals, such as hydrogen peroxide (H_2O_2) (Oliveira, Ferreira, De Freitas, & Silva, 2011).

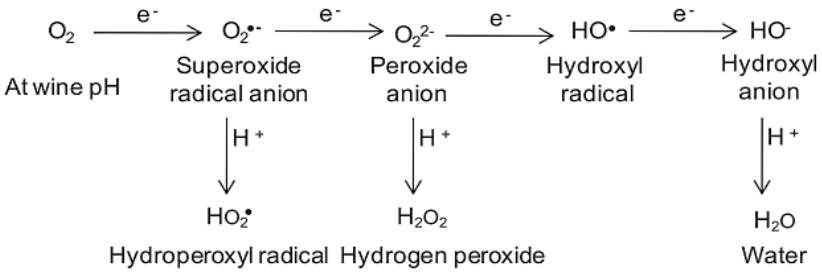

Figure 1.1.1. Reactive oxygen species (ROS) in wine (Waterhouse & Laurie, 2006)

In wine, production of ROS is catalysed by the transfer of a single electron to triplet oxygen (O_2) mediated by the presence of reduced transition metals [e.g Fe (II) and Cu (I)], leading to the formation of superoxide radical anion ($O_2\cdot^-$) which at wine pH exists in the protonated form of hydroperoxyl radical (**Figure 1.1.1.**). A second electron transferring will produce peroxide anion (O_2^{2-}) leading to the formation of hydrogen peroxide after protonation at wine pH. The next reduction step produces hydroxyl radical, one of the most oxidative species able to abstract a hydrogen atom from organic compounds (like polyphenols) producing water in the final step of the oxidation (Waterhouse & Laurie, 2006).

1.1.2. Dissolved oxygen in wine

The role of oxygen during winemaking is crucial, as it can influence the final quality and composition of must and wine.

Oxygen solubility in wines is influenced by the concentration of ethanol and solid particles but depends mainly on the temperature and composition of the gas to which wine is exposed. At low temperatures, about 5ºC, solubility of oxygen increases 10% with respect to room temperature. Therefore, it requires a lot of attention from the winemakers when carrying out low temperature practices (such as pressing cold grapes or cold stabilization) to avoid oxygen uptake (Waterhouse & Laurie, 2006).

During the different steps in the course of winemaking, exposure to oxygen cannot be underestimated. Crushing, pressing, cold stabilization, filtering, centrifugation or pumping the wine lead to dissolution into the liquid matrix. So, a correct control and management of oxygen is crucial to guarantee the quality and stability of the product. (Du Toit, Marais, Pretorius, & Du Toit, 2006)

1.1.3. Polyphenols in wine

Polyphenols, one of the main constituents of white and red wines, are the primary substrates for oxidation. In the case of red wines, a controlled exposure to oxygen can be beneficial from a sensory point of view, since it leads to the stabilization of colour and the reduction of astringency. However, in what relates to white wine, the excessive presence of oxygen generally damages the final quality of the wine (Oliveira et al., 2011).

Polyphenols in wine are divided into two different groups: Flavonoids and non-flavonoid compounds.

- Flavonoids (**Figure 1.1.2.a**): Constituted by a common core, the flavane nucleous, consisting of two benzene rings (A and B) linked by an oxygen-containing pyran ring (C) (C6C3C6). The most common wine flavonoid compounds are:
    - Flavonols: e.g. Kaempferol, quercetin, myricetin, and others
    - Flavan-3-ols: Catechin, epicatechin and tannins
    - Anthocyanins: (cyanidin-3-glucoside, peonidin-3-glucoside, delphinidin-3-glucoside, petunidin-3-glucoside and malvidin-3-glucoside)

- Non-flavonoid (**Figure 1.1.2.b**):
    - Derivates of benzoic and cinnamic acid
    - Stilbenes (e.g. Resveratrol)
    - Volatile phenols (e.g. Guaiacol)

1.1.3.1. Flavonoids

Flavonoids (**Figure 1.1.2a**) constitute the majority of phenols present in red wines and derive from the extraction of skin and seeds during fermentation, since alcohol is a good solvent for the extraction of polyphenols.

Flavanols are the most abundant family of flavonoids of grapes and wine, being found in grapes in both seeds and skins. These compounds are commonly called flavan-3-ols to specify the position of the alcohol group of the C ring. Depending on the position 2 and 3 of the C ring, different stereoisomers can exist, being found two of them in grapes: the *trans* form ((2R,3S) (+)-catechin) and the *cis* form ((2R,3R) (-)-epicatechin. Seed tannins are oligomers and polymers composed of the monomeric flavan-3-ols (+)-catechin, (-)-epicatechin, and (-)-epicatechin gallate. Skin tannins are also constituted by (-)-epigallocatechin and trace amounts of (+)-gallocatechin and (-)-epigallocatechin gallate. However, concentration of monomeric, oligomeric, and polymeric flavan3-ols are higher in the seed than in the skins. Monomeric catechins are bitter, while astringency is the main sensorial character of polymers. (Waterhouse, 2002)

On the other hand, there are flavonols that are found in a wide range of vegetables. These compounds exist in the grape mainly in four different forms: quercetin, myricetin, isorhamnetin and kaempferol, often conjugated with glycosydes or glucuronic acid. Some researchers have demonstrated that the presence of these compounds is correlated to the grape sun exposure, being produced by the plant as a natural sunscreen. (Price, Breen, Valladao, & Watson, 1995)

The last family of flavonoids are anthocyanins, responsible of the colour of red wines and found in the skins of red and black grapes. These compounds can react with tannins, leading to the formation of pigmented tannins which are more stable than the initial form. This reaction stabilizes the colour, increasing its persistence in red wines over the aging. (Waterhouse, 2002)

1.1.3.2. Non-flavonoids

Regarding non-flavonoids (**Figure 1.1.2.b**), hydroxycinnamates are the phenols present in a greater proportion in grapes juice and represent the major class of phenolic in white wines. These compounds are the most susceptible to oxidation, initiating browning phenomena, that will be discussed in the next section. The main hydroxycinnamates found in grapes and wine are based on caffeic acid, coumaric acid and ferulic acid. However, in grape berries, these compounds scarcely found, existing mainly as esters of tartaric acid, forming caftaric acid, p-coutaric acid and fertaric acid, respectively. The levels of hydroxycinnamates vary in grapes, being caftaric acid the most predominant in grapes, at about 170 mg/Kg in *Vitis vinifera* grapes, while *p*-coutaric and fertaric occurs at about 20 and 5 mg/Kg respectively. These naturally occurring esters can be hydrolysed at wine pH, releasing the free hydroxycinnamics acids. Hydrolysis can be carried out by an

enzyme, hydroxycinnamate ester hydrolase. With respect to sensory attributes, at levels found in wines, hydroxycinnamates seems to don't have any impact on bitterness or astringency. (Ong & Nagel, 1978)

Furthermore, derivates of benzoic acid are minor components in new wines. One of the most abundant compounds is gallic acid, which comes from the hydrolysis of gallate esters of hydrolysable and condensed tannins after few months.

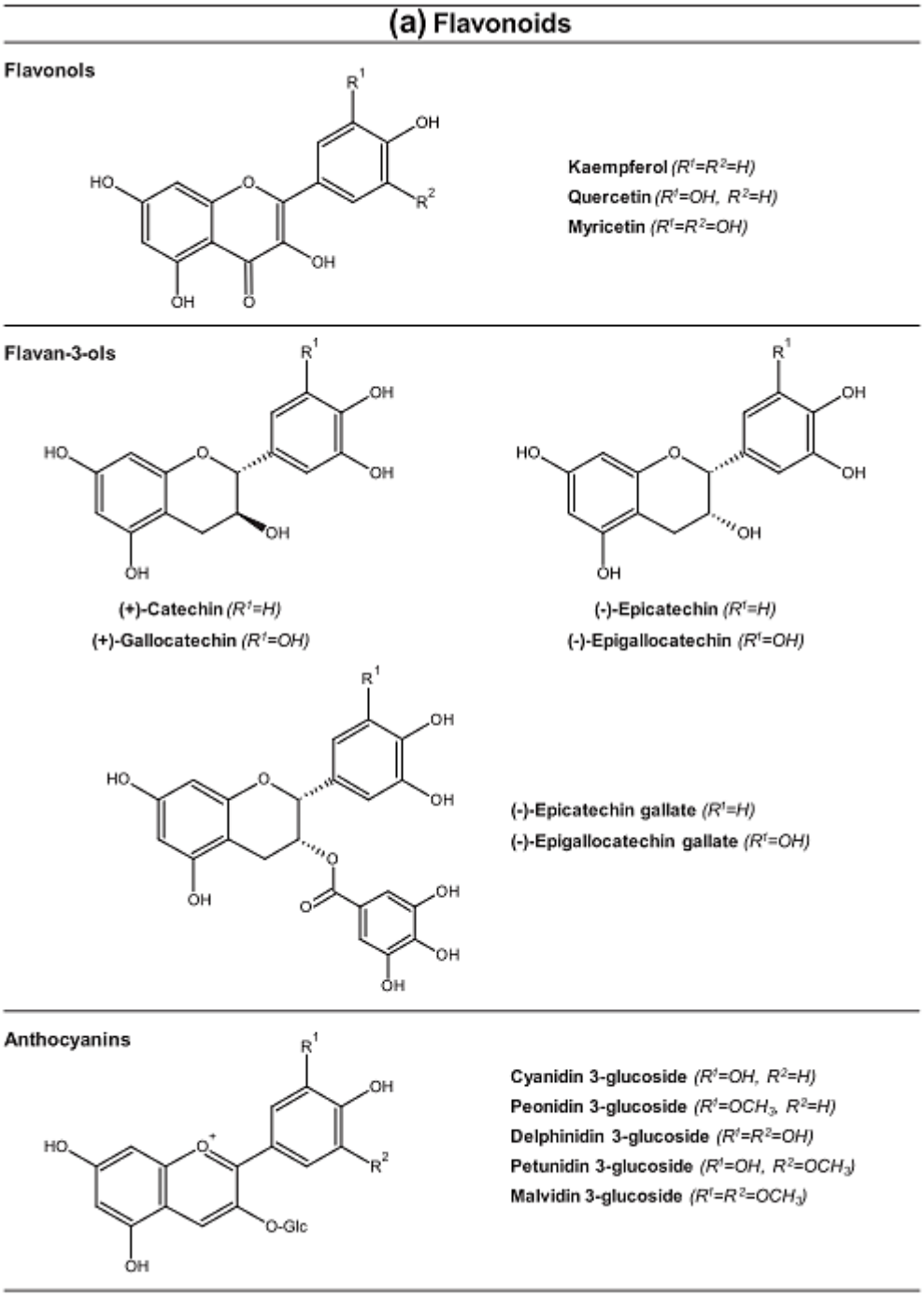

Figure 1.1.2. Flavonoids (a) and non-flavonoids (b) in wines (Oliveira et al., 2011)

Hydrolysable tannins, another family of non-flavonoids are transferred from the oak during wine aging in barrel and levels are near 100 mg/L in white wines aged for at least 6 months ad about 250 mg/L in red wines

after two or more years of aging. This family of phenols are composed of esters of gallic acid and ellagic acids with glucose or related sugars. The term “hydrolysable” is referred to the ester linkage. Depending on their origin, there are two different categories of hydrolysable tannins, gallotannins and ellagitannins, containing gallic acid and ellagic acid respectively.

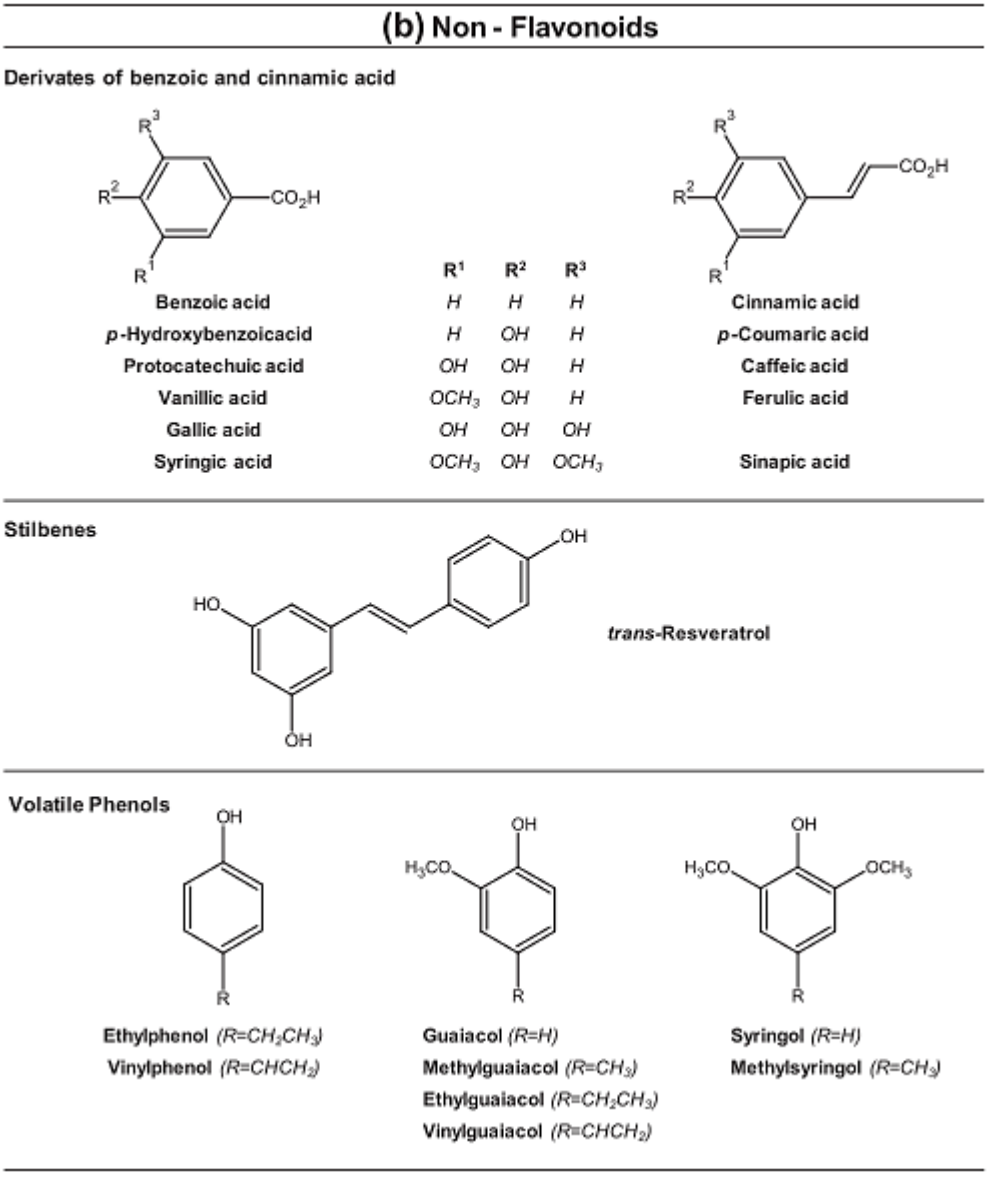

Figure 1.1.2. (continuation). Flavonoids (a) and non-flavonoids (b) in wines (Oliveira et al., 2011)

Stilbenes are another minor compound in wine. One of the main stilbenes in grapes, resveratrol, is produced by vines in response to fungal attacks such as *Botrytis* infection. Resveratrol exists in wines in several forms such as *cis* and *trans* isomers, and the glucosides of both isomers. These derivatives are found only in the skin of the grape being found in much higher concentrations in red wines. The average concentration of the sum of all the forms of resveratrol is 7 mg/L for red wines, 2 mg/l for rosés, and 0.5 mg/L for whites(Lamuela-Raventos, Romero-Perez, Waterhouse, & de la Torre-Boronat, 1995). Furthermore, resveratrol has been reported for its healthy properties such as prevention of heart diseases or cancer (Jang et al., 1997).

1.1.4. Oxidation in white wine.

In red wine, the presence of O_2 induce a decrease in phenolic compounds such as (+)-catechin, (−)-epicatechin, quercetin, caffeic acid and anthocyanins followed by an improvement of the wine colour density due to the increase in red polymeric pigments. In addition, this reactions cause a reduction on astringency, thus affecting both the wine colour and palatability. (J. Bakker, Picinelli, & Bridle, 1993). However, from a healthy point of view, the presence of oxygen could deplete phenolic compounds related with health benefits effects. (Castellari, Matricardi, Arfelli, Galassi, & Amati, 2000).

Regarding white wines, despite containing a significantly lower amounts of polyphenols, being mainly hydroxycinnamic acids, these remain very important for the overall oxidation process and the loss of varietal aromas.

From a sensory standpoint, controlled oxidation could be beneficial for red wines since it enhances colour stabilization and reduce astringency. However, the presence of oxygen is usually detrimental for white wines, because it adversely affects the sensory and nutritional properties of the product. Therefore, being more susceptible to air exposure, this project and the next paragraph will be especially focused on the oxidation of white wines.

Oxidation of white must and wines can be divided into two different mechanisms: 1) Enzymatic oxidation and 2) Non-enzymatic oxidation.

1.1.4.1. Enzymatic oxidation

Enzymatic oxidation of polyphenols in the presence of oxygen takes place only in the early stages of the winemaking process, in grape musts, as the enzymes responsible of the process are inactivated and denatured in the presence of ethanol.

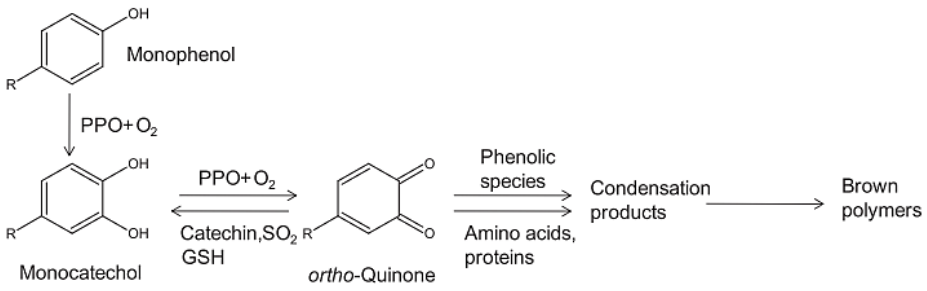

Figure 1.1.3. Enzymatic browning in must (Li, Guo, & Wang, 2008)

During the process (**Figure 1.1.3.**), polyphenols are oxidised by different enzymes via a mechanism that involves ortho hydroxylation of monophenols (cresolase activity) leading to ortho-dihydroxybenzenes (catechol) via the incorporation of an oxygen, followed by oxidation of catechol into o-benzoquinone (catecholase activity) (Oliveira et al., 2011).

These reactions can be catalysed by several classes of enzymes (Li et al., 2008):

- Oxidoreductases
    - Polyphenoloxidase (Catechol oxidase)
    - Laccase (para-diphenoloxidase)
    - Ortho-aminophenoloxidase
- Monophenol monooxygenase
- Peroxidases (POD)

The most important oxidoreductases responsible of browning in grape must are polyphenoloxidase (PPO), also called tyrosinase, and laccase. Tyrosinase is a Cu-containing enzyme, produced naturally in grapes, that catalyse the oxidation of monophenols and catechol. However, laccase, which occurs in grapes infected by the fungus *Botrytis cinerea (grey mold),* represents a greater risk for winemaking due to its resistance to SO_2 and ethanol and ability to oxidise a wide spectrum of substrates, mainly 1,2- and 1,4-dihydroxybenzenes.

Hydroxycinnamates such as caffeoyltartaric acid (caftaric acid) and *p*-coumaroyltartaric acid (coutaric acid) are the main substrates for enzymatic browning in grape must (Cheynier & Van Hulst, 1988). Kinetics of the process, promoted by flavan-3-ols, are directly correlated with their content in must. Grape crushing will promote the release of polyphenoloxidases, which will rapidly oxidise hydroxycinnamates to benzoquinones.

Benzoquinones produced by enzymatic oxidation present a high reactivity and can oxidize other types of substrates with lower redox potential, such as polyphenols or tartaric acid. As electrophiles, they can undergo further reactions with nucleophiles like amino acid derivatives (Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999).

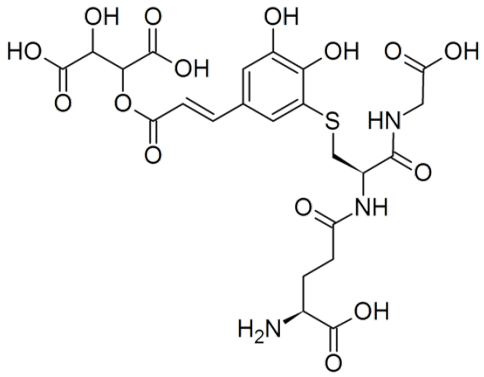

Figure 1.1.4. Structure of GRP

In must oxidation, the presence of thiols like cysteine (cys) and glutathione (GSH) could slow the initial oxygen uptake by ortho-dihydroxybenzenes. In fact, after oxidation of caftaric acid into its corresponding quinone by polyphenoloxidase, GSH is able to react with the quinone forming a colourless compound called “grape reaction product (**Figure 1.1.4.**), (GRP; 2-glutathionlyl caftaric acid) which cannot be further oxidised by PPO (Singleton & Cilliers, 1995) .

Hence, the presence of relative amounts of GSH is a key factor to prevent enzymatic oxidation of must, as GRP formation is believed to limit the browning. GRP has demonstrated to be slowly hydrolysed later into the GSH-caffeic acid (as tartrate ester is hydrolysed) derivative in aged bottled wines. Even if the specific browning products are not well characterised, appear to be the result of the reaction between the hydroxycinnamates and flavan-3-ols to form coloured compounds. However, GRP is susceptible to be oxidised to the corresponding ortho-quinone by laccase (but not tyrosinase) present in grapes affected by *Botrytis cinerea*. When no more glutathione is available, browning developing of must starts due to polymerization of the quinones.

1.1.4.2. Non-enzymatic oxidation

Non-enzymatic oxidation, (e.g. chemical oxidation) of wine, is a process favoured by the presence of polyphenols with an ortho-dihydroxybenzene (catechol ring) or a 1,2,3-trihydroxybenzene (pyrogallol ring) moiety, such as (+)-catechin/ (-)-epicatechin, gallocatechin, gallic acid and caffeic acid which are the most readily oxidised wine components. During the process, polyphenols are sequentially oxidised to semiquinones radicals and benzoquinones, while **hydrogen peroxide** is produced from the reduction of oxygen (**figure 1.1.5.**). The whole process is catalysed by the redox cycle of Fe^{3+}/Fe^{2+} and Cu^{2+}/Cu^{+} (Danilewicz, 2007).

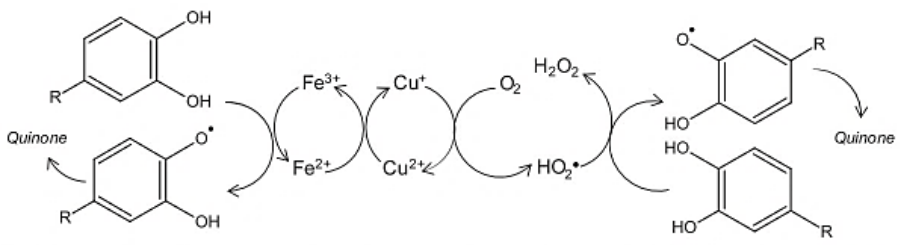

Figure 1.1.5. Metal-catalysed oxidation of catechol leading to *o*-quinones and hydrogen peroxide (H_2O_2) (Danilewicz, Seccombe, & Whelan, 2008)

Waterhouse & Laurie, (2006) demonstrated the decisive role of **transition metal ions** in the proposed mechanism of interaction between oxygen or its intermediate reducing products and wine constituents. The same authors concluded that oxygen does not react directly with phenolic compounds, but the process is catalysed by the presence of transition metals ions such as iron, copper and manganese.

The quinones formed as final product after the oxidation process of the polyphenols, are unstable and will carry out successive reactions leading to the formation of brown pigments. For instance, due to their high electrophilic character, quinones are easily able to undergo nucleophilic additions with other phenols, thiols or amines. Furthermore, the dimers and polymers produced are able to rearrange their structure in an enol-like configuration to form new diphenol moieties, which will present a lower redox potential being much easier to oxidation. Polymerization process is proposed to be accelerated by oxidation of these products (**Figure 1.1.6.**).

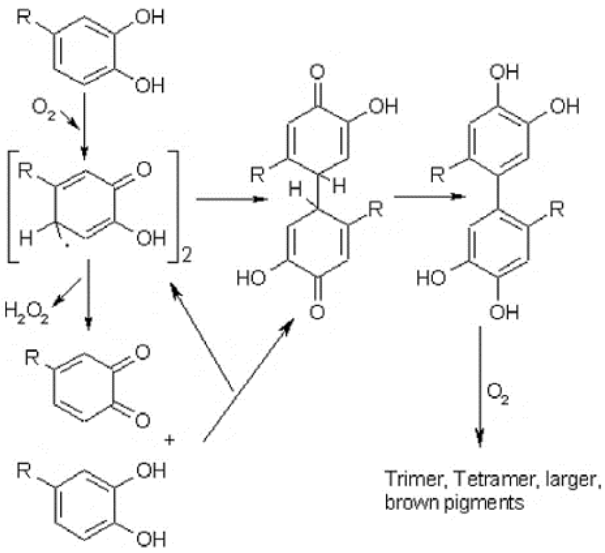

Figure 1.1.6. Reaction between two quinones or a semiquinone and phenol to form brown polymers (Li et al., 2008)

Hydrogen peroxide produced during the process can associate with reduced transition metal ions (Fe^{2+}, Cu^{+}) to undergo a reaction known as Fenton reaction (**figure 1.1.7.**), generating hydroxyl radical (HO·) (Waterhouse & Laurie, 2006). Hydroxyl radical is a reduced product of oxygen known to be capable of oxidise most organic molecules present in wine (Li et al., 2008). Due to its poor selectivity, hydroxyl radical will interact with the first species it encounters depending on their concentration, such as tartaric acid, ethanol, glycerol, sugars and organic acids. Fenton oxidation of ethanol and tartaric acid, the prime substrates to HO· radical due to their abundance in wine, produces acetaldehyde and glyoxylic acid respectively.

$$Fe^{2+} + H_2O_2 \longrightarrow HO^{\bullet} + HO^{-}$$

Figure 1.1.7. Fenton reaction (Waterhouse & Laurie, 2006)

Glyoxylic acid demonstrated to react with (+)-catechin to form yellow pigments identified as xanthylium cations (Clark, 2008; Es-Safi, Le Guernevé, Cheynier, & Moutounet, 2000) .

Additionally, under Fenton conditions, butane-2,3-diol is oxidised to butan-2-one, 3-hydroxybutan-2-one, and butane-2,3-dione. α-hydroxyacids, like L(-)-lactic and L(-)-malic acids are also subjected to oxidation, leading pyruvic and 2-oxobutanedioic acid respectively.

Chemical structure of polyphenols will determine the reaction rate with ROS. As outlined above, polyphenols containing a 1,2-dihydroxybenzene or 1,2,3-trihydroxybenzene moieties are easily oxidised due to the stabilization of the intermediate semiquinone by a second oxygen atom. The majority of polyphenol are susceptible to be oxidised by hydroxyl radical. However, monophenols, and their equivalent meta-dihydroxybenzene rings and substitute phenols are not as readily oxidised due to the instability of the semiquinone radical. Similarly, the main anthocyanin present in red wine, malvidin-3-glucoside is not readily oxidised.

1.1.5. Further mechanisms of browning in white wines.

During wine oxidation, aldehydes, and mostly acetaldehyde, derived from the yeast metabolism during fermentation or after Fenton oxidation of ethanol, are important intermediates in the process of colour and flavour development in wines. Acetaldehyde is characterized by imparting an offensive odour and taste, increasing bitterness and oxidative flavour (Ferreira, Escudero, Fernandez, & Cacho, 1997). Levels of acetaldehyde higher than 50 mg/L are thought to indicate oxidation of wine. It mediates polymerization process reactions between flavanols and anthocyanins in red wines (catechin and condensed tannins) and is also able to cross-link flavanols in white wines, leading to methylmethine linked flavanol adducts.

Browning process starts with the oxidation of ethanol into acetaldehyde by OH· radical (**figure 1.1.8.**). Under wine acidic conditions, protonation of acetaldehyde produce a carbocation, followed by the nucleophilic addition to the position C-8 and less likely C-6 of the A-ring of flavanol, leading to a flavanol intermediate. After losing a water molecule, a secondary carbocation is produced, allowing the nucleophilic addition of another flavanol or anthocyanin, yielding a methylmethine-linked flavanol adduct or anthocyanin-flavanol

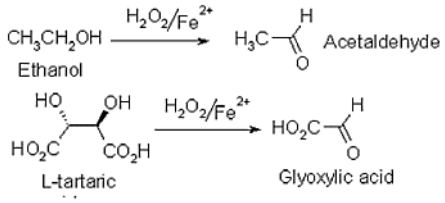

Figure 1.1.8. Fenton oxidation of ethanol and tartaric acid into acetaldehyde and glyoxylic acid (Danilewicz, 2003)

adduct, respectively (Fulcrand, Dueñas, Salas, & Cheynier, 2006) . This reaction starts again from the new formed dimers, leading to polymers in the end. However, methylmethine-linked flavanols generated from acetaldehyde are not stable and cleave into vinylflavanol units. Vinylflavanols can also react with malvidin3-*O*-glucoside and carboxypyrano-malvidin-3-*O*-glucoside (vitisin A) leading to the orange coloured pyranoanthocyanin-flavanols, and also the blue colour pyranoanthocyanin-catechins (Drinkine, Lopes, Kennedy, Teissedre, & Saucier, 2007; Marquez, Serratosa, & Merida, 2013). Self-condensation of anthocyanins may also be mediated by acetaldehyde, leading to methylmethine-linked anthocyanins (Johanna Bakker & Timberlake, 1997). Direct condensation between anthocyanins and tannins or catechin leading anthocyanin-tannin or tannin-anthocyanin adduct could also be carried out by acetaldehyde at very low rates. Final products, yellow xanthylium salts, are the responsible of the orange tonality of red wines (**Figure 1.1.9.**).

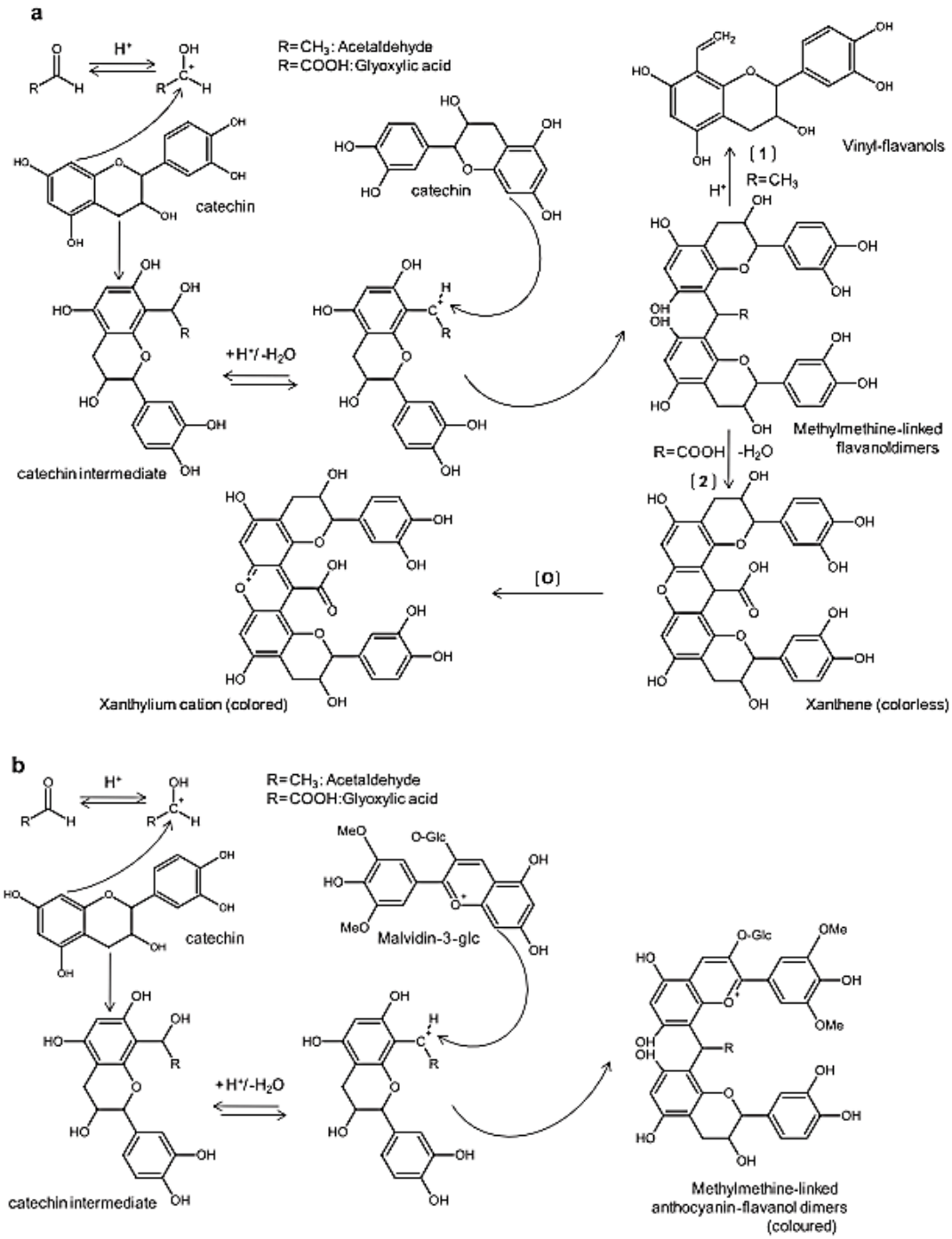

Figure 1.1.9. Mechanisms of glyoxylic acid and acetaldehyde mediated polymerization of flavanols (a) and polymerization of flavanols and anthocyanins (b)

A further browning pathway is the one mediated by glyoxylic acid, the product of Fenton oxidation of tartaric acid (**Figure 1.1.9.**). Once glyoxylic acid is formed, condensation between two flavanol units will be carried out, leading to a colourless carboxymethine-flavanol dimer in a similar mechanism of that of acetaldehyde mediated outlined above. Dehydration of dimers yields the formation of the coloured xanthene, which after an oxidation process, leads to xanthyllium cations pigments, compounds responsible of browning.

1.1.6. Effects of oxidation on wine flavour

Off-flavours production is one of the consequences of wine oxidation. At low concentrations, these flavours can impart some complexity to the wine, however increase of these compounds is detrimental for wine quality. Aromatic degradation of wine occurs rapidly, before colour development. The main sensorial characters are “honey-like”, “farm-feed”, “hay”, and “woody-like”. The most important compounds related to oxidative spoilage aroma of wine are phenylacetaldehyde, with “honey-like” aroma, 3-(methylthio)-propionaldehyde (methional), related to “boiled-potato” odour notes, 3-hydroxy-4,5-dimethyl-2-(5H)-furanone (sotolon) with “nutty” and “spicy” odour notes, and 1,1,6-trimethyl-1,2-dihydroxynaphtalene (TDN), which produces kerosene odour in aged Riesling.

On the other hand, apart from the generation of off-flavors, oxidation causes the loss of aromatic compounds related to positive sensory attributes in wines, affecting therefore, the final quality of the product. One of the most susceptible compounds to oxidation are varietal thiols, produced during alcoholic fermentation from enzymatic cleavage of glutathione and cysteine conjugates by means of yeast activity (Tominaga, Baltenweck-Guyot, Des Gachons, & Dubourdieu, 2000). These compounds are critically important to the sensorial profile, since they contribute to pleasant aroma (e.g., grapefruit, passion fruit) (Roland, Schneider, Razungles, & Cavelier, 2011). However, volatile thiols are labile to oxidation and can rapidly disappear in wines, especially in those bottled in the presence of oxygen or during storage in unsuitable conditions(Nikolantonaki & Waterhouse, 2012). In addition to browning, the main oxidation reactions that take place in must during heating or storage are Maillard reaction and caramelization. Maillard reaction, which occurs during food processing and cooking but also during storage, involves condensation between sugars and amino acids and proteins. It is favoured at 50ºC and pH 4-7. Even if there is little evidence for its occurring in wines, some researchers reported that volatile compounds responsible of typical ageing character seem to be correlated to Maillard-like condensation of sugars and amino acids (Marchand, de Revel, Vercauteren, & Bertrand, 2002).

Another type of reaction, known as Strecker degradation, results from Maillard reaction and includes the condensation of sugar-derived α-dicarbonyl compounds with free amino acids. Decarboxylation and deamination of amino acids in the presence of α-dicarbonyl compound leads to the formation of an aldehyde, with one carbon atom less than the amino acid, known as “Strecker aldehyde”, which also includes the already mentioned phenylacetaldehyde and methional.

Therefore, a thorough control of the oxidation is necessary to guarantee the stability and quality of the final product. In the following chapters the different methods of control of the oxidative processes of wine will be illustrated.

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1.2. Role of Sulfur dioxide in oenology

1.2. Role of Sulfur dioxide in oenology

Sulfur dioxide (SO_2) is the most widely used additives in low pH foods such as fruit juices or fermented beverages. In oenology, SO_2 can be added during all the stages of winemaking process (from harvested grapes, to the bottling,) (Oliveira, Ferreira, De Freitas, & Silva, 2011). Traditionally, SO_2 is used to control the development of unwanted microorganisms and to reduce the activity of enzymatic oxidation. In this way, it carries out a double antioxidant and antimicrobial function, protecting against browning and controlling harmful and undesirable fermentations (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006).

1.2.1. Forms of SO_2

Once added to the wine in the form of potassium metabisulfite ($K_2S_2O_5$) or as gaseous SO_2, this additive takes part of a complex chemical equilibrium, existing in different forms with a specific activity depending on the media conditions. Sulfur dioxide can exist bound to several unsaturated compounds such as aldehydes, phenolic compounds, organic acids, anthocyanins or glucose, constituting the "bound SO_2", or in its "free SO_2" form, referred to SO_2, (molecular SO_2) HSO_3^- (bisulphite ion) and SO_3^{2-}(sulphite ion), which is the active form (**Figure 1.2.1**).

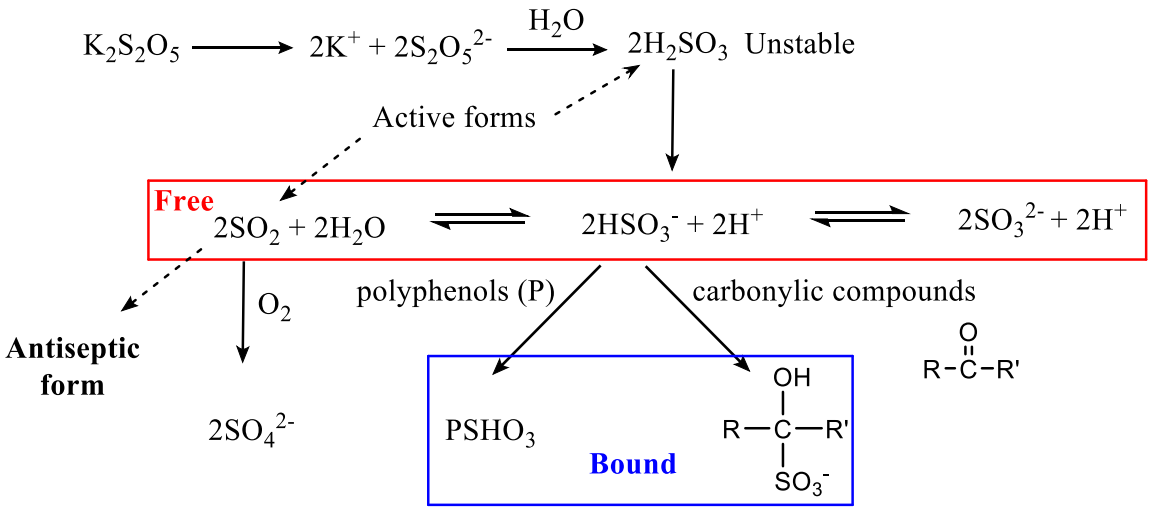

Figure 1.2.1. Chemical equilibrium of SO_2 species after addition of $K_2S_2O_4$

Concentrations of "free" SO_2 species are directly correlated to the pH of the wine. At pH 3-4, the predominant species is the bisulphite form in 90-94% while only a small fraction is in the SO_2 form (**Figure 1.2.2**). **Table 1.2.1.** contains a summary of the properties of sulfur dioxide. As outlined, properties of sulfur dioxide depend on the active form present in the medium, being SO_2 the most effective form, with powerful antioxidant and antimicrobial properties.

Figure 1.2.2 Forms of sulfur dioxide as a function of pH (pink zone represents wine pH region)

Therefore, as molecular SO_2 increases at lower wine pH (**Figure 1.2.2**), a common practice carried out in winemaking is the acidification of must and wine before addition of metabisulfite, in order to maximise the concentration of this chemical species. On the other hand, regarding the second active form of sulfur dioxide, HSO_3^- antimicrobial activity decreases, while antioxidant behaviour is still present. Bound species of SO_2, as discussed above, do not exert any antioxidant or antimicrobial properties. Furthermore, doses added of metabisulfite must be controlled, since different species of sulfur dioxide could impart some unpleasant attributes to the final product.

Property	SO_2	HSO_3^-	$R\text{-}SO_3^-$
Yeast inhibition	+	weak	0
Antibacterial	+	weak	weak
Antioxidant	+	+	0
Antioxidasic	+	+	0
Improvement of sensorial properties:			
• Redox potential	+	+	0
• Neutralizing against ethanal	+	+	+
Organoleptic effect typical of SO_2	Pungent odour, taste of SO_2	Odourless, salty, bitter	Odourless, no taste at normal doses

Table 1.2.1. Properties of the different forms of sulfur dioxide (SO_2, HSO_3^- and $R\text{-}SO_3^-$)

1.2.2. Solubilizing power

SO_2 rapidly produces the death of the grape skin cells, promoting the release of the soluble components contained therein. Due to its acid nature, pH is decreased by the presence of SO_2, increasing its solvent power. In addition, SO_2 forms colourless addition compounds with anthocyanins that are not very stable, being decomposed by heat or simple aeration. At this point, colour returns to its initial tonality in a reversible reaction. (Ribereau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2007)

1.2.3. Antimicrobial action

The control of microbial spoilage in must and wine is one of the most important challenge to be faced by winemakers to avoid economical and quality losses. In order to control the development of these undesirable microorganisms and to limit their activity in wines, the addition of SO_2 is the typical practice to be carried out.

Inhibition of the development of yeast, lactic acid bacteria (LAB) and acetic acid bacteria is the main antimicrobial mechanism of SO_2 (Santos, Nunes, Saraiva, & Coimbra, 2012).

SO_2 is the most widely used additive to control the development of malolactic fermentation (MLF) because of its antimicrobial selectivity, mainly against LAB (Ough & Crowell, 1987). In winemaking processes three main genera of LAB are present, *Oenococcus spp., Pediioccocus spp.* and *Lactobacillus spp*. *Oenococcus oeni* is the main species to develop MLF, because of its ability of growing in difficult winemaking conditions (high ethanol concentration and low pH). The other LAB species such as *Pedioccocus pentosaceous* and *Lactobacillus hilgardii* are related to wine quality changes, including production of biogenic amines (Landete, Ferrer, & Pardo, 2007), generation of off-flavour compounds (Costello & Henschke, 2002) or the so-called "lactic disease". SO_2 is able to avoid the alteration named "piqûre lactique" generated mainly by *Lactobacillus hilgardii*, where residual sugar of the wine is consumed by LAB or acetic acid bacteria during slow alcoholic fermentation, producing an increase of the volatile acidity (acetic acid and lactic acid) resulting in a negative influence of the sensorial profile of the wine (Puértolas, López, Condón, Raso, & Álvarez, 2009).

Regarding yeasts, SO_2 is able to control these microorganisms, acting against undesirable population, mainly the species *Brettanomyces*, present in barrels with poor cleaning, responsible for the unpleasant odour in wines, described as "horse sweat" and "leather", related to the formation of ethylphenols (4-ethylphenol, 4-ethylguaiacol, and 4-vinylphenol) from hydroxycinnamic acids (Suárez, Suárez-Lepe, Morata, & Calderón, 2007).

1.2.4. Antioxidant properties

As outlined in the chapter **1.1**., oxidation phenomena in winemaking can be divided into two mechanisms:

1) Enzymatic oxidation, much faster, which takes places mainly in musts and is carried out by enzymes *Polyphenol oxidase, laccase* and *peroxidase*. SO_2 prevents browning phenomena of must by inactivating enzymes and by inhibiting Maillard reactions (Garde-Cerdán, Marsellés-Fontanet, Arias-Gil, Ancín-Azpilicueta, & Martín-Belloso, 2008)

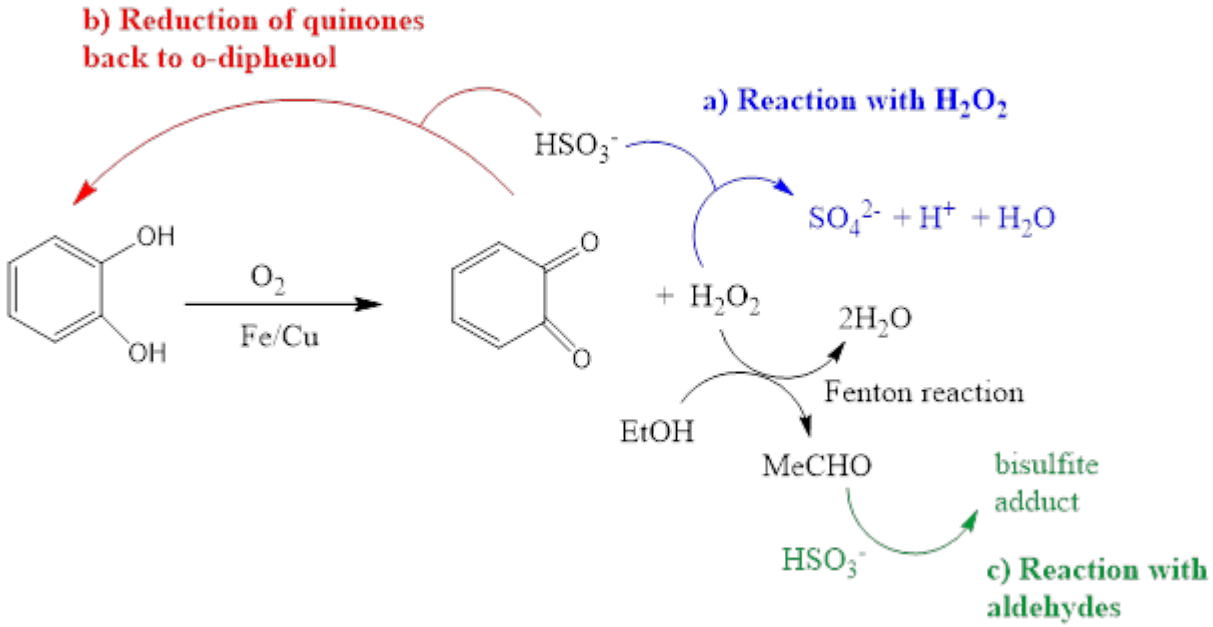

Figure 1.2.3. Antioxidant mechanisms of SO_2

2) Non-enzymatic, a metal mediated cycle of oxidation of polyphenols leading to the formation of quinones and hydrogen peroxide (H_2O_2). Antioxidant activity of SO_2 in wines is based in three different mechanisms (**Figure 1.2.3**): a) Direct reaction with the reduced form of oxygen H_2O_2, (Elias & Waterhouse, 2010) b) reaction with quinones formed during the process and reducing them back to their phenol form, c) direct reaction with aldehydes (Oliveira et al., 2011).

1.2.5. Effect of SO_2 on the quality of wine

Moderate addition of sulfites has demonstrated to enhance and improve the aromatic complexity of grape musts, especially in rotten grapes or those with a scarce primary character. Aroma of young wines may also be protected by the addition of sulfites (Ribereau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2007). Colour stability has been shown to be improved in wines treated with SO_2 (Guerrero & Cantos-Villar, 2014) since sulphating favours the dissolution and extraction of minerals, organic acids and especially phenolic compounds (anthocyanins and tannins) the main components responsible of the colour of red wines. In addition, during wine aging, SO_2 minimizes polymerization rate and consequently the loss of colour. SO_2

present in wines may react with several compounds bearing a carbonyl moiety such as acetaldehyde, pyruvic acid and 2-oxoglutaric acids thus reducing oxidation effects. To a lesser extent, reaction with anthocyanins, hydroxycinnamic acids and reducing sugars can contribute for the modulation of wine properties. The presence of SO_2 during fermentation may promote the consumption of total amino acids by fermenting yeast and hence increase the complexity of flavour and microbiological stability (Cejudo-Bastante et al., 2010; Garde-Cerdán, Marsellés-Fontanet, Arias-Gil, Martín-Belloso, & Ancín-Azpilicueta, 2007)

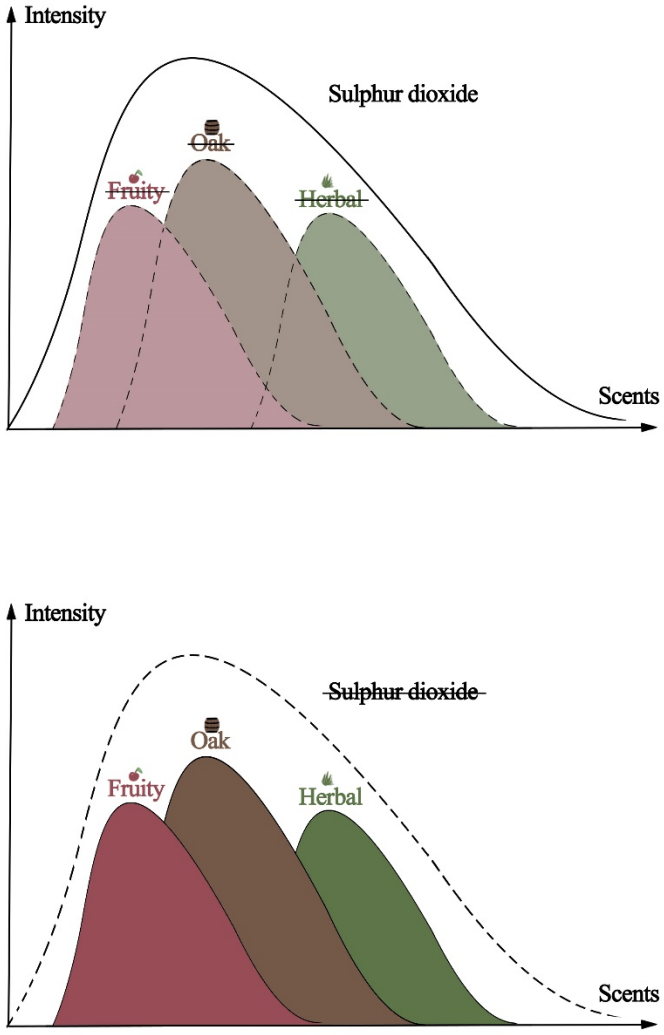

Figure 1.2.4. Effect of SO_2 on wine aroma. Adapted from Guerrero & Cantos-Villar (Guerrero & Cantos-Villar, 2014)

However, excessive amounts of SO_2 may compromise the overall quality of wine, causing unpleasant sensory characteristics. In anaerobic conditions or during long periods of storage on yeas lees, addition of SO_2 can lead to the formation of undesirable compounds such as of hydrogen sulphide or mercaptans formation which confer the "rotten egg" character. SO_2 can actually favour the formation of cloudiness during storage (Li, Guo, & Wang, 2008) and induce protein aggregation by hydrophobic interactions and intermolecular bisulfide bonds (Chagas et al., 2018). In addition, as shown in **figure 1.2.4.**, high doses of SO_2 could neutralize aromatic profile of wines and even impart some defects such as wet wool smell, that becomes suffocating and irritating, together with a burning sensation on the after taste (Guerrero & Cantos-Villar, 2014).

1.2.6. Effects of SO_2 to the human health

Despite all the advantages mentioned above, it has been shown that sulfites produced by adding SO_2 to wines can cause adverse effects to human health such as allergic reactions (Vally, Misso, & Madan, 2009) (dermatitis, urticaria, angioedema, abdominal pain, diarrheal effects, bronchoconstriction and anaphylaxis). Asthmatics who are steroid dependent or who have a higher degree of airway hyper reactivity may be at greater risk of experiencing a reaction to sulphite containing foods, causing activation of proto-oncogenes, inactivation of tumour suppressor genes, and even can play a role in the pathogenesis of SO_2-associated lung cancer (Qin & Meng, 2009). SO_2-sensitive individuals can react negatively to sulphite ingestion in quantities ranging from 10 to 50 mg. Furthermore, some research have reported that excessive doses of SO_2 are toxic for the human health and related with headaches, nausea and asthmatic reactions (Gao et al., 2002), being the amount consumed accumulative in the organisms.

1.2.7. Regulation

Sulphites are found in many food products as a food additive, enclosed with the label "E" and number 220-228 [sulphur dioxide (E 220), sodium sulphite (E 221), sodium hydrogen sulphite (E 222), sodium metabisulphite (E 223), potassium metabisulphite (E 224), calcium sulphite (E 226), calcium hydrogen sulphite (E 227) and potassium hydrogen sulphite (E 228)].

SO_2 has been approved as food preservative by the European Union, establishing a total limited concentration of SO_2 up to 200 mg/L in white and rosé wines, and 150 mg/L in red wines. (European Commission, 2009) Besides, the International Organization of the Vine and Wine (OIV) established the following concentrations depending on the type of wine: 150 mg/L for red wines containing a maximum of 5 g/L of reducing sugars, 200 mg/L for white and rosé wines containing a maximum of 5 g/L of reducing sugars, 300 mg/L for red, rosé and white wines containing more than 5 g/L of reducing substances and 400 mg/L in some exceptions with some sweet white wines(European Commission, 2009). In addition, the acceptable daily intake (ADI) of SO_2 established by the Food and Agriculture Organization of the United Nations (FAO) is 0.7 mg SO_2 equivalent/Kg.

Hence, in order to produce safer wines for the human health, many efforts are being made by winemakers to find alternative to reduced or completely avoid the presence of sulphites in wines.

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1.3. Replacements of sulphites in wine

1.3. Replacement of sulfites in wine

As already mentioned in the previous chapter, there is a growing concern by consumers and producers for the use of sulfites in wine. From an oenological point of view, an excessive dose of sulfur dioxide does not only present a risk to human health, but also cause sensory alterations in the final product, such as neutralization of certain aromas and generation of characteristic defects (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006). However, insufficient concentrations increase the risk of excessive oxidation or microbiological development of undesirable yeasts and bacteria, also compromising the quality of the final product. For this reason, it is therefore necessary to search for alternatives to SO_2 in order to ensure a more natural final product, with no related health problems, that meets the demands of consumers and winemakers.

This chapter will list the potentiality and limitations of the different proposed alternatives to the use of sulfites in oenology, some of them not admitted in oenology yet. **Table 1.3.1.** summarized all the technological approaches discussed on this section.

1.3.1. Physical methods:

1.3.1.1. Pulsed electric fields

Pulsed electric fields (PEF) is a fast, non-thermal and effective technique employed for the inactivation of pathogenic microorganisms in food matrix, without imparting alterations to the quality (Barbosa-Canovas, Fernandez-Molina, & Swanson, 2001) . In this technique, products are placed between two electrodes and short pulses of high electric field strengths (up to 70 KV/cm) are applied. Electric high-voltage pulses generate a transmembrane potential that induces breakdown of membranes and an increase in permeability (Puértolas, López, Condón, Raso, & Álvarez, 2009). High field-strength and sufficient duration of pulses provokes the inactivation of vegetative microorganisms in liquid media due to irreversible membrane destruction.

Several researches on must and juices have shown the efficiency of PEF on the inhibition of yeast and bacteria growth (Marsellés-Fontanet, Puig, Olmos, Mínguez-Sanz, & Martín-Belloso, 2009) reducing completely the spoilage flora of *Brettanomyces* and *Lactobacillus* and ,therefore, protecting wine against the risk of microbial alteration. It has also been reported the effect of PEF treatments on the secondary structure of some enzyme such as PPO or POD, leading to a decrease on their activity. Furthermore, according to Garde-Cerdán and co-workers (Garde-Cerdán, Marsellés-Fontanet, Arias-Gil, Ancín-Azpilicueta, & Martín-Belloso, 2008) wines obtained from musts treated with PEF did not experience any change on the volatile profile. Besides, the absence of SO_2 had no effect on the sensory characteristics of the final product.

Based on the results obtained, PEF technology constitutes a good alternative to the use of sulphites in oenology. However, due to the limited antioxidant capacity and the low complexity in flavour of wines

obtained without addition SO_2, PEFs should be used as a complementary technique and not as a substitute of SO_2 (Piyasena, Mohareb, & McKellar, 2003)

1.3.1.2. Ultrasounds:

Ultrasounds have emerged in the last decade as an alternative to the use of thermal treatments for pasteurization and sterilization of food products (O'Donnell, Tiwari, Bourke, & Cullen, 2010). Principle of inactivation of pathogens, spoilage microorganisms or enzymes by sonication is based on physical (cavitation and other mechanic effects) and/or sonochemical (formation of free radicals) reactions (Piyasena et al., 2003). Propagation of high-power ultrasounds in a liquid provokes pressure changes which generates cavitation bubbles. During successive compression cycles occurring because of a propagated ultrasonic wave, micro-bubbles strongly collapse, resulting in regions with high localized temperature (exceeding 5.500 ºC and pressures of up to 50 MPa) and high shearing effects. Therefore, localized pasteurization is carried out due to the intense local energy and high pressure, without any increase in macrotemperature.

Ultrasounds have been reported to be able to reach the mandatory 5 log reduction of food borne pathogens in fruit juices. However, effectiveness of the treatment is dependent on the bacteria and yeast species to be treated and on the frequency of the ultrasonic wave (Tsukamoto et al., 2004). Unfortunately, spores present high resistance to ultrasounds requiring high periods of treatment and/or the combination of these with other types of treatments or additives to obtain a product free from spores (Piyasena et al., 2003). Apart from its antimicrobial capacity, ultrasounds represent a promising alternative to the use of sulfites as a preservative agent in winemaking. Based on the results obtained by O'Donnell et al. (2010), ultrasounds have the ability to inhibit enzymes such as PPO with minimal effect on quality parameters such as colour or anthocyanin content. Furthermore, ultrasounds have been proposed to be used in must and wines to reduce undesirable microbial population, prior to the inoculation with yeast and/or in wines before the initiation of MLF, as they have the ability to stop or delay MLF or on the contrary to promote MLF by accelerating yeast autolysis (Jiranek, Grbin, Yap, Barnes, & Bates, 2007) . In addition, ultrasounds have the capacity to increase phenolic and flavanol content in wine (Masuzawa, Ohdaira, & Ide, 2000). Nevertheless, despite of the advantages listed above, a practical evaluation is required to proposed ultrasounds as an alternative to completely avoid the used of SO_2 in oenology.

1.3.1.3. Ultraviolet radiation

Ultraviolet (UV) is based on the irradiation with electromagnetic radiation of 100-400 nm, divided into UV-A (320-400nm), UV-B (280-320nm), UV-C (200-280 nm) (Keyser, Műller, Cilliers, Nel, & Gouws, 2008). It is a very effective non-thermal technique for microbial decontamination of surfaces and also used in food processing to inactivate microorganisms (bacteria and yeast) by DNA damage, which disables some cell

functions such as reproductible capability, and to inhibit enzymatic (especially PPO) activity by protein aggregations, without changing the quality parameters (Fredericks, du Toit, & Krügel, 2011).

UV irradiation has certain advantages over other techniques, since it does not produce any type of toxic or by-product during the treatment, it can be used to destroy organic contaminants and requires much less energy than other conventional pasteurization methods (Keyser et al., 2008). Treatments with UV have been shown to be effective against a broad spectrum of wine microorganisms such as *Brettanomyces, Saccharomyces, Lactobacillus, Pediococcus, Oenococcus* and *Acetobacter* (Fredericks, et al., 2011).

However, efficacy of UV irradiation depends on the characteristics of the product such as suspended material, soluble solids, colour, absorbance and density since they can interfere the penetrability of UV radiation, avoiding reaching the microorganisms in the liquid media (Fredericks et al., 2011). Therefore, its low efficacy in red wines due to the high presence of polyphenols and its lack of antioxidant properties, make UV radiation a complement and not a substitute technique of SO_2 in oenology. Further, the effects of this treatment on the activation of Fenton-like cascades caused by the energy transmitted through the UV waves, is still to be investigated.

1.3.1.4. High pressure

High pressure (HP) is another non-thermal, and energetically efficient process in which products are subjected to pressures between 100 and 1000 MPa using water as a compression media (Cao et al., 2011). It is an instant and uniform technique independent of the product size and geometry. HP technology is used to guarantee the microbiological safety of foods. In the HP equipment, the product is placed in high pressure chamber where the vessel is closed and pressurized by pumping pressure transmission medium inside. By applying HP, microbial inactivation is induced due to interferences in the function of cellular components such as membranes, ribosomes and enzymes, leading to cell leakage.

One of the greatest advantages of HP is that it only interferes by destroying the non-covalent bonds without affecting the covalent, so the colour, flavour, taste, or freshness of the final product is not influenced (Daoudi et al., 2002). HP has been shown to inactivate enzymes such as PPO, POD and β-glucosidase (Cao et al., 2011) and to decrease microbial wine population of *Saccharomyces cerevisiae, Brettanomyces bruxellensis*, and *Oenococcus oeni* without any change in the chemical and sensorial profile of wines (Puig, Vilavella, Daoudi, Guamis, & Mínguez, 2003). Furthermore, (Puig et al., 2003) reported that aerobic bacteria were more susceptible to HP treatments than yeast and LAB.

Nevertheless, the main impediment to HP treatment is the impossibility of working in a continuous process, rendering it useful only in the last stages of winemaking, before bottling. In addition, the potentiality of high pressure as an alternative to substitute the use of sulfur dioxide in oenology is still unknown, since there is a lack of information about antioxidant properties, or volatile compounds composition.

1.3.2. Addition of compounds:

Apart from physical treatments, another strategy to reduce the use of sulfur dioxide in oenology is the addition of certain compounds. In this section we will study the main characteristics of the following additives: DMDC, bacteriocins, lysozymes, phenolic compounds, ascorbic acid and glutathione.

1.3.2.1. Dimethyl dicarbonate (DMDC)

DMDC (**figure 1.3.1.**) is a chemical inhibitor of microorganisms recently approved in the European Union for its use in oenology at maximum doses of 200 mg/L at bottling for wines with more than 5g/L of residual sugar (Bartowsky, 2009) . Mechanism of action of DMDC is based on the arrest of cellular growth, by inhibiting some enzymes such as alcohol-dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase and by methoxycarbonylation of the nucleophilic residues (imidazoles, amines, thiols).

When added in wine, DMDC is rapidly converted into methanol (in too low concentration to produce toxicologically significant levels) and very low amounts of methyl carbonate and alkyl carbonates produced after reaction of DMDC with polyphenols.

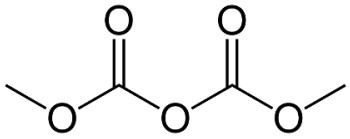

Figure 1.3.1. Structure of DMDC

DMDC has been demonstrated by several studies to be more effective against yeast than against bacteria. Furthermore, DMDC has shown higher inhibitory effect than SO_2 because it kills the cell yeast whereas SO_2 just inhibit microbial growth (Bartowsky, 2009).

However, inability of DMDC to prevent the development of bacteria, its limited ability to protect wine from oxidation and the rapid conversion into methanol make this compound unsuitable to completely replace SO_2, in particular for all the winemaking phases before the bottling or when there is the need of continuous protection against unwanted microorganisms.

1.3.2.2. Bacteriocins

Bacteriocins such as nisin, pediocin and plantaricin are small polypeptides produced by specific LAB which have inhibitory properties to other bacterial species (E.J. Bartowsky, 2009). These compounds have been reported to be specific against gram-positive bacteria, acting against cytoplasmic membrane and rendering the cell permeable to small ionic components, leading to the lysis of the cell (Chun & Hancock, 2000).

Bacteriocins has shown to be very effective against wine LAB being considered ideal preservatives since they have no smell, no colour and are non-toxic. However, its antimicrobial action is selective only against gram-

positive bacteria, with very low effectiveness against gram-negative. In combination with some compound that inhibits yeast development, such as DMDC, bacteriocins could represent a promising alternative to protect wine against microbial spoilage. However, it is necessary to add that the use of bacteriocins in wine has not been authorized yet.

1.3.2.3. Lysozyme

Lysozyme, isolated from egg albumin, is a 129-amino acid protein that has demonstrated high effectiveness against microbial spoilage in food (Azzolini, Tosi, Veneri, & Zapparoli, 2016). It reaches its maximum stability and activity at pH 2.8-4.2, which make it suitable for its use in winemaking. Lysozyme has been found to be very effective against the development of spontaneous LAB growth, responsible of spoilage or undesirable secondary fermentations.

As reported by (Lopez et al., 2009) lysozyme showed high activity against gram-positive bacteria, low activity against gram-negative and is inactive against eukaryotic cells walls. In addition, some studies demonstrated that lysozymes have greater activity in white wine while its capacity suffer an important decrease in red wines, due to the large number of phenolic compounds in the latter that could interact with lysozymes.

Although the wines treated with lysozymes do not show any significant change in the aroma and even the volatile acidity and the content of biogenic amines are reduced(Cejudo-Bastante et al., 2010; Sonni, Moore, et al., 2011; Sonni, Chinnici, Natali, & Riponi, 2011), its action can lead to the formation of wine haze (Eveline J.. Bartowsky, Costello, Villa, & Henzchke, 2004).

However, even though the use of lysozyme has been approved by the OIV, its use generates high additional costs for winemakers. In addition, its presence in the wine could present a risk for consumers who are allergic to the hen's egg, being necessary to indicate their presence in the label.

1.3.2.4. Phenolic compounds

Phenolic compounds (**Figure 1.3.2.**) are one of the main components in wine, being responsible of colour and astringency, so they have a fundamental role in the organoleptic profile of the final product. Those polyphenols in greater proportion are flavonoids, stilbenes and tannins. Furthermore, in red wines, anthocyanins constitute another important family of polyphenols, responsible of the chromatic characteristics. In addition, polyphenols are associated with beneficial effects in the human body, particularly in relation to cardiovascular and degenerative diseases, due to its antioxidant capacity. In fact, they have the ability to scavenge and neutralize free radicals, the main species responsible of wine oxidation processes (P. Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). It has been reported that the addition of oenological tannins can affect the oxidation processes of must and wines, due to a double mechanism of enzymatic and radical-scavenging inhibition (Sonni, Cejudo Bastante, Chinnici, Natali, & Riponi, 2009). Its

antimicrobial activity is based on the ability to increase permeability of cytoplasm plasmatic membrane, leading to the leakage of bacterial cells (Campos et al., 2009). Polyphenols behaviour is directly related with their structure, lipophilic character and especially to the concentration added.

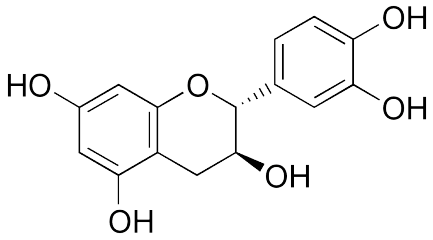

Figure 1.3.2. Example of phenolic compound (structure of (+)-catechin)

Furthermore, a recent study reported a suitable method of SO_2-free winemaking by using grapevine shoots in red wines. Results showed lower contents of free anthocyanins, accompanied by an increase of B-type vitisins after 12 months of storage in bottle, demonstrating better chromatic characteristics. However, organoleptic properties were influenced at higher doses of grapevine shoots, while at low doses, composition of wine was preserved without compromising the final quality (Raposo et al., 2018).

However, despite the promising results, authors demonstrated that the antimicrobial effect of phenolic compounds appears to take place only at higher doses than those normally found in wines. Therefore, it should be considered that the use of polyphenols for antimicrobial purposes would interfere in the quality of the final product, such as viscosity, colour or aroma profile, making polyphenol not a suitable strategy to be used alone for wine preservation instead of SO_2.

1.3.2.5. Ascorbic acid

Ascorbic acid (AA) or vitamin C is a natural compound present in most fruits and vegetables. Due to its antioxidant properties, AA has long been utilized in winemaking as a complement to the addition of sulfur dioxide in white wines (Zoecklein, Fugelsang, Gump, & Nury, 1995). Its ability to rapidly scavenge molecular oxygen being readily oxidised under white wine conditions allows AA to protect other oxidizable compounds such as polyphenols and flavour compounds (Barril, Rutledge, Scollary, & Clark, 2016). Mechanism for the antioxidant role of ascorbic acid is based on the metal-mediated reaction between ascorbic acid and oxygen resulting in dehydroascorbic acid and hydrogen peroxide (Marc P. Bradshaw, Barril, Clark, Prenzler, & Scollary, 2011) (**Figure 1.3.3.**). Furthermore, AA is also suggested to reduce the oxidized o-quinones formed, back to their initial *o*-diphenol state (Boulton, Singleton, Bisson, & Kunkee, 1996). In addition, the efficiency of oxygen consumption by AA makes its presence useful in must and juice in order to compete with oxidative enzymes (polyphenol oxidase, laccase) thereby lowering their activity.

Regarding the impact on the sensory characteristics of the wine, the presence of ascorbic acid during storage have demonstrated a little impact on the aroma on wines (Morozova, Schmidt, & Schwack, 2015). In wines

bottled under closures that allowed a high oxygen intake, the attributes related to oxidation were lower in the presence of AA. In addition, the presence of AA increased the perception of fruity aromas and freshness and reduced the intensity of oxidized aromas.

However, in the absence of sulfur dioxide, the effect known as "crossover" of the ascorbic acid from antioxidant to prooxidant occurs. As mentioned above, the rapid oxidation of ascorbic acid leads to the production of H_2O_2 and dehydroascorbic acid, which is degraded in subsequent carbonyl products. In the absence of sulfur, the yield of H_2O_2 increases and via Fenton reaction with metals, enhances the production of hydroxyl radical and, therefore, oxidation phenomena of wine. In addition, after its reaction with flavan-3-ol, carbonyl degradation products of dehydroascorbic acid contribute to the production of xanthylium cations, leading to the formation of brown pigments (Mark P. Bradshaw, Cheynier, Scollary, & Prenzler, 2003). These phenomena, added to the low effectiveness of ascorbic acid against undesirable microbiological development, show that ascorbic acid should be only used as a complement to sulfur dioxide and not as a substitute.

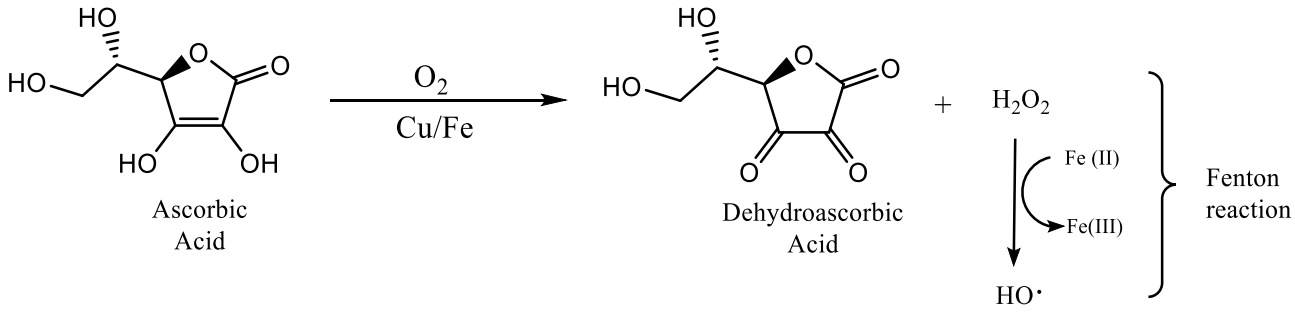

Figure 1.3.3. Reaction of ascorbic acid with oxygen in the absence of SO_2

Nevertheless, despite the promising results, none of the alternatives has proven to be able to completely replace the use of sulfites in wine. Therefore, there is still a challenge to find a health-safe alternative which fulfilled similar effect than SO_2 without affecting sensory profile of wine.

1.3.2.6. Glutathione

Glutathione (GSH) is a relevant constituent of grapes, must and wines, with the structure of a tripeptide of L-glutamate, L-cysteine, and glycine (**figure 1.3.4**). Levels of GSH during winemaking can be managed by winemakers by limiting the exposition to oxygen during the process and the storage period (Kritzinger, Bauer, & Du Toit, 2015).

As illustrated in section 1.1. (chapter 1.1.4.1., paragraph 4) hydroxycinnamates such as caftaric or coutaric acids are converted into their corresponding *o*-quinones during enzymatic oxidation by PPO. GSH, possesses a nucleophilic character due to the presence of a mercapto group, that is able to react with caftaric acid quinones leading to the formation of a thioether known as 2-S-glutathionyl caftaric acid, mostly known as grape reaction product (GRP) which is not a further substrate of oxidation by PPO. Thus, GSH can inhibit browning by trapping o-quinones in a colourless form (Singleton, Salgues, Zaya, & Trousdale, 1985).

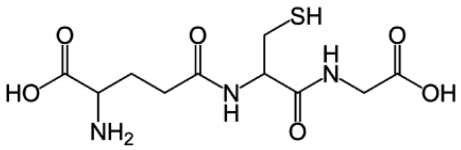

Figure 1.3.4. Structure of Glutathione

GSH has also demonstrated to inhibit oxidative browning by forming addition products with carbonyl compounds, such as glyoxylic acid, one of the main intermediates of non-enzymatic oxidation. Furthermore, some authors reported a second reaction of GSH with and intermediate compound in the formation of the dimers (Sonni, Moore, et al., 2011). These latter authors claimed, in addition, that GSH is able to modulate the polymerization reactions of acetaldehyde and glyoxylic acid, in favour of the latter, in this way influencing the final results of forced microxygentation. Thus, by hindering the carbonyl-derived polymerization reaction, GSH is able to reduce the formation of undesirable coloured compounds such as brown xanthylium cations, protecting white wines during storage period.

Furthermore, GSH has demonstrated to restrict the disappearance of some volatile esters that contribute to wine fruity aromas, such as isoamyl acetate, ethyl hexanoate, ethyl octanoate (Papadopoulou & Roussis, 2008), and terpenes such as linalool or α-terpineol in aromatic white wines (Papadopoulou & Roussis, 2001). The protection effect of GSH against the decline of some aromatic compounds has been attributed to its free sulfhydryl (SH) moiety, conferring unique redox and nucleophilic character.

However, it is postulated that addition of GSH is directly correlated with the production of H_2S, a reductive off-flavour in wines. This phenomenon occurs due to the presence of the amino acid cysteine in the structure of GSH, which can be degraded by the enzyme cysteine desulfhydrase to form H_2S (Tokuyama, Kuraishi, Aida, & Uemura, 1973).

Furthermore, data regarding the antimicrobial effect of GSH are quite scarce and further investigations are still required.

		Advantages	Disadvantages
Physical methods	**Pulsed electric fields**	- Inactivation of enzymes such as PPO and POD. - Effective against undesirable microorganisms. - Reduction of maceration time and increase in phenolic compounds extraction. - No deleterious effect on flavor, color or nutrient value of must and wine. -Low energy consumption.	- No antioxidant properties - Less complex flavour - Not admitted in oenology yet
	Ultrasounds	- Use in must: Reduction of spoilage organisms and enhance of colour and flavour in wine - During fermentation: Reduction of spoilage microorganisms prior to AF or MLF. - MLF: Can be stopped or delayed, or on the contrary, promoted by yeast autolysis. - Increase on phenolic content and acceleration of aging	- High resistance of spores to ultrasound treatments - Necessary to combine with other processing method - No antioxidant properties - Not admitted in oenology yet
	Ultraviolet	- Reduction of LAB population - No-toxic by products - Destruction of organic contaminants - Inactivation of PPO	- No antioxidant properties - Penetration ability and efficacy depend on the characteristic of the product - Less effective in red wines - Not admitted in oenology yet
	High pressure	- 400-600 MPa: Inactivation of PPO, POD, β-glucosidase - 500 MPa: Decrease *S. cerevisiae, B. bruxellensis, O oeni*. - 400 MPa: Killing of *Lactobacillus* spp. *Acetobacter,* and *Botrytis cinereal* - No influence on the original sensory profile	- No antioxidant properties - No possibility in continuous process - Activation of some enzymes depending on the treatments - Not admitted in oenology yet
Addition of compounds	**Dimethyl dicarbonate**	- Inhibition of microorganisms - More effective than SO_2 against yeast	- More effective against yeast than against bacteria - No antioxidant properties -Production of methanol and methyl carbamate - Very limited duration, only useful just before bottling
	Bacteriocins	- Inhibition of LAB - Control of MLF - No colour, no smell and non-toxic	- No influence against yeast growth - No effective against gram-negative bacteria - No antioxidant properties - Not admitted in oenology
	Lysozyme	- Control of MLF - Inhibition of bacterial growth - No important change in aroma	- Low activity against gram-negative bacteria - Less efficient in red wine - Reduction of colour density and phenolic content - No antioxidant properties - Formation of haze - Inactive against yeast
	Polyphenolic compounds	- Some Inhibition of bacterial growth - Antioxidant and antiradical properties	- Changes in physico-chemical and organoleptic properties - Not effective for a number of microorganisms
	Ascorbic acid	- Rapid removal of oxygen - enhance/preservation of aromatic properties of grapes and musts	- Increase in absorbance at 420 nm - Pro-oxidant effect at low concentrations -No antimicrobial properties
	Glutathione	- Antibrowning capacity - Protection against depletion of aromas	- Generation of H_2S - No antimicrobial properties

Table 1.3.1. Properties and limitation of different physico-chemical approaches for the reduction of SO_2 in oenology

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1.4. Chitosan in winemaking

1.4. Chitosan in winemaking

After cellulose, chitin is the second most abundant polysaccharide on earth. This biopolymer, composed of β(1→4)-linked 2-acetamido-2-deoxy-β-D-glucose (N-acetylglucosamine) unit, is synthesized by a great number of living organism in enormous amounts, almost as cellulose. It is a white, inelastic, nitrogen-rich polysaccharide normally presented in nature complexed with other polysaccharide and proteins forming the structure of the exoskeleton of arthropods, crustacean shells or cell walls of fungi and plants. (Rinaudo, 2006)

The number of acetyl groups attached to the backbone of chitin determines the acetylation degree (AD). Depending on the AD, the molecule consists of chitin (unbranched chains of N-acetyl-D-glucosamine), or chitosan (**Figure 1.4.1.**), the mostly deacetylated form of chitin, which is a linear polymer of α (1→4)-linked 2-amino-2-deoxy-β-D-glucopyranose (Dutta, Dutta, & Tripathi, 2004). Both chitin and chitosan are found in nature being chitin the mains source of chitosan, which also occurs naturally in some fungi, but its production is much less widespread than that of chitin. Chitin is transformed into chitosan in a process that involves four steps: deproteinization, demineralization, decolorization and deacetylation (Aranaz et al., 2009).

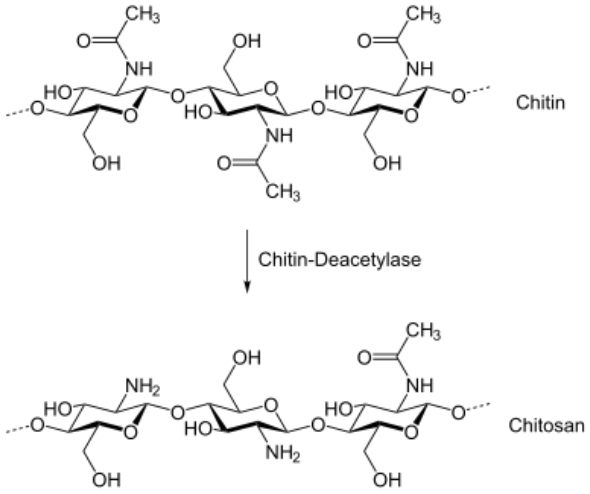

Figure 1.4.1. Structure of chitin and chitosan

With deacetylation, amine groups (-NH_2) are freed, lending to chitosan a cationic character (increasing solubility in acidic media at pH lower than its pH= 6.2), making it a particular compound which differs from the other polysaccharides usually neutral or negatively charged. These properties give to chitosan the capacity of being produced in different forms such as films, gels, beads, nano/micro particles, which together with its biodegradability, biocompatibility and low toxicity makes it a versatile compound with a huge amount of interesting applications in many fields, including food, medicine, cosmetics and pharmaceutical sciences.

Chitosan is of particular interest over synthetic polymers, because is considered as GRAS (Generally Recognised as Safe) by Food and Drug Administration. Due to the versatility of this compound, its application has been widespread in food science with different objectives such as, protection against microbial spoilage,

storage of fruits and vegetables, deacidification and clarification of juices, removal of solid material in water, and against oxidation (Shahidi, Arachchi, & Jeon, 1999).

Chitosan has been accepted in 2011 by the European Commission as an additive in winemaking for reduction of heavy metal content, possible contaminants, especially ochratoxin A and reduction in the population of undesirable micro-organism, in particular *Brettanomyces* ("Commission Regulation (EU) 53/2011 of 21 January 2011," 2011). Even if the distinct structure of chitosan, its reactivity and versatility, is raising a growing interest in oenology, some of its possible applications have not been sufficiently explored and there is a lack information on possible future developments.

This section summarizes the advancements on the use of chitosan and the potentiality of the use of this additive in winemaking technologies, focusing in the following topics: Chelation of metals, microbiological properties, ochratoxin removal, protein stabilization, antioxidant capacity and sensorial influence.

1.4.1. Chelation capacity

During winemaking and storage of wines, the presence of contaminants can cause stabilization and safety problems. High concentration of heavy metals forms insoluble precipitates, being one of the main causes of hazing in wine (Bornet & Teissedre, 2008). Furthermore, as outlined in section 1, metals are one of the main catalysts of non-enzymatic oxidation process, enhancing browning development of wines (Danilewicz, 2003). Therefore, is important to take the appropriate precaution to protect the wine from this spoilage that not only affects the colour but also the sensorial characteristics of the final product. The most effective approach is to limit the metal concentration left in the final wine.

Chitosan and its derivatives have been demonstrated to fix heavy metals compounds. The strong affinity of metal ions for chitosan is correlated with the nitrogen content of the biopolymer.

Different researches (Bornet & Teissedre, 2008; Magomedov & Dagestan, 2014) reported a dose dependence chelation effect by means of chitosan, where higher doses of the polysaccharide resulted in an increase of metallic removal. pH and deacetylation degree (units of free glucosamine) also plays an important role on the chelation capacity of chitosan, exhibiting higher reductions of metals at lower pH and increasing deacetylation degree.

Adsorption behaviour of chitosan against heavy metals and a large number of proposed mechanism have been summarized by Zhang, Zeng, & Cheng, (2016). Two of the most accepted and studied mechanisms are illustrated below (**Figure 1.4.2**):

1. An efficient removal of heavy metal ion may be due to the adsorption of cations into the surface of insoluble chitosan, through the formation of a complex that involves amine and hydroxyl groups. Therefore, the degree of deacetylation and the spatial distribution of the free amino groups determine the binding capacity of chitosan (**Figure 1.4.2**) (Wu, Tseng, & Juang, 2010).

2. Rather than direct chelation to amine groups, an alternative to the high adsorption capacity of chitosan for heavy metals could be the deposition of metal hydroxide produced, into the pores of chitosan particles. (Park, Park, & Park, 1984)

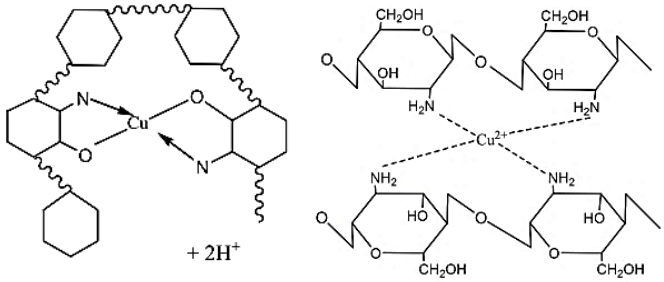

Figure 1.4.2. Mechanisms of metal chelation by chitosan

1.4.2. Antimicrobial capacity

In oenology, it is vital to guarantee the microbiological stability of the final product, since an inefficient control of the microorganism population in wine can lead to the development of alterations of greater or lesser severity such as lactic, acetic stings or the development of undesirable aroma compounds.

Chitosan has become of considerable importance due to its versatile antimicrobial activity against a broad range of microorganism such as gram-positive and negative bacteria, yeast and moulds. Its use extends not only to wine but also to a wide spectrum of food and agriculture matrices, making this product a true natural alternative to other traditionally used preservatives.

Some mechanisms of antimicrobial action of chitosan have been proposed:

1) As already supported in a large amount of literature, polycationic structure of chitosan is of essential importance in microbiological activity. At wine pH, chitosan is positively charged and moreover, the positive charged density is directly correlated with the degree of deacetylation (DD). A higher positive density leads to an intense electrostatic interaction with negatively charged components of cell surface, weakening the membrane, increasing permeability and consequently, leading to a loss of growth capacity and cell death.
2) Chitosan chelation capacity plays an important role in antimicrobial action, as metal ions that combine with cell wall molecules are of great importance for its stability. (Kong, Chen, Xing, & Park, 2010)

3) Surdashan et al., (Sudarshan, Hoover & Knorr, 1992) reported that chitosan could penetrate the cytosol of microorganisms and bind with DNA, inhibiting the synthesis of mRNA and proteins.
4) Chitosan could act as a layer that envelopes the cell, and prevents the uptake of different nutrients present in the medium (Ralston, Tracey, & Wrench, 1964).

1.4.3. Ochratoxin removal

Ochratoxins, produced as secondary metabolites by several moulds of *Aspergillus* or *Penicillium* fungal species, are a group of mycotoxins known for its nephrotoxicity and carcinogenicity in humans. Three types of ochratoxins have been identified, A, B or C, with slightly differences on their chemical structures. However, ochratoxin A (OTA), R-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopy-ran-7-yl) carbonyl] phenylalanine, is the most frequently detected form and also the most toxic of the three species. (Kurtbay, Bekçi, Merdivan, & Yurdakoç, 2008). A wide variety of foods are susceptible of contamination by OTA as a result of fungal infection in the field during growth, harvest and storage period if the conditions of humidity are high. OTA is usually found in cereals. Besides, is also found in coffee, beer, spices, dried fruits and some animal products exposed to contaminated feedstuffs. However, wine and grape juice are estimated to be the second source of OTA in the diet after cereals, representing up to 10% of total OTA intake (Quintela, Villaran, De Armentia, & Elejalde, 2012). Hence, the maximum allowed concentration of OTA in wine, must and grape juice is 2µg/L (ppb).

Some authors (Bornet & Teissedre, 2008; Kurtbay et al., 2008; Quintela et al., 2012) reported than chitin and chitosan present an efficient removal capacity against ochratoxin A when tested in winemaking, showing a direct correlation with the dosage of chitosan applied, increasing with higher concentrations and higher degree of deacetylation. Binding of OTA by means of chitosan depends on the crystal structure and physical properties of the polysaccharide and the physical-chemical properties of the mycotoxin as well. OTA is a weak acid with a pKa of 4.4 of the carboxyl group present on the phenylalanine moiety (Valenta, 1998). Thus, at wine pH, OTA is partially dissociated carrying a negative charge, being able to interact with a positively charged surface. Amino groups of chitin and chitosan are protonated at the wine pH, reacting with negatively charged carboxyl groups of OTA through electrostatic interactions. Furthermore, Bornet and co-workers (Bornet & Teissedre, 2008) propose an alternative mechanism of absorption based on the OTA deposition in pores of chitin or chitosan.

1.4.4. Protein stabilization

Wine limpidity, especially in white wines, is demanded by most consumers, being also the most sensitive to inappropriate shipping or storage conditions. For this reason, controlling wine stability prior to bottling is a key step to be faced in winemaking process (Van Sluyter et al., 2015). A stable wine is characterized by the absence of precipitates at the time of bottling, through transport and storage, to the time of consumption. White wine protein haze formation is caused by the aggregation of residual proteins into light-dispersing particles under high temperature during storage or transport (Chagas, Monteiro, & Boavida Ferreira, 2012).

Due to its physicochemical properties, chitosan can be used as a coagulating agent and to remove proteinaceous materials in food processing operations. The addition of fungal chitosan for fining purposes has been authorised by the international organization of wine (OIV, 2012) to reduce turbidity by precipitating insoluble particles in suspension or excess of proteinaceous matter to prevent protein haze. The maximum dose recommended is 100 g/hl.

Different investigations demonstrated a positive effect of chitosan on the heat stability in white wines, preventing it from protein haze formation (Colangelo, Torchio, De Faveri, & Lambri, 2018; Gassara et al., 2015). Furthermore, some experiments demonstrated dose-dependent behaviour of chitosan, leading to a higher decrease in the juice turbidity when increasing concentration of chitosan (Domingues, Faria Junior, Silva, Cardoso, & Reis, 2012). Again, the influence of degree of deacetylation and molecular weight of chitosan on protein flocculation has been evaluated (Gamage & Shahidi, 2007). Chitosan with the highest degree of deacetylation showed the best protein flocculation ability, evidencing the key role of the presence of free amino groups on the polysaccharide backbone.

The charge density of a polymer results a key factor on the flocculation capacity. Chitosan possess a high charge density compared with common coagulants. As the charge density rises, adsorption capacity of the polymer increases (Ariffin, Shatat, Nik Norulaini, & Mohd Omar, 2005). Chitosan behaviour is also influenced by the pH of the medium. At wine pH (3-4), amino groups of glucosamine units of chitosan are protonated. This cationic polyelectrolyte character allows chitosan to destabilize colloidal suspension and to promote flocculation. Hence, increasing the degree of acetylation increases the availability of amino groups to bind wine negatively charged protein particles (which depends on their PKa) in suspension via ionic or hydrogen bonding. Therefore, mechanism of protein haze prevention by chitosan in wine could be explained by charge neutralization of the particle surface, adsorption, hydrophobic flocculation and inter-particle binding.

1.4.5. Antioxidant and antibrowning activity

In recent years, the evaluation of the antioxidant activity of chitosan and derivatives has aroused great interest in the scientific community. However, investigations about its effect in winemaking is still scarce.

As reported by Chien et al. (Chien, Sheu, Huang, & Su, 2007) in apple juice, chitosan has demonstrated a high antioxidant capacity, by scavenging hydrogen peroxide and chelating metals. Furthermore, data shows that the antioxidant effect was dose dependant and inversely proportional to the molecular weight, with higher effects at maximum concentration (1g/L) and lowest molecular weight. This results were also obtained by Chien and co-workers (Chien, Li, Lee, & Chen, 2013) who postulated an oxidative destruction of chitosan by the presence of hydroxyl radicals generated from degradation of hydrogen peroxide.

In the understanding of the antioxidant mechanism of chitosan, it is important to highlight the fundamental role played by the presence of amino groups along the polysaccharide chain. Indeed, Dong et al. (Dong, Xue, & Liu, 2009), by using different concentrations (0.1-1.6 g/L) of chitosan with various degrees of deacetylation (45-95%) and its carboxymethyl derivatives, observed an increase of hydroxyl radical scavenging ability when increasing concentration and degree of deacetylation, obtaining the highest activity (57.5%) with 95% of degree of deacetylation at 1.6 g/L. The authors also demonstrated that the scavenging ability of chitosan against hydroxyl radical is not exclusively attributed to the amino group. By substituting the C_6 hydroxyl groups with carboxymethyl groups, a reduction in antiradical capacity was observed, concluding that 6-OH was also related to the scavenging of OH radicals. The scavenging mechanism proposed in the study, is the interaction of radicals with hydrogen atoms in chitosan, forming a most stable macromolecule radical. The sources of H atoms of chitosan are NH_2 of C2, and OH of C3 and C6. However, reaction with C3-OH is very difficult because of steric hindrance, being C2-NH_2 and C6-OH the responsible groups of antioxidant capacity of chitosan.

There is still a lack of scientific publications regarding the direct scavenging activity of chitosan against hydrogen peroxide and oxygen radical species in winemaking process. However, some studies that concern prevention of browning by means of reduction of polyphenol content by chitosan in wine have been published. Spagna and co-workers (Spagna et al., 1996) were the first to evaluate the use of chitosan in the clarification of white wine, comparing its effect with other traditional coadjuvants such as potassium caseinate or PVPP, with the aim of preventing browning development and producing a more stable product over time. According to results, chitosan demonstrated an effective adsorption capacity towards phenolic compounds, the main susceptible compounds to oxidation. Efficiency of chitosan resulted to be lower for flavans and proanthocyanins and similar or even higher for hydroxycinnamic acids. Moreover, results obtained showed a positive influence of the presence of amino groups, suggesting an interaction between functional group of chitosan and phenolic compounds through weak interaction such as Van der Waals forces

or hydrogen bonding. In addition, ionic links seem to be formed between free amine groups and carboxylic groups of polyphenols.

Furthermore, Chinnici et al. (Chinnici, Natali, & Riponi, 2014), aimed to evaluate the oxidation of (+)-catechin in wine model solutions spiked with 1 g/L of chitosan during 21 days. Authors, as previously reported by (Spagna et al., 1996), initially observed also a declined of (+)-catechin content likely due to an interaction with the polysaccharide chain. Moreover, authors outlined a reduction in browning development with respect to the control, and apparently comparable with samples added with SO_2. In the paper, analysis of phenolic products of the oxidative decay of (+)-catechin was also carried out, demonstrating a minor content of the latter in chitosan samples, suggesting that the reduction on the trend of browning phenomena could also be due to a lack of the generation of the oxidation products.

A recent study published by Nunes and co-workers, (Nunes et al., 2016) described a novel approach were the film-forming properties of chitosan are exploited to develop a film modified with genipin, an a effective natural antioxidant derived from *Gardenia* fruits, with the aim to evaluate its effectiveness to produce white wines without sulphur dioxide as a preservative. Results showed a lower tendency to browning in wines treated with chitosan-genipin films. In order to find an explanation of the mechanism of chitosan-genipin films to prevent wines from oxidation, authors determined the content of phenolic compounds and metals throughout the storage period. Data showed a slightly reduction (<15%) when compared with untreated or SO_2 added samples, as previously reported by other authors. Fe, Cu and Al, were lowered by 50%, 25% and 20% respectively, as well.

Based on the reported data, some antioxidant mechanisms of chitosan have been elucidated: 1) Chelation of metals, 2) Scavenging of H_2O_2, 3) Direct quenching of hydroxyl radical, 4) Adsorption of phenolic acids. It has also been observed that its effectiveness is improved with a reduction in molecular weight and an increase in the degree of deacetylation. However, despite of the promising results, antioxidant strategies listed above are still to be proved in winemaking.

1.4.6. Sensory impact

A correct management and knowledge of the different treatments carried out in the winery reduces the risk of developing undesirable flavour and aromas.

Despite being a relatively new additive in oenology, some studies about the sensory impact of chitosan have been reported.

Ferrer-Gallego et al. (Ferrer-Gallego, Puxeu, Nart, Martín, & Andorrà, 2017), evaluated the influence of different treatments on the sensory profile of sulfite-free wines. On red wines, chitosan showed an impact on the sensory profile by a reduction on wet vegetal character and increasing the balsamic or black fruits notes. Global parameters were also affected, showing a decrease of burning sensation and an increase on dryness, colour and astringency. Astringency has demonstrated to be significantly influenced by the presence of chitosan (Luck, Varum, Foegeding, Varum, & Foegeding, 2015; Seo, Chang, Lee, & Kwak, 2011). Those authors suggested that astringency resulted from the binding of positively charged moyeties with elements of the oral mucosa and saliva. At wine pH, amino groups of glucosamine units of chitosan are positively charged. Therefore, astringency has been associated with positively charged fraction of chitosan being decreased by decreasing the degree of deacetylation (Luck et al., 2015).

Secondly, chitosan has proved to be an efficient agent in the reduction of volatile phenols (VP`s) produced by *Brettanomyces* Nardi et al. (Nardi, Vagnoli, Minacci, Gautier, & Sieczkowski, 2014) reported the impact of chitosan on long-term application to protect Sangiovese wines against *B. bruxellensis.* The authors observed a double effect of chitosan treatments, by keeping *Brett* population under control and limiting the increase of volatile phenol produced. These data were confirmed by others (Filipe-Ribeiro, Cosme, & Nunes, 2017). whose study aimed to evaluate the potentiality of chitosan to improve sensorial profile of wine contaminated by volatile phenols. Authors found a reduction of red wine VP's headspace content after treatments with chitosan. Even if total VP's were not affected by the treatment, their reduced volatility decreased the negative sensory attributes and bitterness and enhance the positive floral and fruity sensory character. Besides, results demonstrated that efficiency was dependant on the degree of deacetylation and dose applied.

Therefore, based on the published results chitosan represents a powerful approach to reduce negative sensory impact produced by VP's in wines contaminated by *Brett.* However, the understanding of the impact of chitosan in wine sensory properties and mechanism of interaction is still unknown.

FIELD OF USE OIV/336/7/8 A/2009	TARGET	MATRIX	DOSE TESTED	EFFECT	BIBLIOGRAPHY
Reduction of undesirable microorganisms recommended dose OIV: 10 g/Hl	**Yeast:**				
	Brettanomyces spp.	Wine	20-80 mg/L	Fungistatic activity (after 10 days the cells are no longer cultivable).	Pillet, 2010
		Culture medium	100-6000 mg/L	Fungistatic activity (the lag phase lasted up to 80h).	Gomez-Rivas et al. 2004
		Wine	200-750 mg/L	Fungistatic activity (influence of molecular weight on the effects of chitosan).	Ferreira et al. 2013
		Wine in barrique	40-100 mg/L	Fungistatic activity (reduction of the Brettanomyces population).	Petrova et al. 2016
		Wine in barrique	40 mg/L	Fungistatic activity (prevention of the development of Brettanomyces during elevage and influence of batonnage).	Nardi et al. 2014
		Inoculum in vitro	40-400 mg/L	Fungistatic activity (physical and biological effects on Brettanomyces cells).	Taillandier et al. 2015
	S. cerevisiae	Inoculum in vitro	100-6000 mg/L	Increase of the lag phase from 0 to 4 h depending on the concentration of chitosan.	Gomez-Rivas et al. 2004
		Inoculum in vitro	25-50 mg/L	Resistance mechanisms of *Saccharomyces* to the action of chitosan.	Zakrzewska et al. 2007
		Inoculum in vitro	250-10000 mg/L	Influence of chitosan of animal and fungal origin on the growth of two *S. Cerevisiae* strains.	F. Tajdini et al. 2010
		YPG culture medium	600-2000 mg/L	Lag phase increase from 2 to 4 days depending on the concentration.	Elmacı et al. 2015
	Yeast non-Saccharomyces:				
	H.uvarum e Z.baili	YPG culture medium	100-400 mg/L	Growth inhibition and determination of the minimum required concentration (MIC).	Elmacı et al. 2015

	Acetic Bacteria: *A. malorum e A. pasteurianus*	Wine matrix	200 mg/L	Growth inhibition (reduction of Acetobacter spp. activity; effects comparable to the presence of 60 mg / L of SO_2).	Valera et al. 2017
	Lactic acid Bacteria *O. oeni*	YPG culture medium	200-2000 mg/L	Growth inhibition (*O. oeni* was completely inactivated at the lowest concentration, 200 mg/L).	Elmacı et al. 2015
	L. hilgardi	YPG culture medium	200-2000 mg/L	Growth inhibition (*L. hilgardii* was completely inactivated at the lowest concentration, 200 mg/L).	Elmacı et al. 2015
	L. plantarum	YPG culture medium	200-2000 mg/L	Growth inhibition, growth is inhibited at the concentration of 800 mg/L, *L. plantarum* was among the most resistant LAB.	Elmacı et al. 2015
		Peptoned water/Hopped ame	10-1000 mg/L	Growth inhibition, only the test with 1 g / L prevented the development of *L. plantarum*, the effect of the pH and of the medium on the activity of chitosan were also evaluated.	Gil et al. 2004
Clarifying and prevention of protein cases recommended dose OIV: 100g/Hl	Proteins	White wine	1000 mg/L	Protein instability, chitosan at this concentration can not guarantee protein stability (comparative study with other enological clarifying agents).	Chagas et al. 2012 Colangelo et al. 2018
	Browning	White wine	1000 mg/L	Inhibition of browning, chitosan show an excellent affinity for phenolic compounds, only bentonite eliminated more phenols.	Chagas et al. 2012
		Wine matrix	1000 mg/L	Inhibition of browning, comparison between different oenological additives, chitosan at concentrations of 1 g/L showed the same antioxidant power of 80 mg/L of SO_2.	Chinnici et al. 2016

	Clarification	Wine and must	300 mg/L	Clarification of must and wine, action on phenols and caftaric acid. Comparative study between the different clarifiers.	Eder. 2012
		Apple juice	200-1000 mg/L	Increased apple juice clarity up to 73% more than control. Comparison with clarifying gelatin.	Abd, Alaa Jabbar et al. 2012
Reduction of the content in heavy metals	Metals (Cu,Fe)	Model Wine	1000 mg/L	Reduction of Fe and Cu content up to 80% and 56% respectively.	Chinnici et al., 2016
	Metals (Cu,Fe,Pb,Cd)	Wine	100-2000 mg/L	Reduction of metal content up to 90%, depending on the type of chitosan, wine used and pH.	Bornet & Teissedre 2008
	Oxidasic casses	Oxygen satured H_2O	1000-3000 mg/L	Antioxidant, chitosan reduces the amount of molecular oxygen present in solution.	Gyliene et al. 2015
Reduction of contaminants (Ochratoxin) maximum dose OIV: 500g/Hl	Ochratoxin A	Wine	2000-5000 mg/L	Reduction of Ochratoxin A levels from 26 to 86% depending on the type of chitosan, wine used and pH.	Bornet & Teissedre 2008
Other unconventional uses	Reduction of volatile phenols	Red Wine	1000 mg/L	Removal of the content of 4 ethyl-phenol and 4 ethyl guaicol present in the head space, comparative study with different fining agents.	Milheiro et al. 2017
	Aroma protection	Model Wine	1000 mg/L	Lowering of thiol oxidation, reduction of quinone formation.	Chinnici et al. 2016
	Reduction of acidity	White wine	1000 mg/L	Reduction of the titratable acidity of 1 g/L.	Castro et al. 2018
	SO_2 Free wine	White wine	1000 mg/L	Chitosan as a substitute for sulfur dioxide in white wines winemaking	Castro et al. 2018
	SO_2 Free wine	White and red wines	100mg/L	Chitosan as a substitute for sulfur dioxide for the vinification of white and red wines. Comparative study with various additives.	Ferrer-Gallego et al. 2017

Table 1.4.1 Summary of bibliographic data on the effects of chitosan in wine or conditions relevant to wine

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2.Objectives

2. Objectives

Based on the premises illustrated in the introduction, this project is intended to fill the gap in scientific investigation on chitosan in wines with the goal to deepen the possibility for its use in SO_2-free wines, together with other chemical or technological approaches appearing to be a potential good candidate as an alternative to sulphite in winemaking.

During the **first part** of the project, special attention to chitosan characterization will be given, aiming to characterize its antioxidant behaviour in model wine solutions. The use of this simplified matrix will give the opportunity to investigate each specific oxidative mechanism avoiding the interference of other collateral/unwanted phenomena. Parameters to be tested will be:

1. **·OH radical inhibition**
   Photolytic generation of ·OH and determination of chitosan scavenging activity.
2. **H_2O_2 scavenging**
   Quantification of H_2O_2 in model wine solutions after treatments with chitosan.
3. **Inhibition of the oxidation of wine constituents**
   Study of the influence of chitosan on the development of intermediates of oxidation of tartaric acid and ethanol in both model solution and wine. To this purpose, generation of glyoxylic acid and acetaldehyde in the presence of chitosan, together with the evolution of 1-hydroxyethyl radical (1-HER) the intermediate radical of oxidation of ethanol, will be monitored.
4. **Chelation capacity of chitosan**
   Reduction of metal content in both model solution and real wines as affected by the presence of chitosan.

Once characterised the antioxidant and antiradical features of chitosan, its effect on real wines will be studied in the **second part** of the project, by carrying out microvinifications at laboratory scale. Experiments to develop will be:

1. **Influence of the presence of chitosan during alcoholic fermentation, on the chemical-physical parameters of white wines**
2. **Efficacy of pre-bottling treatment of white wine with chitosan in preventing browning phenomena and aroma changes**

In the **third part**, trials at semi-industrial scale will investigate the interaction of chitosan with other well-established wine additives, such as glutathione and ascorbic acid, and other technological intervention, as follow:

1. **Effects of chitosan during flotation and stabilization "sur lies" in white wine.**
2. **In depth investigation on phenolic evolution during winemaking and bottle storage.**

During the entire project, a special attention will be given to the fate of phenolic compounds in order to deepen oxidation kinetics. Due to this, liquid chromatography (HPLC/DAD/FL/MS) will be the analytical technique of choice. Furthermore, antiradical properties of chitosan will be evaluated by Electron Paramagnetic Resonance (EPR). Once at the experimental cave, in real wines, apart from the basic analytical method in oenology (based on titration, distillation or spectrophotometry) a substantial contribute is expected from gas chromatography (GC/MS) in order to describe the aromatic changes of wines treated with the proposed additives. Sensory analysis (on wine produced during the year 2 and 3), will complete the range of analytical techniques exploited.

3.Experimental section

Chapter 3.1.

"Chitosan as an antioxidant alternative to sulphites in oenology: EPR investigation of inhibitory mechanisms"

Chapter 3.1

Chitosan as an antioxidant alternative to sulphites in oenology: EPR investigation of inhibitory mechanisms

3.1.1. Introduction

As outlined in the section 1 of the introduction, trace transition metals, particularly iron and copper, have been shown to play a cardinal role in wine oxidation, notably because they catalyze the reduction of H_2O_2 to HO• by a Fenton-type reaction, being then redox cycled by those polyphenols, such as non-wine like 4-methylcatechol (4-MeC), bearing at least one catechol moiety (Danilewicz, 2003). Finally, HO• will oxidise ethanol and tartaric acid to acetaldehyde CH_3CHO and glyoxylic acid, respectively, the former imparting to white wine a characteristic oxidative odour upon accumulation.

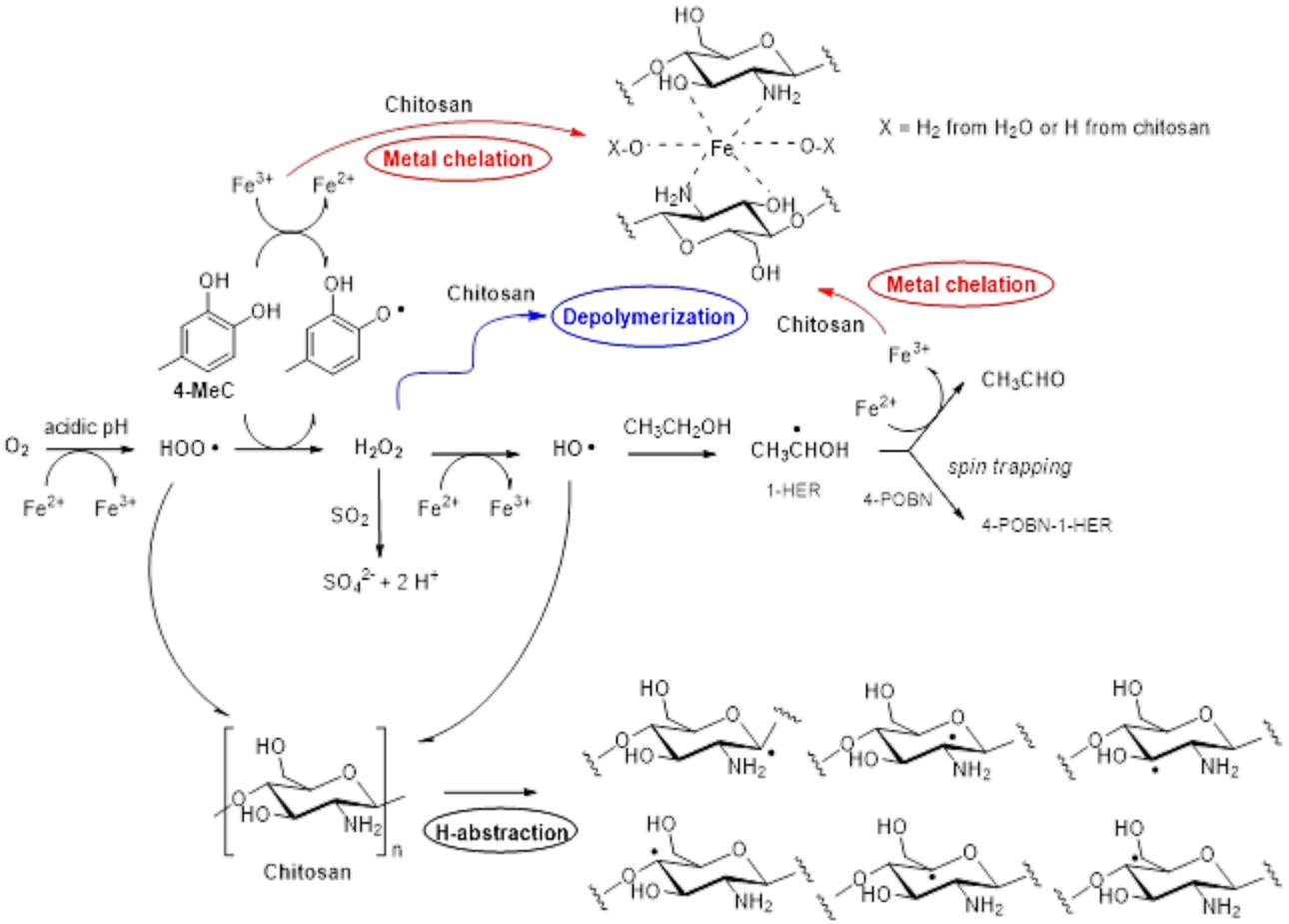

Figure 3.1.1. Free radical mediated formation of acetaldehyde from ethanol during wine oxidation, its assessment using EPR spin trapping, and potential mechanisms of chitosan protection. 4-MeC, 4-methylcatechol.

Of the available methodologies to study the reactivity of HO• in wine oxidation, electron paramagnetic resonance (EPR) spectroscopy coupled to spin-trapping has led to conclusive advances in the understanding of free radical processes. **Fig 3.1.1.** shows that hydroxyl radicals, which non-specifically attack any molecule at diffusion-controlled rates (i.e., with second-order rate constants > 10^9 $M^{-1}{\cdot}s^{-1}$), will oxidise ethanol to the main, thermodynamically stabilized secondary 1-hydroxyethyl radical (1-HER) intermediate. In low O_2

conditions 1-HER is readily oxidized by Fe(III) to yield acetaldehyde. Despite it is also quenched by many wine constituents such as polyphenols and thiols (Kreitman, Laurie & Elias, 2013) enough 1-HER remains available to be spin trapped on nitrones added to a wine oxidation system, giving nitroxide adducts that can sometimes be detected for days (Elias, Andersen, Skibsted & Waterhouse, 2009a, 2009b; Elias & Waterhouse, 2010; Kreitman, Cantu, Waterhouse & Elias, 2013; Zhang, Shen, Fan, García Martín, Wang & Song, 2015; Nikolantonaki et al., 2019).

An attractive route to control wine oxidation could be the inactivation of catalytic metals by potent chelators, as such intervention would, in principle, simultaneously inhibit Fenton chemistry, and the formation of *o*-quinones and acetaldehyde (**Fig. 3.1.1**).

Due to its regular and high density of amino and hydroxy groups (**Fig. 3.1.1**), chitosan has remarkable metal chelation power that could be exploited to control non-enzymatic oxidation. In the present work established EPR spin trapping and wine oxidation relevant techniques were applied for the first time deepening the mechanisms of how an approved, insoluble chitosan protects against white wine spoilage in winemaking conditions.

3.1.2. Materials and methods

3.1.2.1. Chemicals and wine samples

The spin traps α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (4-POBN) and 5,5'-dimethyl-1-pyrroline-*N*-oxide (DMPO) were from TCI (Zwijndrecht, Belgium), and 5-(diethoxyphosphoryl-5-methyl)-1-pyrroline-*N*-oxide (DEPMPO) was synthesized and purified as reported (Culcasi, Rockenbauer, Mercier, Clément & Pietri, 2006) (**Fig. 3.1.2.**). Fluorescein, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were from Acros (Illkirch, France). Phosphate-buffered saline (PBS) and other solvents or chemicals, including ferric chloride, ferrous sulfate heptahydrate, copper(II) sulfate, ferrozine [4,4'-[3-(2-pyridinyl)-1,2,4-triazine-5,6-diyl]bisbenzenesulphonic acid], 2.4-dinitrophenylhydrazine (DNPH), 4-MeC, potassium metabisulfite, (+)-tartaric acid, acetaldehyde, and H_2O_2 were of analytical (> 98.5%) or HPLC grade from Sigma-Adrich (Saint-Quentin Fallavier, France). Doubly distilled deionized water was used throughout.

A 75–85% deacetylated chitosan having a 50–190 kDa molecular weight was purchased from Sigma-Aldrich (CHI-1; product 448869), and a 80–90% deacetylated chitosan having an average molecular weight of 10–30 kDa (CHI-2) of fungal origin (*Aspergillus niger*) obtained from KitoZyme (Herstal, Belgium) were studied.

Commercially available sulphur dioxide-free white wine samples, obtained from Chardonnay grapes (100%; AOP Coteaux Champenois), were kindly provided by Champagne J. de Telmont (Damery, France). These wines had the following oenological characteristics, measured according to standard procedures described in the 'Compendium of international methods of analysis of wines and musts', published in 2018 by the

International Organization of Vine and Wine (OIV): harvest, 2015 (2016); ethanol (% v/v), 11.47 (11.35); pH, 3.22 (3.16); titratable acidity (g/L of sulphuric acid), 4.40 (4.60); volatile acidity (g/L of sulphuric acid), 0.26 (0.45); malic acid content (g/L), <2.0 (< 2.0); free SO_2 (mg/L), < 9 (~4) and total SO_2 (mg/L), 5 (not measurable). After opening, the wine samples were stored under N_2 atmosphere.

3.1.2.2. Model wine solution

One litre of model wine solution consisting of 12% (v/v) ethanol and tartaric acid (8 g/L) was prepared and its pH was adjusted to 3.5 with 10 M NaOH. To guarantee air saturation, samples were stirred for 1 h before carrying out the experiment.

3.1.2.3. Measurement of Fe(II) chelating activity

First, the chelating activity of CHI-2 was determined in model wine (pH 3.5) at room temperature using the ferrozine competition assay (Stookey, 1970) with modifications. Briefly, 0.1 mL of CHI-2 in suspension at different concentrations (0–10 g/L) was mixed with 50 µL of ferrozine solution (0.7–2 mM) in 1.5 mL Eppendorf tubes. Following stirring of the samples for 10 min in darkness, 0.1 mL of Fe(II), as ferrous sulfate, (100 µM) was added and agitation was maintained for 48 h. Following centrifugation (2320 *g*) of the samples for 5 min, 0.2 mL of supernatant was transferred into 96-well microplates and the absorbance was determined at 562 nm using a microplate reader (Tecan Infinite, Männedorf, Switzerland). Plotting absorbance inhibition versus chitosan concentration allowed to determine IC_{50} values, defined as the effective chitosan (or ferrozine) concentration required to chelate 50% of iron (II). Calibration curves (from triplicate measurements) were established in model wine at pH 3.5 by plotting absorbance versus ferrozine concentration (0.25–3 mM) at a given Fe(II) concentration (0.1–0.5 mM) using the same incubation protocol.

Second, the unchelated iron content of real wine samples spiked with 100 µM Fe(II) alone or in the presence of CHI-2 (0.5 and 2 g/L) was also determined by means of flame atomic absorption according to the relevant OIV method (see above). Briefly, samples were saturated with air and aliquots (20 mL) were placed into 50-mL Falcon tubes sealed with stoppers and continuously agitated for 48 h at 20 °C in darkness. Afterwards, samples were centrifugated (45 *g*) and filtered prior to injection for iron analysis. The instrument was an Agilent 240FS AA spectrophotometer, with a deuterium lamp for background radiation correction, a hollow cathode lamp at 248.3 nm, and the air–acetylene flame. Calibration curves were plotted using standard iron diluted with deionized water. All analyses were performed in triplicate.

3.1.2.4. Irradiation and sample analysis

- ***Wine sample preparation***

An additional set of iron-spiked wine samples containing CHI-2 (0.5 and 2 g/L) or SO_2, as potassium metabisulfite, (50 mg/L) were prepared and stored as outlined for flame atomic absorption studies. Samples were placed at 20 °C in a temperature-controlled chamber for 1−6 days at a distance of 5 cm from two cool daylight fluorescent lamps (Sylvania T8 Luxline Plus 36W 840) producing a light at 300−580 nm wavelength and an average intensity of 2000 lux. The light intensity was measured using a 51000 series digital lux meter (Yogokawa, Lyon, France). All samples were shaken for 2 min four times/day throughout. All experiments were performed at least in triplicate.

- ***HPLC-DAD analysis of acetaldehyde***

At the end of the irradiation period the samples were analyzed for their content in acetaldehyde using a Merck Hitachi HPLC system consisting of an Elite LaChrom L-7000 interface module with a diode array detection (DAD) (L-7455) and an EZchrom workstation for data processing. The UV spectra were recorded in the range 220−400 nm. With the aim to detect exclusively the free fraction of aldehydes which take part in oxidation process, no acid hydrolysis of samples was carried out. Samples (800 µL) were incubated with 200 µL of a DNPH solution (10 mM in 2.5 M HCl) for 1 h at 45º C in darkness. After cooling at room temperature separation of the DNPH adducts was achieved on a Nucleodur C18 Htec column (Macherey-Nagel, Düren, Germany; 250 × 4.6 mm; 5 µm) with a flow rate of 0.8 mL/min. Solvent A was acetonitrile; solvent B was water containing 0.05% (v/v) solution of phosphoric acid (pH 2.7). The elution program was the following: 0 min, 40% A, 8 min, 85% A, 9 min, 40% A, 13 min, 40% A, and injection volume was 20 µL. The identification of the observed derivatives was based on their retention time compared with those of standards tested at 360 nm as well as their spectral characteristics. Quantification was based on peak area.

3.1.2.5. Oxygen radical absorbance capacity (ORAC) assay

The assay was performed in microplates as previously described (Kandouli et al., 2017). Briefly, a fluorescein stock solution (821 µM) was prepared in PBS and stored at 4 °C. Prior to use, the following solutions in KH_2PO_4 buffer (0.1 M, pH 7.4) were prepared: fluorescein stock solution rediluted as to reach 82.1 nM, and AAPH (153 mM). Test samples, Trolox calibration solution or the blank (25 µL/well) were added to the wells of a 96-well plate, diluted with fluorescein solution (150 µL/well) and incubated for 10 min at 37 °C. AAPH solution (25 µL/well, 19.12 mM, final concentration) was then added and the fluorescence intensity (excitation at 485 nm, emission at 530 nm) was monitored every 2 min for 70 min with a microplate reader. The ORAC value was calculated using the net area-under-curve and expressed as µmol Trolox/mL.

3.1.2.6. Preparation of solutions and suspensions for EPR spin trapping analysis

Samples to be scanned by EPR were aspirated into 50 µL glass capillary tubes (Hirschmann Lab., Eberstadt, Germany), as to fill them completely, and sealed with Critoseal (McCormick Scientific, St Louis, MO) at lower (nucleophilic addition and Fenton reaction with 4-MeC) or both ends (remaining studies).

- ***Solutions for in situ photolysis and Fenton reaction in model wine and calculation of rate constants***

Hydrogen peroxide (3% v/v) was used as photolytic precursor of HO•. Solutions of chitosan (0.1–2 g/L) dissolved in water containing 0.5% (v/v) acetic acid (pH 3.18) and DMPO (3.33 mM, final) were continuously illuminated using a 1000 W xenon-mercury UV–Vis light source (Oriel, Newport Corp., Irvine, CA) guided within the EPR cavity through an optical glass fiber. The corresponding blank spectra were substracted from experimental spectra before data processing.

The apparent second-order rate constant k_{CHI} for the reaction of HO• with chitosan was calculated using the equation:

$$I_0/I = 1 + [(k_{CHI}/(k_{DMPO} \times C_{DMPO}) \times C_{CHI}]$$

where I_0 and I is the intensity of the EPR signal recorded in the control and in presence of chitosan, respectively, C_{DMPO} and C_{CHI} are the concentrations of DMPO and chitosan, respectively, and k_{DMPO} is the second-order rate constant for the trapping of HO• on DMPO. The slope of the regression plot of I_0/I against C_{CHI} for a constant value of C_{DMPO} was used to estimate k_{CHI} (Finkelstein, Rosen & Rauckman, 1980):

$$k_{CHI} = \text{slope} \times C_{DMPO} \times k_{DMPO}$$

assuming that $k_{DMPO} = 3.4 \times 10^9$ $M^{-1}.s^{-1}$ using the above conditions and photolytic system (Finkelstein et al., 1980).

To estimate the effect of hydrogen peroxide and iron(II) on Fenton-driven 4-POBN spin adduct formation H_2O_2 (0.25–25 µg/mL) was added to a freshly prepared solution of the nitrone (15 mM) and Fe(II) (0.1 or 0.2 mM) in model wine. EPR spectra were acquired 130 s or 10 min after addition of H_2O_2.

- ***Suspensions for nucleophilic addition assays***

A suspension of tested chitosan (0.5–2 g/L) in water containing a wine relevant concentration of Fe(III), as $FeCl_3$, of 30 mg/L and Cu(II), as $CuSO_4$, of 12.5 mg/L was stirred for 1 h at room temperature to allow for metal complexation by chitosan. EPR spectra were recorded 1 min following addition of aqueous DEPMPO (55 mM) to the suspension.

- ***Suspensions for Fenton reaction assays and incubations***

All experiments described below (incubations and EPR spectrometry) were conducted at 20–22 °C in darkness.

To assess the effect of CHI-2 (0.5 or 2 g/L) or SO_2 (50 mg/L) on 4-POBN adduct formation the tested inhibitor was pre-incubated with 100 µM Fe(II) for 48 h in model wine. Following pre-incubation of suspensions/solutions were added the nitrone (15 mM) and H_2O_2 (2.5 µg/mL) dissolved in model wine. EPR spectra were then sequentially recorded up to 1 h following addition of H_2O_2. In some experiments 4-MeC (1 mM) was also added after pre-incubation.

To extend the above experiments to wine oxidation under winemaking conditions the incubations were prolonged up to 3 or 6 days in model and real wine, respectively. A 48-h pre-incubation with 100 µM of Fe(II) and varying concentrations of the tested inhibitor (i.e., chitosan, SO_2 or ferrozine) was first applied. Then 4-POBN (15 mM) alone (real wine) or mixed with 4-MeC (1 mM; model wine) were added and the EPR signal intensity was followed over time. In experiments performed in real wine samples were gently decanted by centrifugation at 25 *g* for 5 min before adding the nitrone in the clear supernatant.

Throughout incubation the solutions/suspensions were stored in capped Eppendorf tubes with a ~10 times air volume above and stirred at 5–10 rpm with a Stuart SB3 rotator (Cole-Parmer, Vernon Hills, IL).

3.1.2.7. Acquisition of EPR spectra and apparatus

EPR signals were obtained with a Bruker ESP 300 spectrometer (Karlsruhe, Germany) operating at X-band (9.79 GHz) with 100 kHz modulation frequency and a microwave power of 10 mW. Typical settings in DMPO and 4-POBN (DEPMPO) spin trapping were: modulation amplitude, 0.625 (0.279) G; time constant, 81.92 (40.96) ms; gain, 1×10^5 (1.25×10^5); sweep width, 60 (140) G; sweep time/scan, 41.94 (41.94) s; number of accumulated scans, 10 (1). To determine *g*-factors the magnetic field strength and microwave frequency were measured with a Bruker ER 035M NMR gaussmeter and a Hewlett Packard 5350B frequency counter, respectively. Spin adduct intensities were determined by double integration of simulated spectra using WinSim software (Duling, 1994). Area-under-curve of spin adduct variations were obained using Prism sofware (GraphPad Software, San Diego, CA).

3.1.2.8. Statistical analysis

Data are given as mean ± SEM for the indicated number of independent experiments. Evaluation of statistical significance was conducted by one-way analysis of variance (ANOVA) followed, if significant ($p < 0.05$), by a posteriori Duncan test. Differences between groups were considered significant when $p < 0.05$.

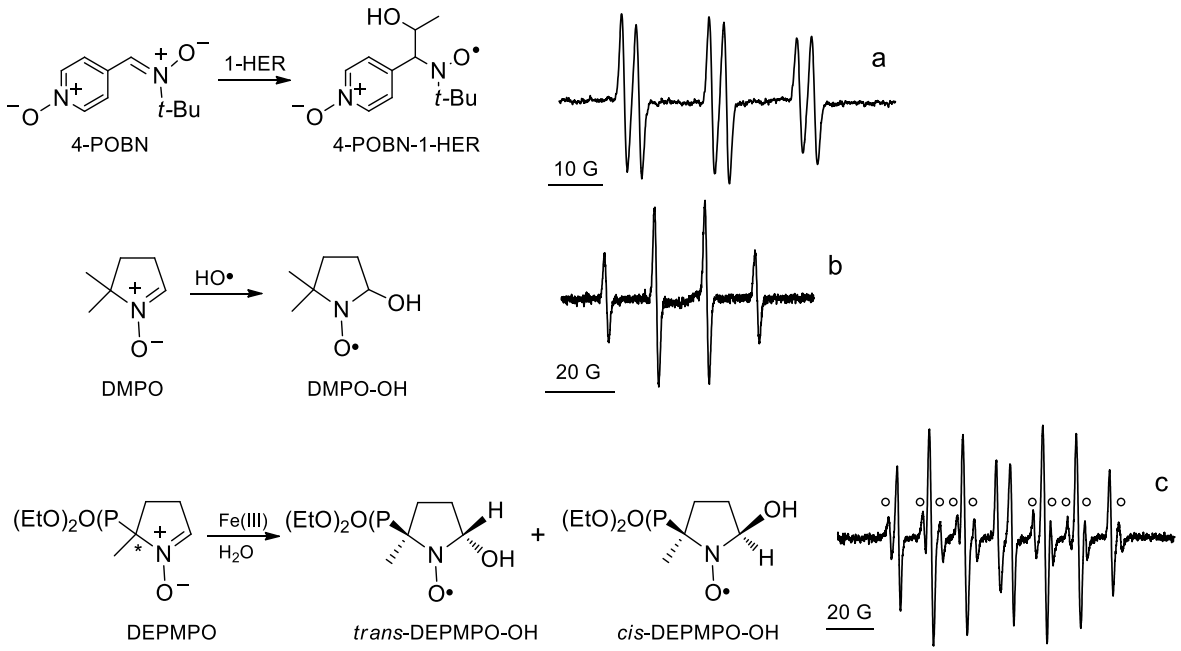

Figure 3.1.2. Spin traps used in the study, spin adducts detected, and representative EPR spectra in the absence of inhibitor. Experimental conditions: (a) incubation of wine for 96 h at room temperature in darkness in the presence of 4-POBN (15 mM); (b) photolysis of H_2O_2 (3% v/v in 0.5% acetic acid solution) in the presence of DMPO (3.33 mM); (c) nucleophilic addition of water in the presence of $FeCl_3$ (30 mg/L), $CuSO_4$ (12.5 mg/L) and DEPMPO (55 mM). The asterisk indicates the asymmetric carbon of DEPMPO. Open circles indicate lines from a minor DEPMPO carbon centered radical adduct.

3.1.3. Results and discussion

Owing to its insolubility at wine pH and known metal chelation property, suspensions of chitosan were stirred for 2 days in darkness with 100 µM (5.5 mg/L) Fe(II), a typical concentration found in white wine, to ensure maximum metal chelation before oxidation under wine conditions was induced. Under these pre-incubation conditions 500 µM ferrozine were found to chelate 142 µM of Fe(II) in model wine at pH 3.5, while increasing the pH to 4.5 resulted in a 30% increase of the chelating power, in agreement with previous observations (Stookey, 1970).

3.1.3.1. EPR evidence that chitosan slowers free radical formation during wine oxidation

A general method to follow 1-HER formation in oenology as an intermediate in non-enzymatic wine oxidation (**Fig. 3.1.1.**) is to apply EPR spin trapping using the linear arylnitrone 4-POBN as the spin trap (Elias et al., 2009a, 2009b; Nikolantonaki et al. 2019). Thus, in 4-POBN (15 mM)-containing model wine (12% v/v ethanol, 8 g/L tartaric acid, pH 3.5) challenged by HO• radicals formed via a Fenton reaction, the strong six-lines

spectrum of 4-POBN-1-HER spin adduct was detected, with hyperfine splittings: a_N = 15.62 G, a_H = 2.55 G, $a_{^{13}C}$ = 5.22 G, and *g* = 2.0054 in good agreement with previous data, including the coupling value of the ^{13}C satellite lines (Halpern, Yu, Barth, Peric & Rosen, 1995; Nakao & Augusto, 1998; Pou et al., 1994). In the same system removal of ethanol allowed transient detection of the 4-POBN/hydroxyl radical adduct (4-POBN-OH) as a sextet with slightly different EPR parameters: a_N = 14.99 G, a_H = 1.65 G, and *g* = 2.0057, consistent with early data (Pou et al., 1994). The fact that 1-HER, and not the primarily formed HO• (**Fig. 3.1.1.**), is the major species trapped in 4-POBN spin trapping studies on alcoholic beverages mainly relies on: (i) the very low stability of 4-POBN-OH versus 4-POBN-1-HER (Halpern et al., 1995; Pou et al., 1994), and (ii) the large excess of ethanol (molar range) with respect to the nitrone (millimolar range) in the system to compete with HO•. Indeed, detection of nitrone/HO• adducts in oxidizing wine required a molar concentration of the trap (Elias et al., 2009a).

As depicted in **Fig. 3.1.1.** endogenous wine's phenolics can be considered suitable Fe(II)/Fe(III) redox recyclers to sustain the Fenton reaction involved in wine oxidation (Elias et al., 2009a; Elias & Waterhouse, 2010). When the model wine system above was added 1 mM of 4-MeC (taken as a model for wine's catechols), a wine's typical concentration with respect to total phenolics (Kreitman, et al., 2013b), 4-POBN-1-HER adducts developed over 3 days at ambient temperature and in darkness, provided that incubating samples were always well aerated. Given the known high stability and resistance to redox-active agents of 4-POBN-1-HER adducts (Halpern et al., 1995) a sensitive EPR acquisition method was applied here, where accumulating signals in ~7-min blocks allowed detection of weak signal intensities since 1 h in control samples (**Fig. 3.1.3A**). Both chitosans added in suspension up to 2 g/L significantly inhibited oxidative formation of 4-POBN-1-HER with similar profiles (but no clear dose-dependence), CHI-2 being the most effective after 48 h of action. A very significant inhibitory effect of the best compound, CHI-2, added at 1 or 2 g/L was also seen when incubations were carried out in sulphite-free Chardonnay wine for up to 6 days at ambient temperature, again with no significant dose effect except in the early oxidation phase (**Fig. 3.1.3B**).

In order to address the mechanisms by which chitosans protect synthetic and real wine against free radical mediated ethanol oxidation, i.e., by delaying the formation of 1-HER radical intermediate, incubations in both matrices were carried out in the presence of SO_2 at a winemaking dose (50 mg/L), or the strong iron (II) chelator ferrozine. By interacting with two main components of the Fenton system (**Fig. 3.1.1.**), SO_2 and ferrozine can inhibit 1-HER formation by removing H_2O_2 or forming iron complexes with no catalytic power, respectively (Elias et al., 2009b; Elias & Waterhouse, 2010; Kreitman et al. 2013b). The strong decreases in 4-POBN-1-HER formation seen with both types of treatments seem to confirm the pertinence of these two mechanisms (**Fig. 3.1.3.**). Thus, spin adduct formation re-increased in SO_2 added samples after 48 h incubation, possibly because decreased levels of free SO_2 (i.e., the scavenging-active SO_2 fraction not linked

to acetaldehyde and not already oxidized to sulfate) could no more efficiently eliminate the continuous H_2O_2 formation in the system.

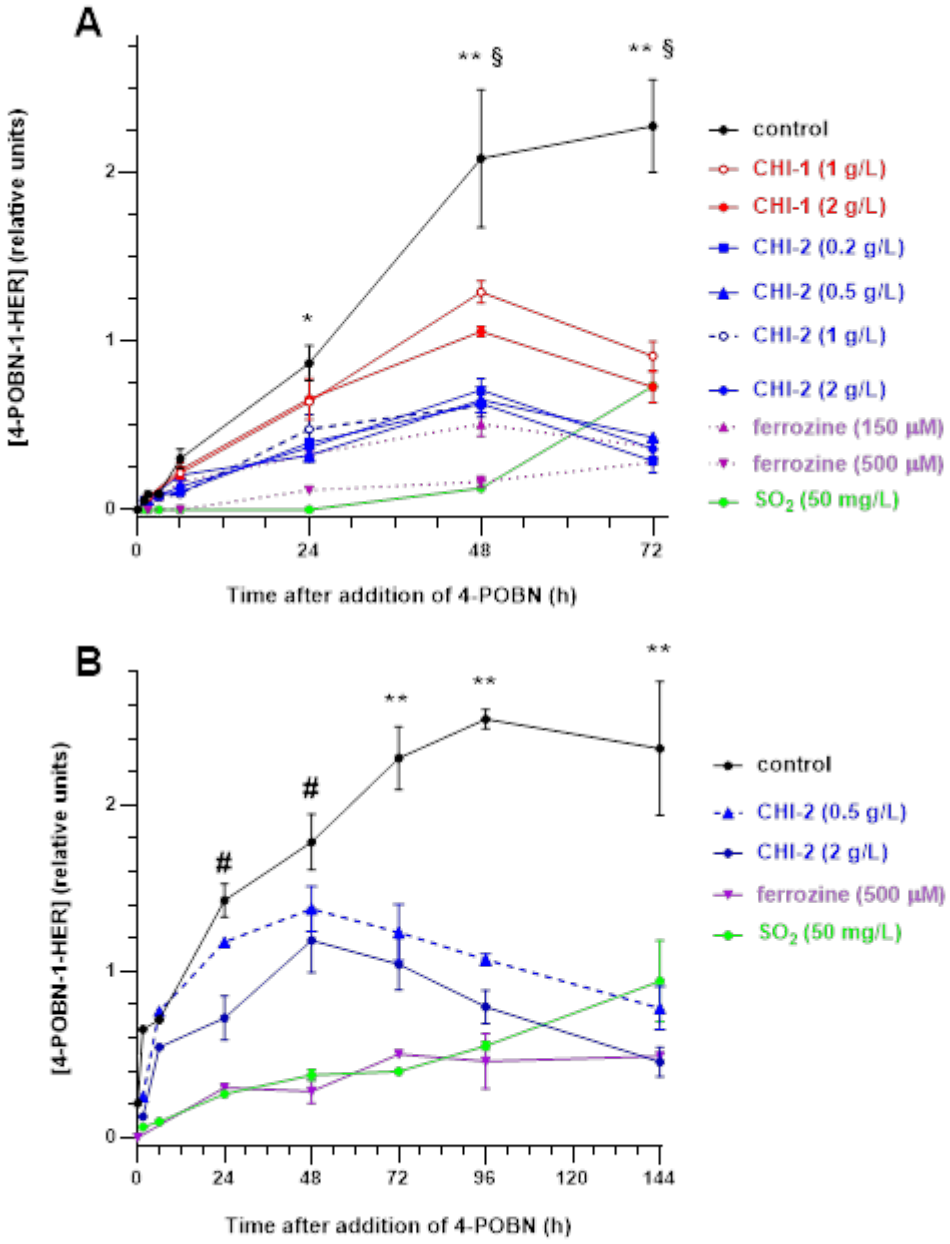

Figure 3.1.3. Effect of treatments on 4-POBN-1-HER spin adduct formation at room temperature during oxidation under air of (**A**) model wine, and (**B**) SO_2 free Chardonnay wine. Treatments and 100 µM of Fe(II) as the oxidant were first applied for 48 h, followed by addition of 4-POBN (15 mM) alone (real wine) or with 1 mM of 4-methylcatechol (model wine). Continuous agitation was applied throughout. CHI, chitosan. Level of significance vs. control (by one-way ANOVA followed by Duncan test): (A): **p < 0.01 vs. CHI-2; *p < 0.05 vs. CHI-2; §p < 0.05 vs. CHI-1 (all at any dose); (B): **p < 0.01 vs. CHI-2 (any dose); $^{\#}$p < 0.05 vs. CHI-2 (2 g/L). Vertical bars represent SEM (n = 3–10).

EPR signals from all samples pre-treated with high ferrozine (500 µM) exhibited the lowest intensities throughout the incubation time frame (**Fig. 3.1.3.**). This is consistent with the results of the Fe(II) activity assay above suggesting that practically all of the 100 µM iron (II) added should have been complexed by ferrozine into a Fenton-inactive species. Moreover, from the IC_{50} values obtained by the ferrozine assay in model wine it was found that 168 µM ferrozine and 2.4 g/L CHI-2 exhibited similar chelating effects toward

100 µM of Fe(II). This could explain the similarity of 4-POBN-1-HER inhibition profiles between samples supplied with 150 µM ferrozine and those added CHI-2, but not CHI-1 (**Fig. 3.1.3A**).

The extent to which pre-treating the real wine with CHI-2 had reduced the amount of catalytic Fe(II) before the spin trapping reaction shown in **Fig. 3.1.3B** was started was quantified by flame atomic absorption. The endogenous concentration of Fe in the wine was only of 6.8 $\pm$ 0.1 µM (both vintages combined). Following addition of 100 µM Fe(II) and incubation for 2 days in darkness, 98.9 $\pm$ 1.1 µM of iron was detected, with a small loss consequent to, e.g., adsorption onto the labware or wine proteins, or chelation by tartaric or citric acids. In the presence of CHI-2 at 0.5 and 2 g/L, the free iron content of the wine samples was significantly decreased to 48.5 $\pm$ 0.4 and 31.2 $\pm$ 0.8 µM, respectively.

It is therefore possible that part of the effect of chitosans found in the above EPR experiments may be due to Fe(II) chelation properties. Chelation capacity of chitosan in oenology has already been reported (Bornet & Teissedre, 2008; Chinnici et al., 2014; Colangelo et al., 2018). Since for these compounds metal removal is based on the formation of a complex involving amine or hydroxyl groups (**Fig. 3.1.1**), chelation capacity increases with increasing degree of deacetylation and decreasing molecular weight as a consequence of greater availability of amino groups toward metal ions (Bornet & Teissedre, 2008). These structural features may explain the lower effectiveness of CHI-1 versus CHI-2 (**Fig. 3.1.3A**). Furthermore, chitosan can adsorb polyphenols into its matrix, decreasing their level in wine (Chinnici et al., 2014; Spagna et al., 1996). Hence, such a decrease in the 4-MeC (model wine) or oxidizable polyphenols (real wine) contents would indirectly inhibit spin adduct formation by lowering H_2O_2 levels.

3.1.3.2. Mechanistic understanding of the inhibitory effect of chitosan

- ***Quantification of H_2O_2 levels occurring during wine oxidation***

In the above experiments the relative EPR intensities were found similar over the incubation time frame regardless experiments were performed in 4-MeC-supplemented model or real wine (**Fig. 3.1.3.**). To estimate the H_2O_2 concentrations implicated, a Fenton assay was run in unsupplemented model wine by measuring 4-POBN-1-HER levels, obtained using an identical temperature and acquisition protocol, as a function of Fe(II) and H_2O_2 constituents. As seen in **Fig. 3.1.4**, spin adduct concentration, which increased with any of these two constituents, was more dramatically affected upon doubling iron(II) content than when H_2O_2 concentration was increased 10 times. This substantiates herein and previous findings (Bornet & Teissedre, 2008; Elias et al., 2009b; Kreitman et al., 2013b) that decreasing metal ion content in wine may be a more sustainable strategy against oxidation than temporarily scavenging H_2O_2 by adding SO_2. Furthermore, in completely filled and stopped capillaries, 4-POBN-1-HER intensities only moderately augmented 10 min versus ~2 min after triggering the Fenton reaction, and consequently the generation/detection system run here can be considered as a controlled one.

As seen in **Fig. 3.1.3**, accumulation of long-lived 1-POBN-1-HER adducts resulted in average EPR intensities peaking at ~2.5 relative units in the controls when 100 μM Fe(II) was used to start oxidation. According to the results of **Fig. 3.1.4** where H_2O_2 was added at once in the system, this suggests that the total H_2O_2 concentration decomposed by the Fenton reaction over 3–6 days under wine oxidation conditions was very low, ranging 0.25–2.5 μg/mL (7–75 μM), as visualized by the dashed line in **Fig. 3.1.4**. Fenton generators commonly used in wine oxidation spin trapping studies involve similar Fe(II) concentrations but at least fourfold higher H_2O_2 concentrations (Elias et al., 2009a; Nikolantonaki et al., 2019). Obviously, the EPR spin trapping technique applied here underestimates H_2O_2 levels produced in wine oxidation because a variety of scavenging mechanisms are operating, e.g., reactions with SO_2. Thus, in a set of red wines oxidized in air at 40°C using 100 μM Fe(II), a rate of H_2O_2 formation of ~14 μM/30 min was reported (Héritier, Bach, Schönberger, Gaillard, Ducruet & Segura, 2016).

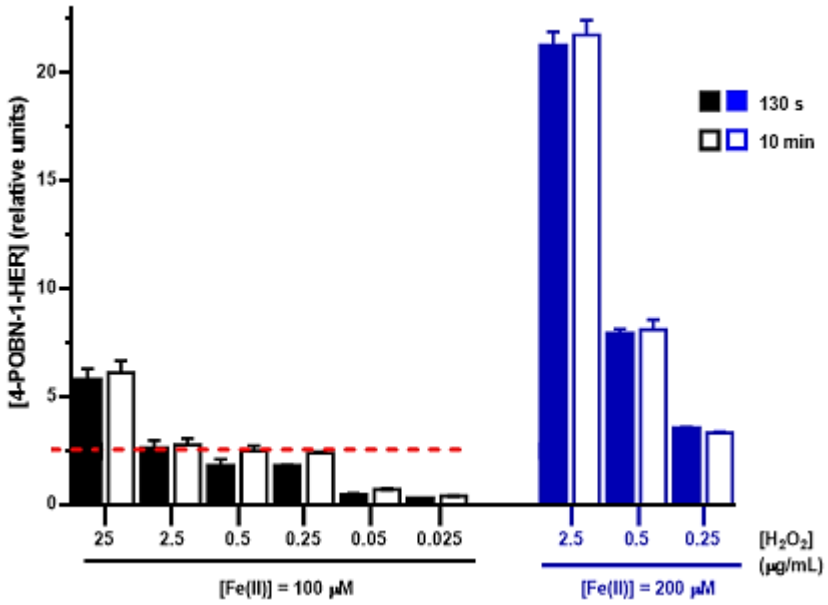

Figure 3.1.4. Effect of varying H_2O_2 on the EPR signal detected in model wine 130 s (filled bars) or 10 min (empty bars) after induction of a Fenton reaction in the presence of wine-like concentrations of iron(II). The dashed line visualizes the maximum of spin adduct levels obtained in incubations (**Fig. 3.1.3**). Vertical bars represent SEM (n = 3).

- ***Effect on Fenton-derived 1-HER***

Having defined the combination of 100 μM Fe(II) + 2.5 μg/mL H_2O_2 (74 μM) as a wine-like Fenton system to model incubations of **Fig. 3.1.3A**, it was applied in model wine ± 4-MeC (1 mM), alone or in the presence of inhibitors, and the effects on 4-POBN-1-HER formation were monitored for up to 1 h. In unsupplemented medium EPR signals, which expectedly increased along with the continuous formation of HO• radicals, showed a 2.5–3.5 amplification in the presence of 4-MeC (**Fig. 3.1.5A**). A similar effect has been reported by Elias and Waterhouse (2010) who suggested that the recycling of Fe(III) to Fe(II) by 4-MeC may increase adventitiously the amount of 1-HER available for spin trapping (**Fig. 3.1.1**). The control EPR signals in **Fig. 3.1.5A** were decreased up to 89% (unsupplemented) or 95% (with 4-MeC) in samples pre-incubated with

CHI-2 suspensions (0.5 or 2 g/L) for 2 days, with no clear dose-response effect. In these experiments background 4-POBN-1-HER adducts were detected in SO_2 added samples up to 30 min.

- ***Effect on photochemically generated hydroxyl radicals***

Having investigated the iron(II) chelating property of chitosans as a key step of the inhibition of HO•-mediated wine oxidation, additional spin trapping experiments were carried out in attempt to delineate the specific HO• scavenging behaviour of these compounds. Previous EPR investigations of the antioxidant properties of chitosan have generally focused on hydrosoluble derivatives and standard assays, including Fenton reaction-based tests on HO• scavenging (see, e.g., (Park, Je & Kim, 2003)) for which there is clear interference with Fe(II) chelation property. Here, an iron independent method for producing HO• spin adducts was used, where in situ photolysis of 3% H_2O_2 in the presence of the cyclic nitrone DMPO (ca. 3 mM) in 0.5% acetic acid solution (pH 3.2) afforded the known DMPO/hydroxyl radical adduct (DMPO-OH), giving a characteristic 1:2:2:1 EPR quartet with $a_N = a_H = 14.95$ G and $g = 2.0053$ (**Fig. 3.1.2B**).

Both chitosans, soluble in the medium up to 2 g/L, dose-dependently inhibited the formation of DMPO-OH. Using the kinetic analysis of (Finkelstein et al., 1980) plots of I_0/I against concentration were obtained (see Methods), exhibiting satisfactory linear fits (**Fig. 3.1.5B**). Assuming an average molecular weight of 120 and 20 kDa for CHI-1 and CHI-2, respectively, second-order rate constants for the reaction of HO• were calculated as 7×10^{12} and 10^{12} $M^{-1}.s^{-1}$ for CHI-1 and CHI-2, respectively. Such high values, reflecting diffusion-controlled processes, have been reported for many macromolecules, including proteins (Bailey et al., 2014). Using pulse radiolysis, a technique more specific for determining HO• rate constants, a value of 6.3×10^8 $M^{-1}.s^{-1}$ has been reported for deacetylated chitosan from a crustacean, krill (*Euphausia superba*) at pH 3 (Ulanski & von Sonntag, 2000).

As displayed in **Fig. 3.1.1**, molecular mechanisms for HO• scavenging by chitosan backbone can involve either free amine groups and/or their ammonium derivatives, or typical H-abstraction reactions along the polysaccharide unit (Xie, Xu & Liu, 2001). Moreover, earlier EPR and pulse radiolysis studies have revealed a low selectivity for H-abstraction onto the chitosan unit, i.e., these compounds would behave as if a single preferred site was submitted to HO• attack (Ulanski & von Sonntag, 2000). This could explain the linear variations of **Fig. 3.1.5B**, with intercepts of 1.1 and 1.5 for CHI-1 and CHI-2, respectively, close to the theoretical value of 1 in the kinetic model of (Finkelstein et al., 1980).

In another approach to discriminate between iron chelation and free radical scavenging in the inhibitions seen in **Fig. 3.1.3** the ORAC-fluorescein values were calculated for tested wines. This method, which measures the scavenging efficacy against a peroxyl radical formed by thermal scission of an azo initiator, AAPH, has been widely applied to assess the antioxidant capacity of wine (Sánchez-Moreno, Cao, Ou, & Prior, 2003; Stockham et al., 2013), a high ORAC showing a better antioxidative power. In this study the ORAC found for

experimental wine, typical of Chardonnay wines (Sánchez-Moreno et al., 2003; Stockham et al., 2013), is yet twice as low as that for 4-MeC containing synthetic wine (**Table 3.1.1**). If radical scavenging was the dominant mechanism this would lead in principle to lower levels of 4-POBN-1-HER in the model vs. the real wine in the incubation controls with no added iron chelator. To estimate these levels the area-under-curves (AUC) of **Fig. 3.1.3A** were calculated and the expected [2 (real wine):1 (model wine)] ratio was obtained in control experiments only during the first incubation day, consistent with differences in ORACs (**Table 3.1.1**). Therefore, further decrease of this ratio found up to day 3 may suggest a shift from dominant radical scavenging to other inhibitory mechanisms, e.g., delayed Fe(II) chelation by 4-MeC or other wine phenolics. In samples incubated with CHI-2 (2 g/L) the same AUC analysis, yielding expected lower values, also demonstrated a nearly constant 2:1 ratio. This suggests that, once iron had been removed by the 2-days pre-treatment, it is the antioxidant property of chitosan that could have caused the lower spin adduct levels observed. It is worth mentioning, however, that good correlations between ORAC assay and EPR spin trapping have been only reported for peroxyl, but not hydroxyl radicals (Kameya, Watanabe, Takano-Ishikawa & Todoriki, 2014).

- ***Effect on cupric and ferric ions***

Adding copper, as Cu(II), during the winemaking process is a common practice, in particular to decrease the levels of sulfur containing compounds responsible for off-flavors before bottling of white wines. Since cupric ions can catalyze H_2O_2 degradation by a Fenton-like mechanism (Hanna & Mason, 1992), they would potentiate the effect of Fe(III) in wine oxidation (Danilewicz, 2003). In wine studies, however, use of spin trapping is complicated because Cu(II) often induce degradation of nitrones into unwanted nitroxides and/or lead to paradoxical 1-HER formation profiles (Elias et al., 2009b).

In this study EPR has been used to assess indirectly the effect of chitosans on iron and copper at wine-like concentrations, by measuring the impact on nucleophilic induced spin adduct formation. Thus, by forming a transient complex at the nitronyl oxygen of spin traps such as DMPO or its phosphorylated analog, DEPMPO, Fe(III) or Cu(II) catalyze the nucleophilic addition of water to form the corresponding hydroxyl radical adduct, and this reaction is inhibited by Fe(III) (Culcasi et al., 2006a) and Cu(II) (Hanna & Mason, 1992) chelators. When an aqueous solution of DEPMPO (55 mM), a chiral molecule (**Fig. 3.1.2**), was added to a mixture of wine-like 30 mg/L Fe(III) and 12.5 mg/L Cu(II), a major 8-lines EPR spectrum was observed (**Fig. 3.1.2C**). A satisfactory fit was obtained assuming a mixture of EPR-distinguishable diastereoisomeric DEPMPO/hydroxyl radical adducts (DEPMPO-OH) with the following parameters (couplings in G): *cis*-DEPMPO-OH (a_N = 14.05, a_P = 47.25, a_H = 14.13), and *trans*-DEPMPO-OH (a_N = 14.05, a_P = 47.22, and a_H = 12.73), and g = 2.0057. A minor carbon-centered DEPMPO adduct was also detected (a_N = 14.32 G, a_P = 45.91 G, a_H = 21.36 G), possibly due to some degradation of the trap by Cu(II), and accounting for 24% of the total signal. The *cis*:*trans* ratio in **Fig. 3.1.2C** was of 36:64, consistent with a HO• scavenging-unrelated, nucleophilic addition mechanism

(Culcasi et al., 2006b). When metal added solutions were stirred for 1 h at ambient temperature in the presence of varying amounts of chitosan, further addition of DEPMPO to the suspensions led to dose dependently decreased DEPMPO-OH levels, by 73–92%, with no differences between chitosans (**Fig. 3.1.5C**). Altogether these results demonstrated sequestration of Fe(III) / Cu(II) as another facet of the inhibitory action of chitosan in wine oxidation. Accordingly, chitosan has been shown to adsorb iron from wines spiked with ferric ions (Bornet & Teissedre, 2008).

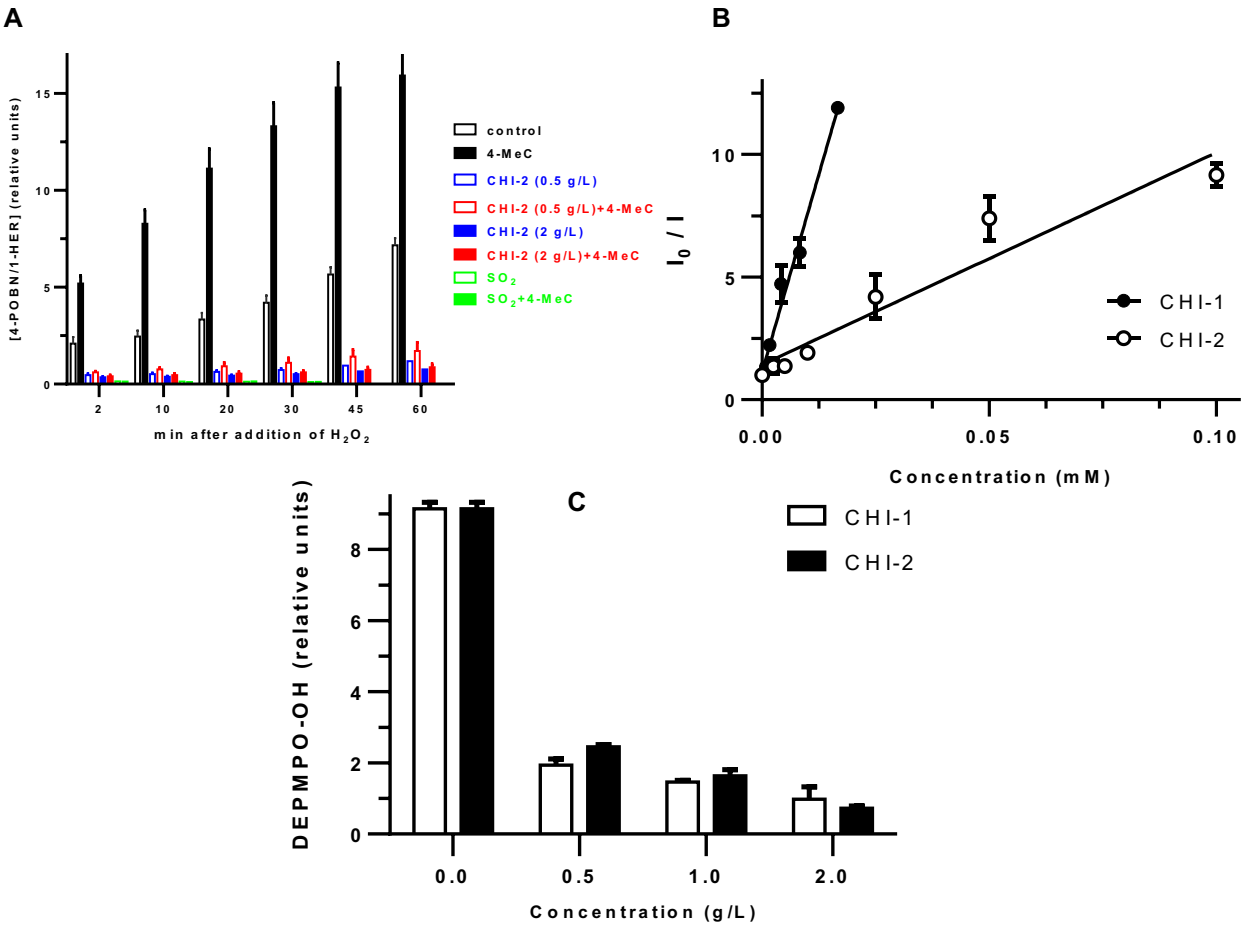

Figure 3.1.5. Assessment by EPR using various spin traps of the inhibitory effect of chitosan (CHI) on some potential mechanisms involved in wine oxidation. (**A**) Effect on [100 µM Fe(II)/ 2.5 µg/mL H_2O_2] Fenton reagent in model wine; (**B**) Determination of apparent rate constants for direct hydroxyl radical scavenging in acidic water (pH 3.17). I_0, I: intensity of EPR signal in unsupplemented and test sample, respectively. Concentrations are estimates from mean molecular weights; (**C**) Inhibition of metal-catalyzed nucleophilic addition in water, in the presence of a wine-relevant metal concentration [30 mg/L of Fe(II) + 12.5 mg/L of Cu(II)]. Nitrones used were: (**A**) 4-POBN (15 mM); (**B**) DMPO (3.33 mM); and (**C**) DEPMPO (55 mM). Samples in A and C were pre-incubated with metals as described in the legend of **Fig. 3**. 4-MeC, 4-methylcatechol at 1 mM. Vertical bars represent SEM (n = 3).

- ***Effect on photooxidation-induced acetaldehyde formation***

To substantiate the effect of CHI-2 seen in **Fig. 3.1.3B** the production of acetaldehyde was monitored by HPLC-DAD in experimental wine spiked with 100 µM Fe(II) and irradiated with fluorescent light (300–580 nm) for up to 6 days at ambient temperature. Long term exposure to sunlight or fluorescent tubes has been shown to contribute to the development of browning and the formation of off-odors in white wine. In wine

conditions (pH and metals) a main proportion of carboxylic acids in wine, such as tartaric and lactic acids, exist as Fe(III) carboxylate complexes, the irradiation of which leads to a range of carbonyls, including acetaldehyde. This in turn will release free Fe(II), providing an additional source of catalytic iron to fuel the Fenton system, a forced oxidation mechanism termed as 'photo-Fenton' (Grant-Preece et al., 2017).

At opening, acetaldehyde concentration of experimental SO_2 free wine was 7.6 $\pm$ 0.7 mg/L (mean value from both vintages), falling within the lowest acetaldehyde concentrations reported in just finished, sulphited dry white wines (Jackowetz & de Orduña, 2013). Following initial production by yeasts during fermentation acetaldehyde can be further synthesized from ethanol through Fenton oxidative degradation (**Fig. 3.1.1**). Here this second source of acetaldehyde is likely poorly active since when the wine was stored for 2 days in darkness with a 1.5 times air volume in the headspace, a non-significant increase to 9.1 $\pm$ 0.2 mg/L was found, possibly because catalytic iron present in the wine was only ~0.4 mg/L. Wine samples added 5.5 mg/L Fe(II) and incubated in darkness for 2 days, which retained 5.4 mg/L iron after filtration, showed, however, non-significantly increased acetaldehyde levels of 10.8 $\pm$ 0.4 mg/L (**Table 3.1.1**). Under photo-Fenton conditions acetaldehyde in the controls increased significantly afterwards, doubling after 6 days irradiation. In wine samples spiked with 5.5 mg/L Fe(II) and having had their iron content lowered by 51% and 68% after 2 days in contact with CHI-2 at 0.5 and 2 g/L, respectively, this irradiation-induced elevation of acetaldehyde concentration was significantly inhibited, with decreases of 19% and 38%, respectively, at day 6 (**Table 3.1.1**). Being a strong binder for sulphur dioxide (Oliveira et al., 2011) free acetaldehyde expectedly exhibited the lowest concentrations in irradiated wine added SO_2 (50 mg/L). However, after 6 days of light exposure, once complete oxidation and/or binding of SO_2 was reached, acetaldehyde production in those samples was not statistically different from that in samples containing 2 g/L CHI-2 (**Table 3.1.1**) and therefore the acetaldehyde inhibition pattern in CHI-2 added wine paralleled that seen for 1-HER formation in **Fig. 3.1.3B**.

3.1.4. Conclusion

The results of this study strengthen current interest in using chitosan as a substitute for and/or complement to lower sulphur dioxide and suphites in winemaking. By monitoring the formation of spin trapped 1-HER, a pivotal intermediate of wine oxidation, EPR analysis sought to establish a chronology of chitosan antioxidant action under wine relevant doses, application and aging conditions. It was found that once the catalytic activity of the metal pool in wine, especially Fe(II)Fe(III), has been partly deactivated by chelation, direct scavenging of oxidizing species such as HO• continuing to form at slow rates may represent a significant inhibitory mechanism of chitosan. In this regard, the well documented metal ions-sensitive depolymerization of chitosan by H_2O_2 (Chang, Tai & Cheng, 2001) could be an additional protective effect against wine oxidation as depicted in **Fig. 3.1.1**. Studies are in progress to verify, using specific tests, if a related free radical-independent mechanism could participate in the effects seen in the present study.

Of note, the significant impact of chitosans against free radical formation seen here was obtained as the compounds were directly added in suspension in the finished wine. This will encourage designing future spin trapping studies using EPR techniques specific for large heterogenous samples to follow in situ oxidation of white musts during the alcoholic fermentation.

Table 3.1.1.
Radical scavenging activity and effect of treatments on spin adduct and fluorescent lightning induced acetaldehyde formation[a,b].

Sample and treatment	ORAC (µmol Trolox/mL)	AUC/ incubation time[c]						acetaldehyde (mg/L)			
		1 day		2 days		3 days		control	1 day	4 days	6 days
		Ctr	CHI-2 (2 g/L)	Ctr	CHI-2 (2 g/L)	Ctr	CHI-2 (2 g/L)				
MW[e]	not measurable										
MW + 4-MeC[f]	6.24 ± 0.27	11	5	47	18	99	31				
Chardonnay wine	3.18 ± 0.12	23	11	61	36	110	63	10.8 ± 0.4	14.1 ± 0.7***	16.2 ± 0.9***	21.6 ± 1.7***
+ CHI-2 (0.5 g/L)								10.0 ± 0.3	11.8 ± 0.5*§	13.0 ± 0.3*§	17.4 ± 1.2*§
+ CHI-2 (2 g/L)								9.6 ± 0.3§	10.3 ± 0.3+	11.5 ± 0.3*+	13.4 ± 1.0*+
+ SO_2 (50 mg/L)								5.0 ± 0.1§	5.4 ± 0.3+	6.7 ± 0.5*+	11.2 ± 0.8*+

Table 3.1.1 (continued)

[a].In the presence of 100 µM of Fe(II).

[b] Mean ± SEM (*n* = 3–5).

[c] AUC, area-under-curve (arbitrary units) calculated from the curves in **Fig. 3**. Ctr, unsupplemented sample.

[d] Before illumination, after 2-days incubation in darkness.

[e] Model wine (12% v/v, 8 g/L tartaric acid, pH 3.5).

[f] 4-methylcatechol at 1 mM.

Statistics: (*$p < 0.05$ and ***$p < 0.001$) vs. pre-illuminated control; ($^{§}p < 0.05$ and $^{+}p < 0.001$) vs. untreated wine after the same illumination time.

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Chapter 3.2.

"Insights on the effect of chitosan in the generation of aldehydes relevant to wine oxidation"

Chapter 3.2

Insights on the effect of chitosan in the generation of aldehydes relevant to wine oxidation

3.2.1. Introduction

Studies on chemical oxidation mechanisms in wines report that glyoxylic acid (Gly) and acetaldehyde (Ace) obtained from the oxidation of ethanol and tartaric acid respectively, are two of the main intermediates in the oxidative evolution of wine. These aldehydic products are able to cross-link flavanols such as (+)-catechin, leading to the production of yellow xanthylium cation pigments, which are thought to be intermediate products that react to more complex compounds, responsible of the wine browning of wines (Bührle, Gohl, & Weber, 2017; Es-Safı, Le Guernevé, Fulcrand, Cheynier, & Moutounet, 1999). Thus, controlling the development of aldehydic oxidation intermediates could be one of the strategies to reduce browning spoilage of wines.

On the previous chapter, antiradical mechanisms of chitosan against hydroxyl radical and 1-hydroxyethyl radical were outlined with satisfactory results. Stimulated by the intriguing results, and based on the previous studies on antibrowning capacity of chitosan (Chinnici, Natali, & Riponi, 2014; Spagna et al., 1996) , the aim of this work was to further deepen into the antioxidant mechanism of chitosan against browning oxidative spoilage in white wines, focusing, in particular, on the study of the generation of aldehydic oxidative intermediates. To this aim, glyoxylic acid and acetaldehyde were photolitically generated in both air saturated model wine solutions or sulphite-free white wines and monitored by means of high-performance liquid chromatography with diode array detection (HPLC-DAD). In addition, chitosan metal-chelation capacity, and H_2O_2 scavenging properties together with browning development, were also evaluated both in model or real white wines.

3.2.2. Material and methods

3.2.2.1. Reagents

All the reagents were of analytical grade provided by Sigma Chemical (St Louis, MO, USA). A 80-90% deacetylated chitosan of fungal origin (*Aspergillus niger*) having a 10-30 KDa was supplied by KitoZyme (Herstal, Belgium).

3.2.2.2. Development and analysis of glyoxylic acid and acetaldehyde

The development of oxidation-related aldehydes was monitored in both model wines and sulfite-free white wines. For model wine solution, a total of 2 L of a solution containing 5 g/L (+)-tartaric acid and 12% (v/v) ethanol was prepared. The pH was adjusted to 3.2 with 5 M NaOH before bringing to the mark progressively by adding water. The solution was stirred overnight at room temperature in open-air to reach oxygen saturation. Sulfites-free white wines from Chardonnay grapes, harvest 2017, were supplied by the Institut Oenologique de Champagne (IOC). White wines were centrifuged at 2400 g during 5 minutes before carrying out the experiments.

- ***Light exposure:***

Trials were arranged in triplicate by transferring aliquots (20mL) of model wine or white wine in 50 mL conical centrifuge plastic tubes. A total of 250 µL of an aqueous solution of Fe^{II} sulfate heptahydrate (1 g/L) was added to each tube to give a final concentration of 2.5 mg/L of Fe (II). When appropriate, 25, 50 and 100 mg/L sulfur dioxide (as potassium metabisulfite) and 0.2, 0.5, 1 and 2 g/L of chitosan were separately added, to constitute the following solutions: Control (Model wine or white wine), SO_2 (model solution or wine + SO_2), CSG (model solution or wine + chitosan). Because of chitosan insolubility, once closed, CsG samples were vortexed 2 min 4 times a day all along the experiment. Samples were stored 48 h at room temperature (20 ºC) in the dark before light exposure. After incubation in the dark, model matrixes were exposed to two Sylvania T8Luxline Plus F36 W/840 cool daylight fluorescent lamps for 24h and 48h at a distance of 5 cm and 2000 Lux of luminance. The light intensity was measured using a digital lux meter (Yogokawa, 51000 series).

Spectral distribution of light emitted by the fluorescent lamps was not assessed. However, commonly used "cool white" fluorescent lamps show emission peaks centered at approximately 313, 365, 405, 436, 546, and 578 nm and emission peaks with maxima at around 480 and 580 nm (Spikes, 1981)

Samples of white wines were exposed up to 10 days to study the long-term effect. In parallel, for comparison, a second experiment was carried out where, after incubation in the dark, white wines treated with different concentrations of chitosan were filtered with a 0.45µm nylon filter to guarantee the absence of chitosan during exposure to light.

- ***HPLC analysis of free fraction of glyoxylic acid and acetaldehyde***

Chromatographic analyses were performed with a Merck Hitachi HPLC System consisting of a LaChrom L-7000 interface module and a L-7455 photodiode array detector controlled by the EZChrome Chromatography manager software. The UV spectra were recorded in the range 220-400 nm. DNPH solutions were freshly prepared at 10 mM in 2.5 M HCL. Samples (800 µl) were filtered (0.45 µm nylon filters) and mixed with 200 µl of DNPH solution for 1 hour at 45 ºC in the dark. No hydrolysis of samples was carried out. Separation of the DNPH adducts was achieved at room temperature on a Nucleodur C18 Htec (250 x 4.6 mm; 5µm) column, with a flow rate of 0.8 $mL \cdot min^{-1}$. Solvent A was acetonitrile; solvent B was water containing 0.05% v/v of phosphoric acid (pH 2.7). The elution program was the following: 0 min, 40% A, 8 min, 85% A, 9 min, 40% A, 13 min, 40% A. Injection volume was 20 µl. The identification of the derivatized compounds was based on their retention time compared with those of standards, detected at 360 nm as well as their spectral characteristics. Quantification was performed by means of the external standard technique taking into consideration the peak areas.

3.2.2.3. Chelation effect of chitosan

Sulfite-free white wines were centrifuged at 2400 g during 5 minutes before carrying out the experiment. A 2.5 $mg \cdot L^{-1}$ of iron (II) was added to 20 mL of wine sample with different concentrations of chitosan (0.2-2 $g\ L^{-1}$) in 50 mL conical centrifuge plastic tubes. Samples were mixed and incubated in the darkness at room temperature without agitation. After 48 h of incubation, analysis of iron was carried out.

For the determination of iron, an Agilent 240FS AA model flame atomic absorption spectrometer with deuterium lamp background correction and an air acetylene burner was used. The instrumental parameters were adjusted according to the manufacturer's recommendations. An Fe hollow cathode lamp operating at 248.3 nm was used as the radiation source. Run blank consisting of 12 %(vol/vol) ethanol in deionized water to set zero absorbance. Fe was determined by using standard calibration curves.

3.2.2.4. Scavenging activity of H_2O_2

Reduction of H_2O_2 towards chitosan was evaluated by chemiluminescence with lucigenin. A 20 mL of wine model solution was added in 50 mL conical centrifuge plastic tubes with different concentrations of chitosan (0.2-2 $g \cdot L^{-1}$) and spiked with 100µm or 500 µm of H_2O_2. After 10 minutes of agitation, analysis was carried out. Lucigenin was dissolved in 0.2 M glycine buffer (pH 10) to a final concentration of 0.2mg/mL^{-1}. 20µl of each sample was incubated 10 minutes with 200µl of lucigenin solution in 96 wells. The chemiluminescence measurements were performed with a microplate reader Tecan Infinite M200 (Tecan, Männedorf, Switzerland). Concentration of H_2O_2 was calculated by extrapolation from a calibration curve performed with increasing amounts of H_2O_2. Reduction in the content of H_2O_2 was measured by comparing the signal of chitosan treated samples with a control without the presence of chitosan which corresponded to the

maximum chemiluminescence signal. Blank values were measured without H_2O_2. All measurements were performed in triplicate.

3.2.2.5. Browning development of samples

A 20 mL of wine model solution containing 100 mg·L^{-1} of (+)-catechin and 2,5 mg·L^{-1} of Iron II were added in 50 mL conical centrifuge plastic tubes with different concentrations of chitosan (0.2-2 g·L^{-1}) or SO_2 (25, 50, 100 mg·L^{-1}). After 48 h of incubation in the dark, samples were exposed at a distance of 5 cm to a light source of 2000 lux during 1, 2, 7, 10, 14 and 21 days. Browning development measurements were performed with a microplate reader TECAN Infinite M200 (TECAN, Männedorf, Switzerland) at 420 nm wavelength, to measure the contribution to colour given by xanthylium cations, the oxidation products of (+)-catechin responsible of browning development.

3.2.2.6. Statistics

Statistical analysis was performed using the XLSTAT Software package (Version 2013.2, France). One-way analysis of variance (ANOVA) followed by a post hoc comparison (Tukey's HSD test) were carried out. Graphics were made through GraphPad Prism (version 6.01, 2012). Data are presented as mean ± SD for the indicated number of independently performed experiments.

3.2.3. Results and discussion

3.2.3.1. Studies in model white wines

- ***Development of aldehydes during light exposure***

Under our chromatographic conditions both aldehydic intermediates (Gly and Ace) were detected. The generation of these intermediates is initially due to the oxidation of Fe (II) to Fe (III) and the subsequent production of hydrogen peroxide (Danilewicz, 2012). Via the Fenton chemistry, this latter generates the hydroxyl radical that successively oxidize tartaric acid and ethanol into the corresponding aldehydes. As previously reported by Grant-Preece at al., (Grant-Preece, Schmidtke, Barril, & Clark, 2017) in model wine solution only containing tartaric acid and ethanol (and not *o*-diphenols), an important role is played by tartaric acid since it can recycle iron (III) back to iron (II) by forming an iron (III) complex susceptible to be (photo)degraded to iron (II) and glyoxylic acid (**Figure 3.2.1**). The adoption of photo-Fenton conditions further could speed up the process thanks to the faster recycling of Fe(III) to Fe(II) and the additional generation of hydroxyl radical (Machulek et al., 2012).

While commenting results, it should be considered that our data only relates to the non-bounded fraction of aldehydes which is the one that actively participates to wine browning phenomena. This is particularly relevant for SO_2 added samples, being largely known the ability of sulfites to binds carbonyl compounds, impeding their involvement in further browning reactions (Danilewicz 2012). In control samples, the increase of aldehydes was directly related to the duration of light exposure. After incubation in the dark, in fact, none of the oxidation intermediates was detected (data not shown), while an exposition of 24 hours resulted to

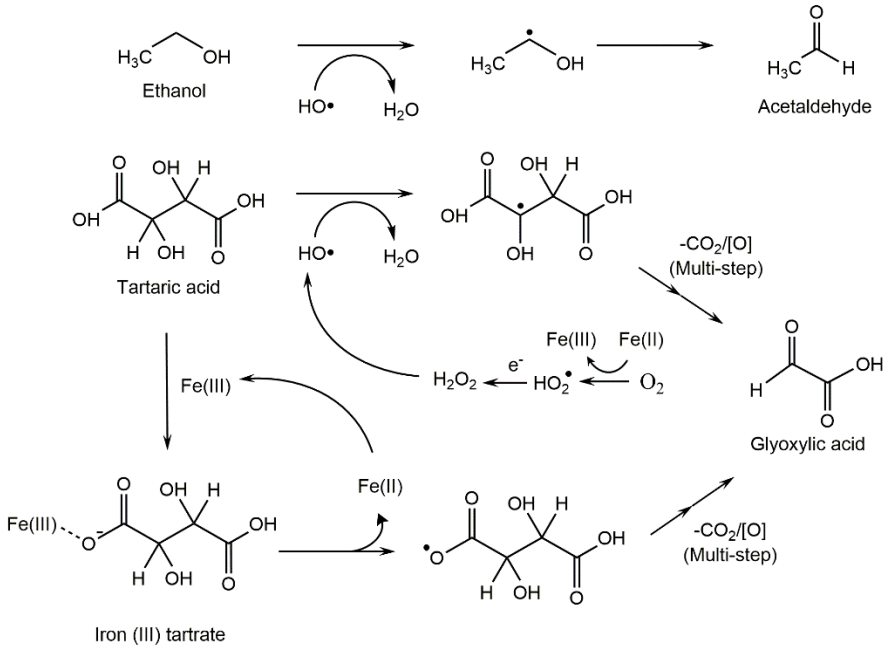

Figure 3.2.1. Mechanism of generation of glyoxylic acid and acetaldehyde in light exposed solutions lacking of o-diphenols, and role of tartaric acid. (Adapted from Grant-Preece et al., 2017)

be enough to stimulate the generation, in the control solution, of 1,5 mM of Gly and 0.3 mM of Ace (**Figure 3.2.2**). This result agreed with that obtained by Clark et al. (Clark, Prenzler, & Scollary, 2007) who detected glyoxylic acid only after direct sunlight exposure of model wines containing tartaric acid. As shown in **Figure 3.2.2**, after 24 h of irradiation, in samples treated either with CsG or SO_2, the level of free aldehydic intermediates was reduced when compared to the control. A dose-dependent effect was observed for CsG samples, where Gly amounts varied from 0.96 mM to 0.31 mM (corresponding to a reduction from 34% to 79% - **Table 3.2.1**-) for 0.2 $g \cdot L^{-1}$ and 2 $g \cdot L^{-1}$ of added CsG respectively. In SO_2 solutions, at 24h unbounded Gly content was independent from sulfite concentration, being about 0.22mM for all the treatments. This reduction of about 85% could be attributed to a combined effect of the additive, capable to scavenges H_2O_2 and binds aldehydes. Similar trends were observed for acetaldehyde, with amounts varying from 0.20 mM (reduction of 36%) at the lowest concentrations of CsG, to 0.08 mM (77% inhibition) at 2 $g \cdot L^{-1}$ and about 0.018 mM (92%) in all SO_2 treated solutions. Interestingly, in SO_2 samples, both aldehydes were reduced to a similar extent independently from the amount of sulphite added. This done could be linked to similarities in apparent equilibrium constants for hydroxysulfonates adducts (3.7×10^{-6} and 1.5×10^{-6} for Gly acid and Ace

respectively (Sonni, Moore, et al., 2011) suggesting that this specific inhibitory mechanism might prevails over H_2O_2 blocking mechanism in those samples.

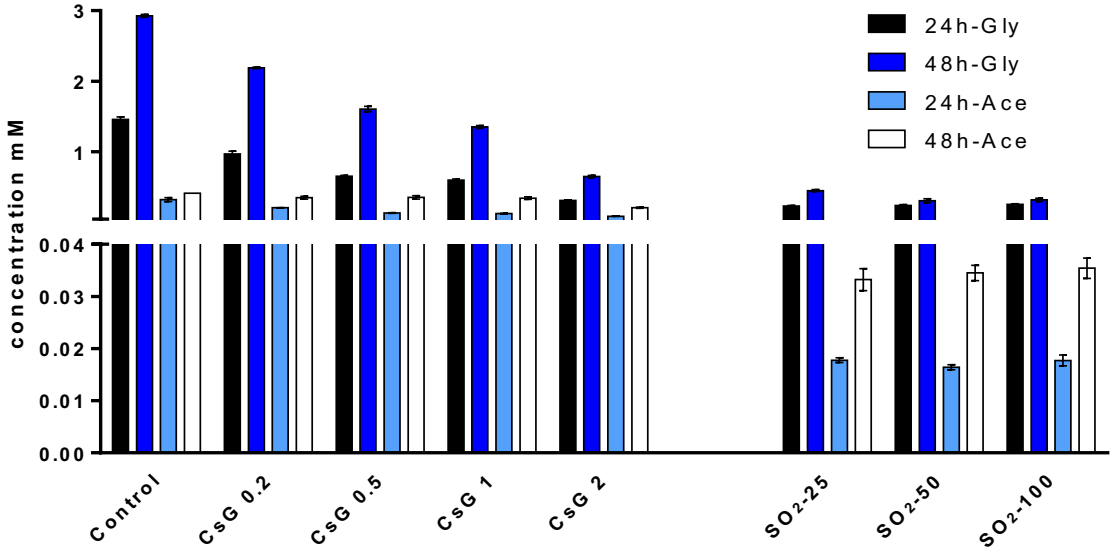

Figure 3.2.2. Unbound glyoxylic acid and acetaldehyde concentrations in model wines after 24h or 48h of light exposure, as affected by the presence of chitosan (Csg) or sulfur dioxide (SO_2) at increasing doses. Gly: Glyoxylic acid, Acet: Acetaldehyde

The evolution of the aldehydic intermediates after 48 h of light exposure is also shown in **figure 3.2.2**. The inhibitory effect of chitosan against the generation of Gly was maintained even if the % inhibition seemed to slightly decay at the lower doses **(table 3.2.1).** For Ace, the effect of chitosan was reduced over time and only the highest dose maintained an inhibition of about 50%. A different result was observed in samples treated with sulfur dioxide, where the unbound fractions of both Gly and Ace did not seem to significantly change with time or added amounts (the increase of acetaldehyde after 48h of light exposure - **figure 3.2.2** - is significant but small in magnitude **-table 3.2.1-**).

- ***Scavenging of H_2O_2***

As already mentioned, the scavenging of hydrogen peroxide (H_2O_2) is thought to be one of the mechanisms by which antioxidants exert their action with respect to the chemical oxidation of wines. For sulfites, this has been the subject of several researches (Danilewicz, 2007, Danilewicz 2012, Waterhouse and Laurie 2006). However, for what concern chitosan, information is scarce, especially at pH and for addition modes (e.g. without dissolution) relevant to wine. To get some insights on this matter, model wines containing increasing amounts of CsG, were added of H_2O_2 at two doses and the disappearance of hydrogen peroxide was monitored. At both the concentrations of hydrogen peroxide (100 and 500 µM/L), chitosan exerted a dose-dependent effect with 76% of scavenging at 2 $g \cdot L^{-1}$ of chitosan and 100 µM of H_2O_2 and 21.4% at 500 µM (**Figure 3.2.3**). Chien, Sheu, Huang, & Su, (2007) obtained somewhat higher scavenging activities, in both model solutions at pH 5 and apple juices but, in their work, chitosan was preventively dissolved in glacial acetic acid, in this way enhancing its specific surface.

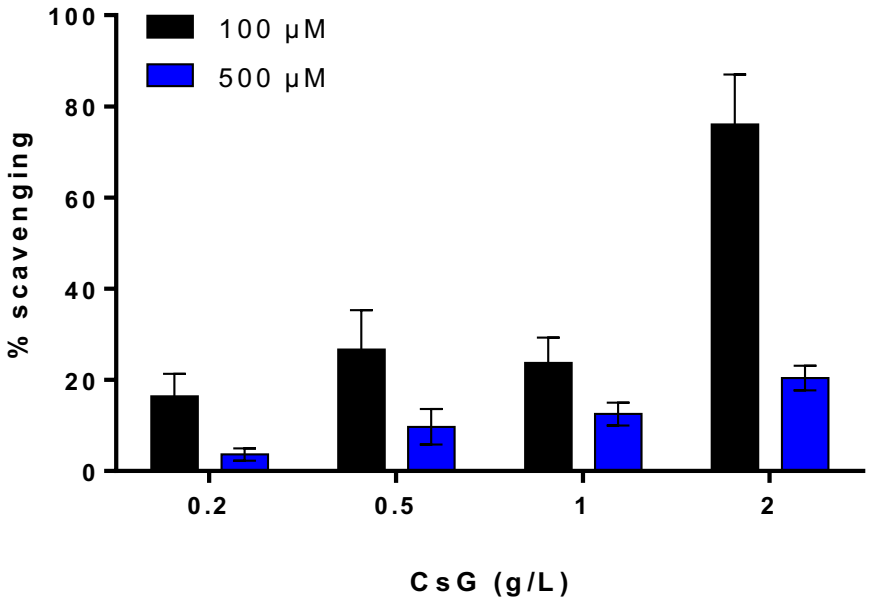

Figure 3.2.3. Scavenging effect of increasing doses of chitosan on hydrogen peroxide added in model wine solutions

Scavenging activity toward H_2O_2 and the resulting chitosan degradation, has been previously reported by Qin et al. (2002). As suggested by the authors, chitosan could scavenge H_2O_2 by an oxidative breakdown of the β-1,4 glycosidic linkages of the polysaccharide backbone. Decrease of chitosan MW, structural changes of the polymer and increased solubility are the effects of this activity. Our results further suggest that when concentration of H_2O_2 increased from 100 to 500 µM, the absolute amount of scavenged H_2O_2 was also increased (for samples at 2 g/L CsG, from 76 µM to 107 µM H_2O_2 respectively). This results agreed with those obtained by Chang et al., (2001) who further hypothesize that transition metals present in the medium may play a role.

- ***Browning development***

The tendency to develop oxidative browning of model wines containing (+)-catechin, as affected by the presence of chitosan or SO_2 was evaluated by measuring the optical densities at 420nm (A_{420}). As shown in **figure 3.2.4**, samples treated with both chitosan or SO_2 were variably protected against browning with respect to the control, where no treatment had been carried out. SO_2 proved to be more effective in the first 7 days of exposure to light due to its ability to reacts with oxygen radical species and to reduce back or quench *o*-quinones (Danilewicz, 2007). However, after 10 days, the samples treated with SO_2 showed a steady increase of brown nuances, largely higher than in those solutions containing chitosan.

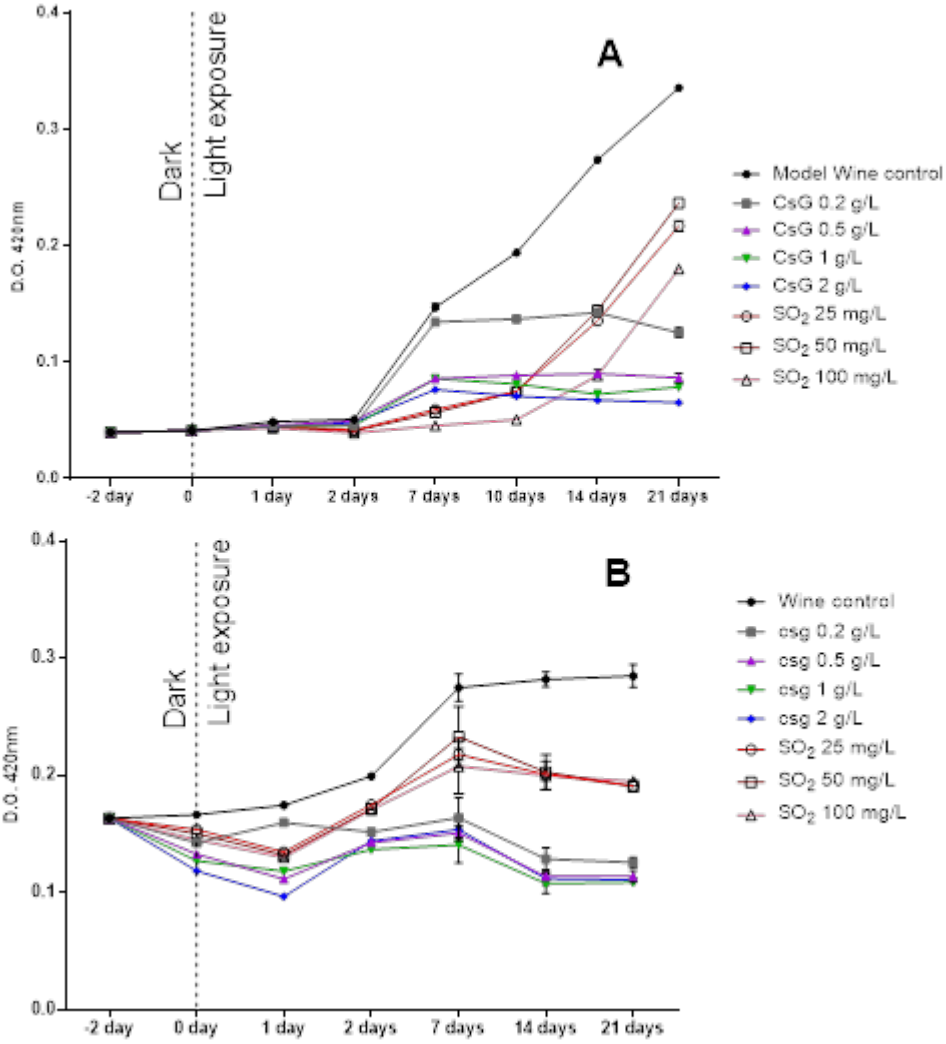

Figure 3.2.4. Browning development of model wines (A) white wines (B) exposed to light as affected by different concentrations of chitosan (CsG) or Sulfur dioxide (SO_2)

This trend, as outlined in chapter 1 as well, could be due to the complete oxidation of sulfites, since these would be oxidized by H_2O_2 generated after the oxidation of (+)-catechin, or reacts with ACE and/or GLY as already mentioned in the experiments of paragraph 3.1.1 For CsG samples and up to 7 days, browning followed a trend similar to the rest of treatments and control, with an increase in A_{420} inversely proportional

to the dose of chitosan used. However, at day 7 and onward, further oxidation was stopped as demonstrated by the plateau in the graph of **figure 3.2.4A.** Chinnici et al. (2014), also observed a lower browning tendency of model wines treated with $1g \cdot L^{-1}$ of chitosan, claiming that up to 70% of the xantylium yellow pigments generated during (+)-catechin oxidation and relevant percentages of intermediate dimers may absorbs onto the polymer surface. Based on these assumptions, hence, our done is probably the result of an initially faster oxidative process (that, mediated by ACE and GLY, generates carboxymethine dimers and brown pigments) followed by a subsequent phase were the progressive accumulation of dimeric intermediates and pigments is counterbalanced by their absorption on CsG.

3.2.3.2. Studies in sulphite-free white wines

- ***Development of aldehydes during light exposure***

When applied to white wines, photo-Fenton oxidation produced a remarkably lower amounts of aldehydes if compared to model solutions (compare sample "control" in **figures 3.2.2** and **3.2.5**). This could be due to the articulate composition of real wines, where molecules with reducing or antiradical features, such as phenolic acids or flavanols, may have quenched a portion of oxidizing species from the medium, decreasing the extent of ethanol or tartaric acid oxidation. In wines, both the additives under investigation exerted a reduced impact on the presence of aldehydes. Indeed, once in white wine, sulphur dioxide could binds several molecules such as monosaccharides, carbonyls or phenolic compounds, in this way reducing the free (e.g. antioxidant) fraction of this additive (Oliveira, Ferreira, De Freitas, & Silva, 2011). Similarly, chitosan may interact with other wine components not present in the model solution, such as polyphenols, proteins, sugars, and organic acids, which could interfere in the net charge of the outer layer of its crystalline structure. Overall, in real wines chitosan demonstrated to be more effective in preventing the generation of Gly rather than Ace (**Figure 3.2.5** and **table 3.2.1**). In fact, at 2 g/L and after 48h of light exposure, the amount of free Gly in CsG samples was comparable or slightly lower than what found in wines added of 100 mg/L of SO_2. Further, when light exposure was prolonged up to 10 days, glyoxylic acid clearly increased in all the wines (**Figure 3.2.5**) but, once more, chitosan at the highest doses guaranteed a significantly better control of its generation when compared to SO_2 wines. In these latter samples, Gly dramatically increased after 10 days under photo-Fenton environment, probably because of the almost complete oxidation of sulphur dioxide. Regarding acetaldehyde, as already commented for model solutions, sulphites had the highest capacity in limiting the presence of the unbound form of such carbonyl compound, accomplishing reductions of about 60% also after 240 h of light exposure while CgS diminished its efficacy with time, reaching reduction as low as 16%.

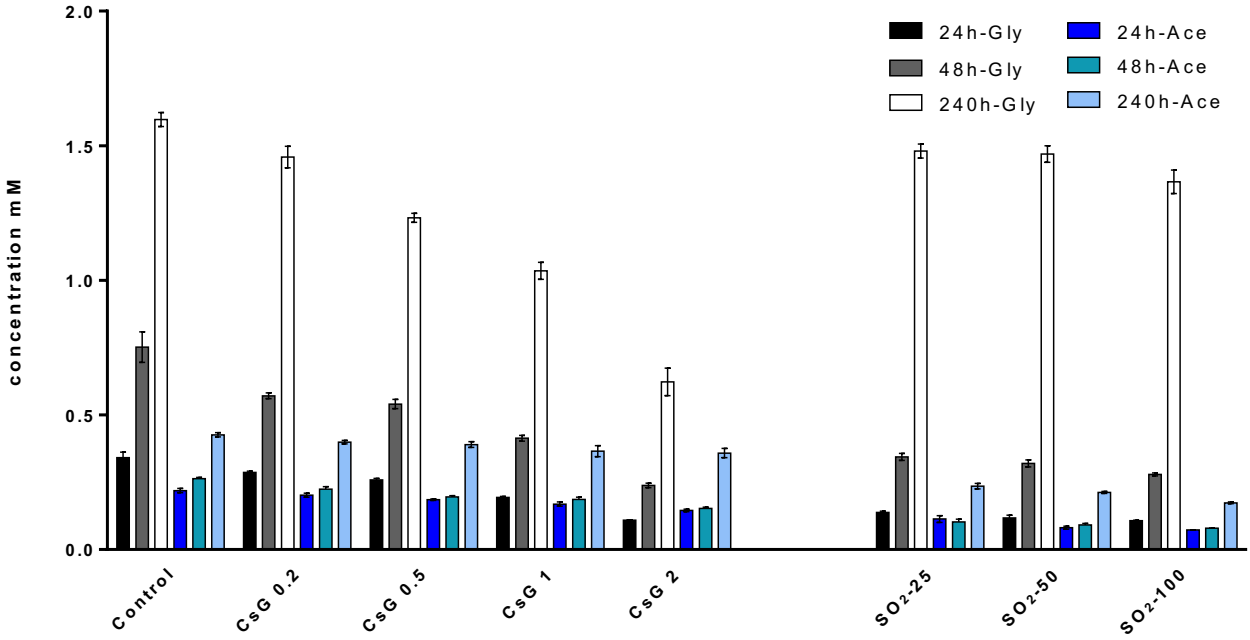

Figure 3.2.5. Unbound glyoxylic acid and acetaldehyde concentrations in sulfite-free white wines after 24h or 48h of light exposure, as affected by the presence of chitosan (Csg) or sulfur dioxide (SO_2) at increasing doses. Gly: Glyoxylic acid, Ace: Acetaldehyde

Chitosan is claimed not to dissolve in wine. However, it seemed interesting to investigate whether the effects of its addition on oxidative mechanisms may last also once removed from the medium. Hence, an experiment was conducted where the wine was treated with chitosan for 48 hours in the dark and then filtered to remove Csg before the exposure to light.

In control wines, after 24 hours of exposure, Gly and Ace were quantified to be 0.65mM and 0.27mM respectively (**figure 3.2.6**), values higher than what we found in previous experiments (see control sample of **figure 3.2.5**). This is probably a consequence of the forced aeration caused by wine filtration. Even without the presence of chitosan during light exposure, glyoxylic acid was reduced from 33% to 46% depending on the CsG initial amount. Surprisingly, for the lowest dose (0.5 g/L), these figures are almost the same found in samples containing the polysaccharide **(table 3.2.1)** and may suggest that, in those conditions, the inhibition is only marginally due to the actual presence of chitosan during the oxidation. One hypothesis is that at low CsG amounts, iron chelation that occurred before light exposure might have prevailed over other antioxidant mechanisms. Further insights on chitosan chelating properties are given in a next paragraph. At higher CsG doses, the inhibition on the generation of Gly slightly increased **(figure 3.2.6)** but, as expected, it was largely lower than when in the presence of chitosan **(figure 3.2.5),** in this case demonstrating the active antioxidant role of chitosan when present at concentration >0.5 g/L. For what concern Ace, the pretreatment of wines with 1 or 2 g/L of chitosan reduced the subsequent generation of such aldehyde up to the same concentration (about 0.20 mM), after both 24 and 48 hours. This corresponds to inhibitions ranging from 20% to 32% at 24 and 48 hours respectively, independently from the amount of added chitosan. These data seem to further corroborate the hypothesis that the kinetics underlying the inhibition of Gly and Ace by Csg are not the same

since, as opposed to Gly, for Ace the metal chelation acted by chitosan before the light exposure, did not appear to be directly reflected by the aldehyde residual levels in wines.

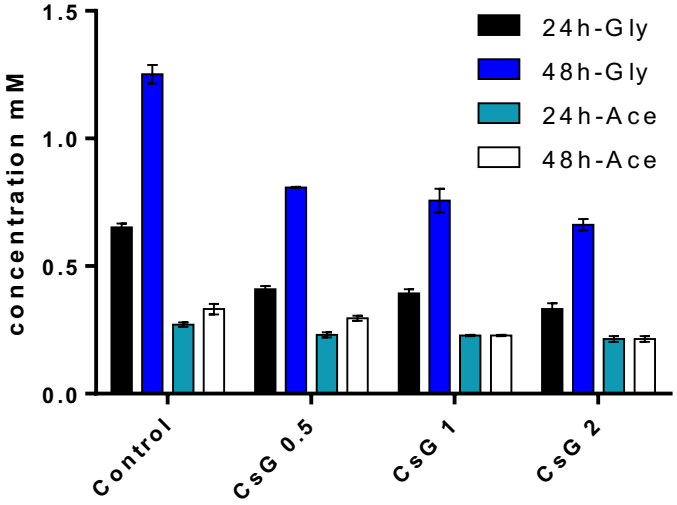

Figure 3.2.6. Free glyoxylic acid and acetaldehyde concentrations in white wines pre-incubated with chitosan at increasing doses (chitosan was removed by filtration before light irradiation)

- ***Browning development***

The browning development of the wines used for experiments of paragraph 3.1 was investigated. **Figure 3.2.4B** shows that, during the 48 hr of incubation in the dark, the presence of sulfites or chitosan reduced the yellow color of wines. This is not surprising since the bleaching properties of SO_2 have already been reported (Bradshaw, Scollary, and Prenzler, 2004). For Csg, instead, the evidence is certainly related to the absorption of phenolics and brown pigments that the adjuvant has (Chinnici et al. 2014; Spagna et al. 1996). The discolouring effect of chitosan is proportional to its amount and, at least at higher doses, significantly higher than sulphites. Once submitted to photo-fenton conditions, the control wine started to get browner while, similarly to what we found in model solutions (**Figure 3.2.4A**), csg samples developed little or no brown nuances up to 21 days, especially when added of > 0.5 g/L of the polysaccharide. In real wines the effect of sulphur dioxide appeared to be less effective than in model wines as, since the very first day, it was not comparable to the one exerted by chitosan. As mentioned before, this behaviour is likely due to the presence in wines of quinones, phenols, sugars and carbonyls that may quench or oxidize sulphites, diminishing their anti-browning efficacy.

- ***Iron chelating capacity of chitosan***

A final experiment was conducted on metal chelating capacity of chitosan. Results confirmed that, after incubation in the darkness, wines treated with chitosan had a reduced iron content also as a function of the dose of chitosan. These reductions varied from 38% at 0.2 $g \cdot L^{-1}$ to 66% in samples treated with 2 $g \cdot L^{-1}$ (**Figure 3.2.7**). Results roughly agreed in magnitude with those already obtained by Bornet & Teissedre, (2007) and Chinnici et al. (2014). Two different mechanisms of interaction between chitosan and iron have been

reported: 1) adsorption onto the surface of insoluble chitosan, involving a complexation carried out by hydroxyl groups (Wu, Tseng, & Juang, 2010) or 2) deposition of metal hydroxide into the pores of chitosan particles (Park, Park, & Park, 1984). Interestingly, Gyliene and co-workers (Gyliene et al., 2014) pointed out that Fe(II) chelation by chitosan always drives to relevant consumption of dissolved oxygen, needed to oxidize iron ions to Fe(III). According to these authors, in fact, this latter is the only Fe form sorbed by the polymer and, in wine like conditions, the reduced amount of oxygen would further contribute in limiting the chemical oxidation of wines. This subject is currently under further investigation in our laboratory.

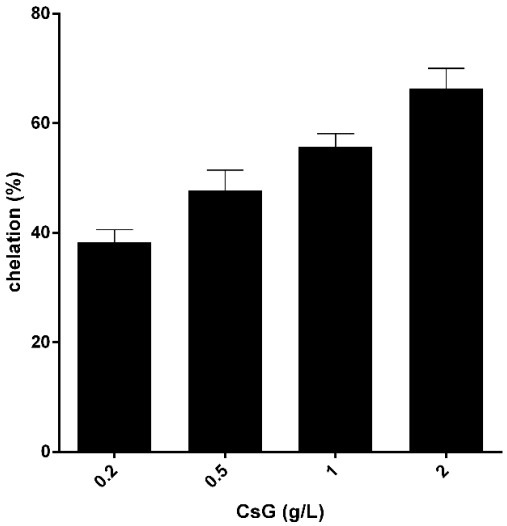

Figure 3.2.7. Chelation effect of different concentrations ($g \cdot L^{-1}$) of chitosan in sulfite free white wines

3.2.4. Conclusion

Overall, on this work it has been demonstrated that chitosan may reduce the production of aldehydes related to wine oxidation. Inhibitions percentages as high as 78% were found for glyoxylic acid, while acetaldehyde was somewhat less affected by the polymer, especially in real wines. Particularly if added at >0.5 g/L, Csg significantly reduced the oxidative browning of samples to a comparable or even higher extent than 100 mg/L of sulphur dioxide. Further, the action of chitosan on oxidation and browning was found to persist also after the removal of the polysaccharide. Apart from aldehyde reduction, we gave evidence that these effects may be attributed to a series of concurrent mechanisms such as: i) phenolic absorption, ii) metal chelation iii) scavenging of H_2O_2 and as obtained in chapter 1, quenching properties of chitosan against hydroxyl and 1-hydroxyethyl radicals.

	Gly-24h		Gly-48h		Gly-240h	Ace-24h		Ace-48h		Ace-240h
	Model	Wine	Model	Wine	Wine	Model	Wine	Model	Wine	Wine
CsG 0.2	34 e	16 de	25 f	24 de	9 de	36 d	8 e	16 c	15 de	6 d
CsG 0.5	55 d	24 d	45 e	28 de	23 c	60 c	15 de	15 c	26 cd	8 cd
CsG 1	59 c	43 c	54 d	45 cd	35 b	62 c	23 cd	17 c	29 cd	14 cd
CsG 2	79 b	68 ab	78 c	68 a	61 a	77 b	34 cd	50 b	42 bc	16 c
SO_2-25	84 a	59 b	85 b	54 bc	7 e	94 a	48 b	92 a	61 ab	45 b
SO_2-50	83 a	65 ab	90 a	57 bc	8 de	95 a	63 a	92 a	65 ab	50 b
SO_2-100	83 a	69 a	89 a	63 ab	14 d	94 a	67 a	91 a	70 a	59 a

Table 3.2.1. Reduction (%) in the presence of free glyoxylic acid (Gly) and acetaldehyde (Ace) in model solutions (Model) and wines as affected by the presence of chitosan (CsG) or sulfite (SO_2) and light exposure (24, 48 and 240 hours). In the same column, different letters indicate significant differences at p<= 0.05 (n=3)

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Chapter 3.3

"Interaction of chitosan with other antioxidant preservatives during the oxidative evolution of model white wines"

Chapter 3.3.

Interaction of chitosan with other antioxidant preservatives during the oxidative evolution of model white wine

3.3.1. Introduction

The main alternatives to the addition of sulfites in winemaking have been listed in section 3 of the introduction. Glutathione (GSH) and ascorbic acid (AA) have been described for their efficiency to control the oxidative development of colour by limiting the formation of coloured condensation products such as xanthyllium cations (Barril, Clark, & Scollary, 2012; Sonni, Clark, Prenzler, Riponi, & Scollary, 2011). However, for a number of reasons already mentioned in the same section, none of them is able to completely substitute the use of SO_2 in wines.

On previous works (chapters 1 and 2), antiradical and antioxidant properties of chitosan in wine-like conditions were investigated. Based on the results, several mechanisms were proposed, such as metal chelation, quenching of ·OH and 1-HER radical (chapter 1), scavenging of H_2O_2, inhibition of the generation of aldehydic intermediates of oxidation (chapter 2) and absorption of phenols and oxidised compound (Chinnici, Natali, & Riponi, 2014).

One intriguing question could be if the antibrowning activity of chitosan could benefit from the simultaneous presence of other antioxidants whose use is well established in oenology. This would change the way and the doses of those preservatives and, potentially, represents a further way to substitute sulphites in winemaking.

The aim of this work was, hence, to evaluate the synergism or antagonism of the interaction between chitosan and other three widely used additives SO_2, ascorbic acid and glutathione. To this purpose, the study was focused on browning development and the follow-up of the formation of brownish xanthyllium cations and phenolic intermediates during storage in model wine solutions containing (+)-catechin, tartaric acid and metals.

3.3.2. Materials and Methods

3.3.2.1. Chemicals

HPLC-grade acetonitrile, acetic acid hydrochloric acid and ethanol were obtained from Merck (Darmstadt, Germany). Water was of Milli-Q quality. (+)-Catechin, (+)-tartaric acid, Fe^{II} sulfate heptahydrate, Cu^{II} sulfate pentahydrate, potassium metabisulfite, low-molecular-weight 75−85% deacylated chitosan (product 448869), SO_2, glutathione, and ascorbic acid, glucosamine and N-acetylglucosamine were purchased from Sigma-Aldrich (Milan, Italy).

3.3.2.2. Model wine solutions

A total of 4 L of a solution containing 4 g/L (+)-tartaric acid and 10% (v/v) ethanol was prepared. The pH was adjusted to 3.2 with 2N NaOH before bringing to the mark progressively by adding water. The solution was stirred at room temperature in open air until saturated with O_2 (2 h). To this solution, 100 mg/L of (+)-catechin, 5 mg/L of Fe(II) in the form of Fe^{II} sulfate heptahydrate and 0.35 mg/L of Cu (II) in the form of Cu^{II} sulfate pentahydrate were added.

Trials were arranged by transferring aliquots (20 mL) of the above cited solution in 50 mL glass bottles (Supelco, Bellefonte, PA) leaving 30 mL of air in the headspace with the aim to accelerate the oxidation process. One g/L of chitosan was separately added to all the samples, except the control and, when appropriate, 20 mg/L glutathione, 80 mg/L SO_2 and 100 mg/L ascorbic acid were added to constitute the following solutions: Control (model wine solution), GSH (model solution + glutathione + chitosan), AA (model solution + ascorbic acid + chitosan), SO_2 (model solution + sulfur dioxide + chitosan) and KT (model solution + chitosan). Samples, closed with a silicone septum, were stored at room temperature (22 °C), in the dark.

Because of chitosan insolubility, once closed, samples were mechanically shacked (Roller 6, IKA, Germany) for 5 minutes to permit a homogeneous distribution of the polysaccharide. A total of 5 mins of shaking was also performed each day along the experiment.

3.3.2.3. pH

The pH was determined by using a pH-meter (Mettler Toledo, Spain).

3.3.2.4. Deacetylation degree

Acetylation degree of chitosan was determined following the method of Liu et al. (Liu, Wei, Yao, & Jiang, 2006).

- ***Calibration curve***

Mixed solutions with varying glucosamine (GlcN)/N-acetylglucosamine (GlCNAc) ratios were prepared in 0.1M HCl to simulate increasing deacetylation degrees. GlcN and GlCNAc mixture presents the same UV spectrum than chitosan in 0.1M HCl solution, with λ_{max} at 201nm. Therefore, calibration curve with different DA was constructed by measuring absorbance at 201nm of GlcN + GlCNAc solutions. The plot of A/c_t (absorbance/(c_{GlcN}+c_{GLcNAc})) against DA is shown in **figure 3.3.1**. Equation obtained by linear regression was: A/ct = 1.2446DA + 0.0055, R^2 = 0.985.

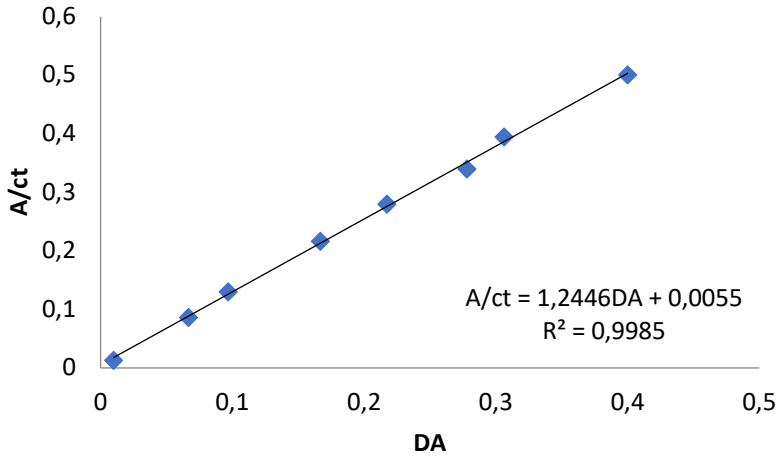

Figure 3.3.1. Degree of acetylation (DA) Calibration curve

- ***Determination of DA of chitosan***

17.2 mg of chitosan was dissolved in 100mL of 0.1M HCl. DA was determined by means of the following equation:

$$\mathrm{DA} = \frac{161.1 \cdot A \cdot V - C \cdot m}{k \cdot m - 42.1 \cdot A \cdot V}$$

were A is the absorbance of the sample, V is the volume of the solution, C is intercept of the equation, k is the slope and m is the weighted chitosan. By means this procedure, DA of the chitosan batch used for the experiment was established to be 11%.

3.3.2.5. Browning and SO_2 measurement

The experimentation lasted 21 days in the darkness at ambient temperature (22°C), during which three bottles, prepared identically for each trial (MS, GSH, AA, SO_2, and KT), were taken at time points 0, 2, 7, 14, and 21 days (75 bottles, as a total), for the analytical determinations to be carried out in triplicate. In this way, for each time point and for each trial, a new and stopped bottle has been sampled. The browning development of the samples was followed measuring the increase in absorbance at 440 nm (1 cm optical path) using a Jasco 810 spectrophotometer (Tokyo, Japan), after filtration (0.45 µm, cellulose filters). Further, the optical density of model solution at initial time (time 0) was recorded to be equal to 0.008. Total SO_2 concentrations were determined in accordance with the official International Organization of Vine and Wine (OIV) method.

3.3.2.6. Reversed-Phase (RP)-HPLC/Electrospray Ionization (ESI)-MS Analysis of (+)-catechin and other derivatives generated during its oxidation

HPLC separation and identification of (+)-catechin and phenolic compounds generated during its oxidation were performed on a Agilent LC-MSD 1100 series single quadrupole (Agilent Technologies, Palo Alto, CA), equipped with an autosampler and a diode array UV−vis detector, according to a previous method (Chinnici, Sonni, Natali, & Riponi, 2013). The column was a Poroshell 120 SB C18, 2.7 µm, 150 × 4.6 mm inner diameter, operating at 30 °C with a flow of 0.9 mL/min. Elution solvents were 2% acetic acid in HPLC-grade water (eluent A) and 2% acetic acid in HPLC-grade acetonitrile (eluent B). Gradient elution was as follow: from 98 to 95% A in 9 min, from 95 to 90% A in 6 min, from 90 to 82% A in 4 min, from 82 to 80% A in 3 min, from 80 to 70% A in 3 min, from 70 to 50% A in 3 min, from 50 to 0% A in 2 min, and from 0 to 98% A in 3 min. A post-run time of 5 min was further applied. Mass detection was carried out using an ESI interface operating in positive mode, scanning from m/z 100 to 1000 and using the following conditions: drying gas flow, 9.0 L/min; nebulizer pressure, 50 psi; gas drying temperature, 350 °C; capillary voltage, 4000 V; and fragmentor voltage, 15 V. Identification of (+)-catechin was accomplished by comparison of the UV spectrum and retention time of the standard compound. Phenolic compounds generated by (+)-catechin oxidative decay, which lacked of available standards, were identified on the basis of the UV and MS spectrum already reported in other studies (Chinnici et al., 2014). Accordingly, carboxymethyne bridged (+)-catechin isomers were identified at m/z 635, xanthene at m/z 617, xanthylium isomers at m/z 615 and xanthylium ethyl esters at m/z 643 (all in negative ion mode). Quantification of residual (+)-catechin in the samples was performed at 280 nm using an external calibration curve built by injecting solutions of known concentrations. Other phenols were monitored according to their maximum absorption wavelength (280 nm for carboxymethine-linked dimers and xantene and 440 nm for xanthylium ions and their esters) and quantified in a semi- quantitative fashion as peak area. Before injection, synthetic wines were filtered at 0.45 µm with a cellulose filter. All analyses were performed in duplicate.

3.3.2.7. Chitosan solubility

Solubility was evaluated by testing chitosan in hydro-alcoholic solutions composed by 10% (v/v) ethanol in distilled water and pH 3.2 adjusted with hydrochloric acid 1 M to mimic white wine conditions. A 40 mg of fungal chitosan was weighted and added to 40 ml of solution in closed glass flaks at 20ºC under stirring (200 rpm) for 10, 30, 60, 120, 1200 min to study the solubility of chitosan with time. After contact time, samples were filtered on 0.45 µm filters (Millipore, Darmstat, Germany). Membranes containing chitosan were put in the oven for 24h at 100ºC and then placed in vacuum-dryer at 20ºC until constant weight. Once dried, % solubility of chitosan was calculated by differences of weight before and after treatments.

3.3.3. Results and discussion

3.3.3.1. Browning of white wine model solutions

The colour development of samples was monitored spectrophotometrically at 440 nm (**Figure 3.3.2 upper**), which corresponds to the maximum absorption wavelength of brown polymers generated by the oxidative degradation of (+)-catechin (Es-Safi, Guernevé, Fulcrand, Cheynier, & Moutounet, 2000). After 2 days, browning of control samples steadily increased while only a slightly increment was observed in the rest of the treatments.

All the KT added samples experienced a substantial reduction of browning with respect to the control. Based on our previous experiments (chapter 1 and 2) this trend of absorbances could be related to different mechanisms: 1) Absorption of (+)-catechin and its intermediates of oxidation on the chitosan backbone, reducing therefore their generation (Chinnici et al., 2014) 2) chelation of transition metals by means of chitosan and thus, blocking Fenton chemistry. 3) Inhibition on the generation of glyoxylic acid and acetaldehyde during oxidation 4) Scavenging effect against H_2O_2 produced after oxidation of polyphenols (experiment 1), 5) Hydroxyl radical (·OH) scavenging of KT.

When compared to KT samples, combined addition of SO_2 + KT resulted in a greater protection against brown polymers generation. This phenomenon may be due to the sum of both antioxidant actions, those previously mentioned, and the direct reaction of SO_2 with aldehyde compounds and H_2O_2.

On the contrary, AA + KT samples suffered an even greater colour increase. In this case, as already reported by Bradshaw et al., (Mark P. Bradshaw, Cheynier, Scollary, & Prenzler, 2003) in the absence of SO_2, ascorbic acid could undergo a “crossover” phenomenon from anti-oxidant to pro-oxidant leading to the production of (H_2O_2) during its oxidation. The greater proportion of H_2O_2 leads not only to a greater oxidation of phenolic compounds, but also to the degradation of chitosan, producing changes on its structure and therefore in its physical and chemical properties, such as antioxidant capacity (Qin, Du, & Xiao, 2002).

Regarding GSH, treatments with KT did not exerted any significant difference compared to KT alone. It could be hypothesized that glutathione was absorbed to some extent by chitosan, due to the peptidic structure of the former and protein affinity of the latter. Thus, by absorbing it, KT may have interfered with the antibrowing effect of GSH.

In **figure 3.3.2** (**bottom**) are shown the changes in absorbance at 280 nm of the different treatments. Absorbance at 280nm mainly corresponds to (+)-catechin and other colourless oxidation intermediates (carboxymethine-linked dimers and xanthene) (Chinnici et al., 2014). It can be observed that, up to 7 days, all the samples containing KT showed an important reduction of the absorbance mainly due to the absorption of phenolic compounds present in the media. However, control solution slightly reduced as well, because of (+)-catechin oxidation. Therefore, based on the latter result, it is worth to mention that the decrease in absorbance at 280 nm of KT samples may be due to the sum of two events: 1) absorption of phenolic compounds (e.g. (+)-catechin dimers intermediates) on chitosan backbone and 2) oxidation of (+)-catechin.

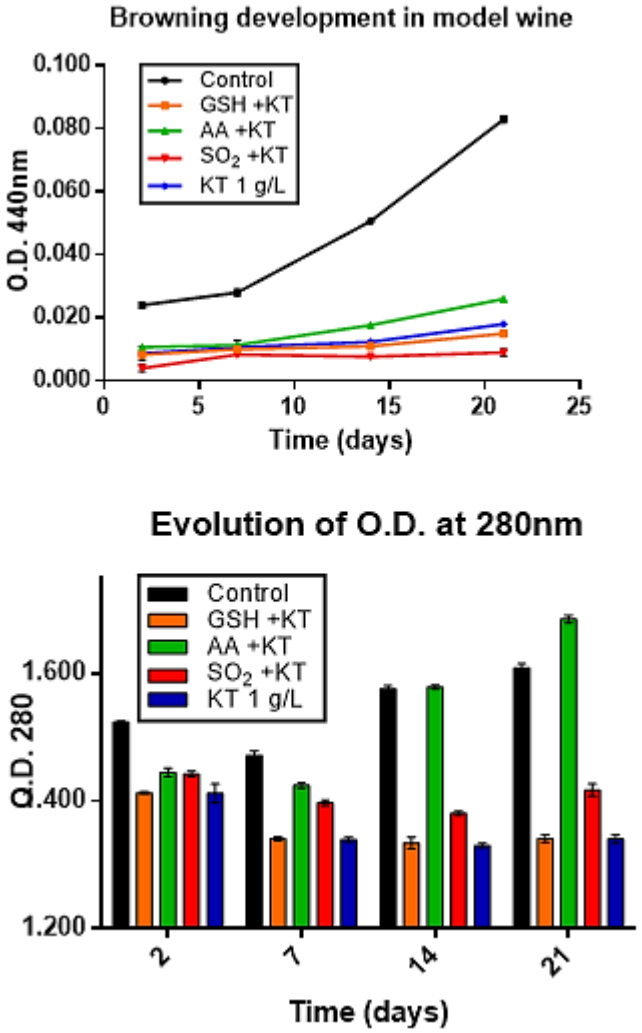

Figure 3.3.2. Evolution of browning (abs 440 nm) and oxidation intermediates (280 nm) of control, GSH (glutathione), AA (ascorbic acid), SO_2 (sulfur dioxide) and KT (chitosan)

In fact, significative minor decrease in absorbance of SO_2 +KT samples ($p < 0.05$) could be due to the protecting effect of SO_2 against (+)-catechin oxidation. Furthermore, after the 7^{th} day onwards, control samples and AA +KT samples increased their absorbance at 280 nm while the rest of the samples remained substantially stable. Regarding control, absorbance raised likely due to the progressive production of oxidized dimers. However, after 21 days AA +KT samples resulted in a greater absorbance at 280 nm than the control. This fact is likely to be linked to the production of ascorbic acid degradation products (Marc P. Bradshaw, Barril, Clark, Prenzler, & Scollary, 2011). These data will be further commented in the continuing of the discussion.

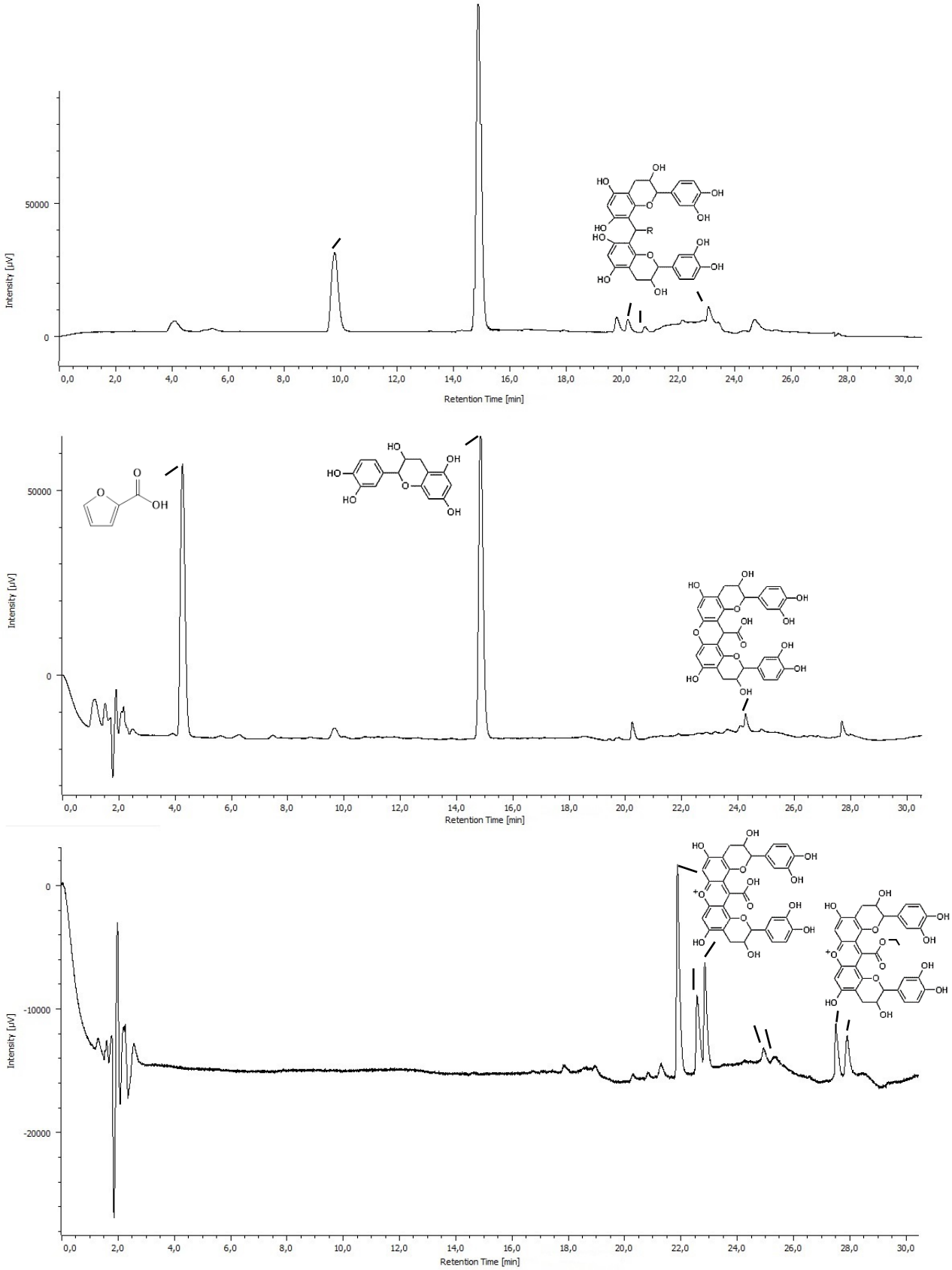

Figure 3.3.3. HPLC traces of phenolic compounds presents in model white wines after 21 days of oxidation

In order to deepen the previous subject, evolution of polyphenolic compounds such as (+)-catechin and its oxidation products were analysed by means of HPLC-DAD/MS all over the experience. According to what already obtained in a previous work (Chinnici et al., 2014) and based on UV and mass spectrum, a total of 12 compounds were identified (**Figure 3.3.3**), as follows:

- (+)-catechin
- carboxymethine-linked isomers (4 isomers)
- Xanthene
- xanthillium cations (2 isomers)
- Xanthilium esters (2 isomers)
- Ascorbic acid degradation product

The monitoring of the above compounds would give a better understanding of the browning development on model wines in the presence of chitosan and other antioxidants. As shown in **figure 3.3.4A**, after 2 days, dimers were formed except for samples treated with SO_2 + KT. Control samples experienced a fast generation of browning precursors reaching the maximum amount at day 14, in coincidence with the significant increase in browning (**figure 3.3.2**). Regarding KT treated samples, even if to a much lesser extent, (+)-catechin dimers started to be formed after 2 days. However, after reaching the maximum levels at day 7, dimers remained unchanged except for AA and SO_2 samples as already reported by Chinnici et al., (Chinnici et al., 2014). Furthermore, SO_2 in combination with KT effectively inhibited oxidation of model wines up to 21 days. This interesting phenomenon could be attributed to a combining antioxidant effect of SO_2 (trapping of glyoxylic acid and acetaldehyde and scavenging of hydrogen peroxide)(Danilewicz, Seccombe, & Whelan, 2008) and KT (chelation of metal catalyst of oxidation, scavenging of ·OH and 1-HER radicals (chapter 1) reduction of H_2O_2 content, and inhibition of development of glyoxylic acid and acetaldehyde (chapter 2)).

Generation of xanthenes and xanthylium cations are depicted in **Figure 3.3.4B and 3.3.4C** respectively. Excepting for AA+KT samples, these compounds followed a common trend, being generated after 7 days and increasing over the course of the incubation, directly correlated with the amounts of carboxymethine-linked dimers (**Figure 3.3.4A**). According with data outlined in **figure 3.3.4A**, KT samples displayed a reduction in the generation of Xanthenes and Xanthillium cations, with a complete inhibition of their presence in samples treated with both SO_2 and KT. Apparently, this data fit completely with browning development, shown in **figure 3.3.2**.

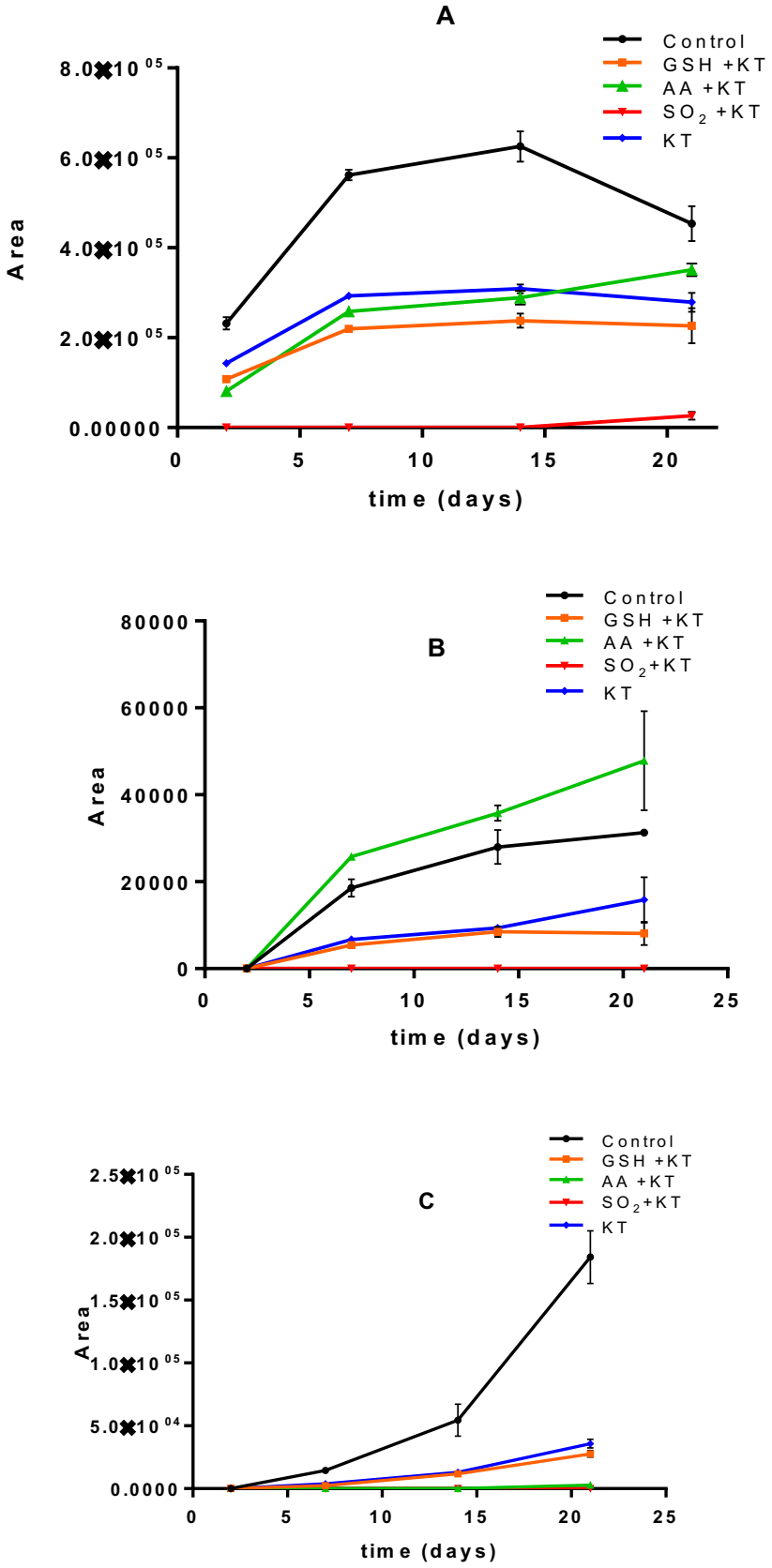

Figure 3.3.4. Monitoring of peak area of **A**) carboxymethin-linked (+)-catechin dimers (Sum of peaks) **B**) Xanthene and **C**) Xanthillium cations (sum of peaks)

On the contrary, samples treated with AA + KT experienced an increase in carboxymethine-linked dimers content reaching their maximum level after 21 days of incubation (**Figure 3.3.4A).** Besides, xanthene generation seemed to be enhanced by the presence of AA increasing even higher than in control samples (**Figure 3.3.4B**). Surprisingly, the rise in colour in AA samples was not correlated with the amount of brown pigments since no xanthillium cations were detected in samples treated with AA (**Figure 3.3.4C**). This result agreed with those obtained by Clark et. al, (Clark, Pedretti, Prenzler, & Scollary, 2008) which postulated that although yellow colouration (Abs 440) was enhanced by the degradation products of ascorbic acid, its was able to supress the generation of brown pigments.

As already mentioned in the previous chapter, oxidation of ascorbic acids leads to the generation of dehydroascorbic acid and H_2O_2. Dehydroascorbic acid is further degraded into 2-furoic acid, 3-hydroxy-2-pyrone (Barril et al., 2012) , and by reacting with (+)-Catechin, leads to the formation of (+)-1”-methylene-6”-hydroxy-2H-furan-5”-one-8-catechin ((+)-MHF-8-catechin) (**Figure 3.3.5**).

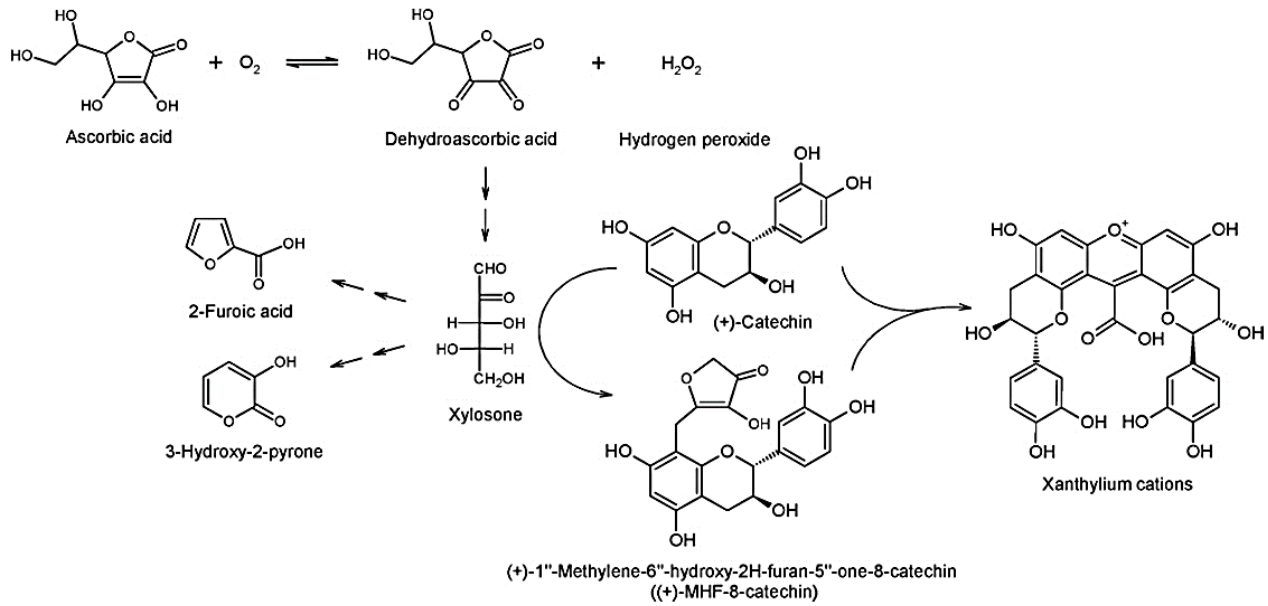

Figure 3.3.5. Ascorbic acid oxidation and further reactions in wine conditions (Barril et al., 2012)

Indeed, in samples containing AA, compounds mentioned above were identified (**Figure 3.3.6**). 3-hydroxy-2-pyrone and furoic acid were found to increase progressively over time, while (+)-MHF-8-catechin raised in concentration up to the day 14, followed by a decline which corresponded to the increase in the browning development of AA +KT samples. 3-hydroxy-2-pyrone and (+)-MHF-8-catechin presented maximum wavelength at λ= 280nm. Thus, rise in absorbance at 280nm (**figure 3.3.2 bottom**) of AA+ KT could be justified by the presence of degradation products of oxidation of AA.

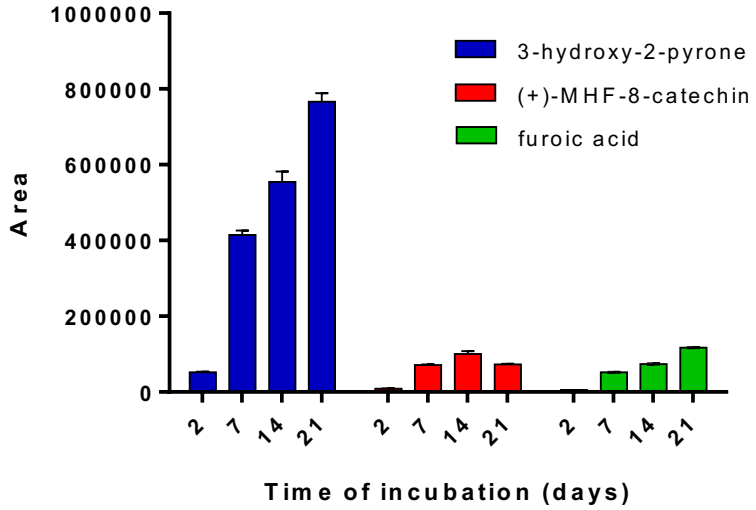

Figure 3.3.6. Oxidation products derived from AA oxidation

3.3.3.2. Influence of chitosan on the pH of model wine solutions

Samples treated with chitosan showed a significant increase in pH (augmented by 0.06 units) throughout the whole incubation (**table 3.3.1**). This trend may be attributable to the ability of chitosan to reduce the organic acid content (tartaric acid in model wine conditions of this experiment). The acid binding properties of chitosan have already been proposed for the treatment of acid beverages such as vegetable or fruit juices and coffee (Imeri & Knorr, 1988; Scheruhn, Wille, & Knorr, 1999).

	Time (days)			
	2	**7**	**14**	**21**
Control	3,21 ^{a}	3,2 ^{b}	3,18 ^{b}	3,19 ^{c}
GSH + KT	3,23 ^{a}	3,25 ^{a}	3,24 ^{a}	3,26 ^{b}
AA + KT	3,21 ^{a}	3,24 ^{a}	3,25 ^{a}	3,27 ^{b}
SO_2 + KT	3,22 ^{a}	3,23 ^{ab}	3,24 ^{a}	3,25 ^{b}
KT	3,23 ^{a}	3,24 ^{a}	3,27 ^{a}	3,29 ^{a}

Table 3.3.1. pH of different samples on each timepoint. In the same row, different letters indicate significant differences according to Tukey's test ($p < 0.05$). n=3.

Mechanism of acid reduction in wine media is based on the electrostatic interaction between positive charged amino groups ($-NH_2$) of glucosamine units of chitosan and the anionic form of organic acid in wine (Mitani, Yamashita, Okumura, & Ishii, 1995), resulting in a higher pH acid due to acid-base neutralization:

$$\textbf{CHIT}\text{-}NH_2 \leftrightarrow \textbf{CHIT}\text{-}NH_3^+$$

$$AH + H_2O \leftrightarrow A^- + H_3O^+$$

$$\textbf{CHIT}\text{-}NH_2 + AH \leftrightarrow \textbf{CHIT}\text{-}NH_3^+A^-$$

In addition, at wine pH, primary amino groups (-NH_2) of chitosan, with a pKa of 6.3, get positively charged (-NH_3) by hydrogen abstraction from water, leading to the formation of OH^- groups, resulting in an increase of pH as already reported Gliene and co-workers (Gyliene et al., 2014)

$$\textbf{CHIT-}NH_2 + H_2O \leftrightarrow \textbf{CHIT-}NH_3^+ + OH^-$$

3.3.3.3. Solubility of chitosan on hydroalcoholic solutions

Data obtained from solubility test are depicted in **table 3.3.2**. As can be appreciated, no difference was observed among times of agitation. This result agrees with those obtained by Colangelo et al., (Colangelo, Torchio, De Faveri, & Lambri, 2018), which reported that solubility of chitosan was independent of the alcohol level and time of contact. Regarding solubility levels of ~16% independent of any treatment, this fact could be due to the loss of a portion of soluble glucan content of chitosan. This hypothesis is based on data reported in the patent developed by *Kitozyme* (Haute & Hauts-sarts, n.d.) where fungal glucans was quantified ~13%. Therefore, even if not with a conclusive proof, is highly probable that no chitosan was dissolved during the experiment and that it can be entirely removed by filtering before bottling operation in cellars.

Time of agitation (min)	% Sol
10	16,35 ± 0,5
30	16,93 ± 0,38
60	15,87 ± 0,77
120	17,19 ± 0,34
1200	15,98 ± 2,25

Table 3.3.2. % Solubility of chitosan at different times of stirring (mean ±SD) n=3.

3.3.4. Conclusions

Overall, results obtained demonstrated the efficacy of chitosan in inhibiting the oxidative degradation of (+)-catechin. According to what was obtained in the two previous works (chapter 1&2) mechanism of action of chitosan against browning development is based on several pathways:

- Chelation of metals
- Direct absorption of polyphenols and oxidised compounds
- Antiradical activity against hydroxyl and 1-hydroxyethyl radical
- Scavenging effect against H_2O_2
- Inhibition of the generation of aldehydic intermediates of oxydation

Furthermore, addition of KT and SO_2 resulted in a higher control against oxidative decay mainly due to the combination of antioxidant properties of both additives. These results suggested that the dose of SO_2 could be reduced in winemaking if combined with a treatment with KT. On the other hand, combination with GSH did not exerted any significant differences when compared with KT alone as discussed above in section 3.1. However, the presence of AA resulted in a reduction of the antibrowning capacity of KT, due to a pro-oxidant effect of AA, leading to the production of H_2O_2 and thus enhancing oxidative decay of (+)-catechin.

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Chapter 3.4.

"Volatile and fixed composition of sulphite-free white wines obtained after fermentation in the presence of chitosan"

Chapter 3.4.

Volatile and fixed composition of sulphite-free white wines obtained after fermentation in the presence of chitosan

3.4.1. Introduction

As commented in previous chapters, for what concern oxidation, sulphite effectively counteracts both the phenolic and aromatic decay of wines (Bueno, Culleré, Cacho, & Ferreira, 2010; Waterhouse & Laurie, 2006), otherwise resulting in a decreased attractiveness of final products.

Chitosan can be used in several steps along the winemaking process, from initial must clarification, to final wine stabilization just before bottling. Unprotected (e.g. sulphite-free) white musts are prone to enzymatic oxidation or unwanted yeast and bacterial proliferation, which may drive to early browning development and sluggish fermentations (Bisson, 1999).

Interestingly, the addition of chitosan to free-run juices or during fermentation could acts as both an antioxidant and antimicrobial, in this way reproducing the two main functions that sulphites are called upon to play in the very first phases of winemaking. However, very little is known about the influence of the use of this polymer on musts, on fermentation kinetics and on the volatile composition of the obtained wines.

The aim of this work was, hence, to study the effects of the fermentative addition of chitosan on fixed and volatile compounds of sulphite-free white wines.

Chitosan was added just before yeast inoculation of white musts and resulting wines were evaluated after 12 months of storage in bottles and compared to wines treated with sulphur dioxide in the same step of the production process.

3.4.2. Material and Methods

3.4.2.1. Chemicals

Pure standards of volatile compounds, internal standard (2-octanol) and potassium metabisulphite were purchased from Sigma-Aldrich (Milano, Italy).

Dichloromethane and methanol (SupraSolv) were supplied by Merck (Darmstadt, Germany), absolute ethanol (ACS grade) was obtained from Scharlau Chemie (Sentmenat, Spain), and pure water was obtained from a Milli-Q purification system (Millipore, USA). LiChrolut EN resin for solid-phase extraction (SPE) prepacked in 200 mg cartridges (3 ml total volume) were purchased from Merck (Darmstadt, Germany). Chitosan (low MW, 75-85% deacetylated, product #448869) was obtained from Sigma-Aldrich (Milano-Italy).

3.4.2.2. Microvinifications

Sulphite-free white musts were obtained at the experimental winery of the University of Bologna, from grapes cv. Trebbiano. Grapes were destemmed, crushed, pressed at 0.9 bars in a bladder press and cold-settled at 4°C for 24 h. The racked must was then filtered with Seitz-Supra EK1 filters from Seitz (Bad Kreuznach, Germany). The analytical parameters of the obtained must were as follow: sugars 205 g L^{-1}; pH 3.05; titratable acidity 6.8 g L^{-1}; total phenolics 107 mg L^{-1}; O.D. 420 nm 0.146. Filtered must was placed in two litres laboratory glass fermentors, at room temperature, to start the fermentation. Trials were arranged in triplicate, before yeast inoculation, by adding potassium metabisulphite or chitosan to the musts at dosage of 60 mg L^{-1} and 1 g L^{-1} respectively. A further control fermentation (in triplicate) with no additions was also prepared. To avoid microbial contamination and oxygen entrance during fermentation, each fermentor was provided of a glass trap filled with 37% H_2SO_4. A *Saccharomyces cerevisiae* strain already characterized for its low SO_2 production (strain 1042 from University of Bologna – ESAVE collection) (Sonni, Cejudo Bastante, Chinnici, Natali, & Riponi, 2009) was inoculated after the rehydration of about 1.5×10^6 CFU mL^{-1} into 25 mL of sterilized must in 250-mL Erlenmeyer flasks plugged with cotton wool, incubated for 24 h. Fermentations were monitored by following the weight loss of samples. Once the weight loss stopped, chitosan and yeasts lees were left to settle down and the clarified wines were transferred by means of a peristaltic pump (VWR international, Milano, Italy) in 50 mL bottles, without headspace, and stored for 12 months at room temperature and in the darkness. Before the filling, air in the bottles was evacuated by a gentle nitrogen stream.

3.4.2.3. Oenological parameters

All the oenological parameters were determined according to OIV methods (International Organisation of Vine and Wine (OIV), 2015).

The pH was determined by using a pH-meter (Mettler Toledo, Spain). The alcoholic strength of wines was determined by using an oenochemical distilling unit (Gibertini, Italy). Total polyphenolics were spectrophotometrically determined (after wine filtration at 0.45 nm with PTFE filters) at 280 nm using an Uvidec 610 spectrophotometer (Jasco, Japan) and results were expressed as mg L^{-1} of gallic acid (GAE). All the analyses were carried out in duplicate.

3.4.2.4. Organic acids, sugars and glycerol

Quantification of organic acids, sugars and glycerol was conducted following the procedure described by Chinnici et al. (Chinnici, Spinabelli, Riponi, & Amati, 2005).

The HPLC used was a Jasco apparatus (Tokyo, Japan) equipped with a binary pump (PU 1580), a 20 µL loop, a Rheodyne valve (Cotati, CA, USA), a photodiode detector (PU MD 910; Tokyo, Japan), and a column oven (Hengoed Mid Glamorgan, UK). The column was a Bio-Rad Aminex HPX 87H (300 mm×7.8 mm), thermostated at 35 °C. Isocratic elution was carried out with 0.005 N phosphoric acid at flow 0.4 mL/min. All the analyses were carried out in duplicate.

3.4.2.5. Wine volatile compounds

Volatile compounds were extracted according to the method described and validated by Lopez et al. (López, Aznar, Cacho, & Ferreira, 2002). A 20 ml wine sample was added of 100 µL of a 2-octanol solution at 500 mgL^{-1} as internal standard and deposed on an Lichrolut EN cartridge previously activated. Analytes were eluted with 5 mL of dichloromethane, and concentrated to a final volume of 200 µL under a stream of pure nitrogen (N_2), prior to GC-MS analysis.

The Trace GC ultra-apparatus coupled with a Trace DSQ mass selective detector (Thermo Fisher Scientific, Milan, Italy) was equipped with a fused silica capillary column Stabilwax DA (Restek, Bellefonte, PA, USA; 30 m, 0.25mm i.d., and 0.25 µm film thickness). The carrier gas was He at a constant flow of 1.0 mL/min.

The GC programmed temperature was: 45 °C (held for 3 min) to 100 °C (held for 1 min) at 3 °C/min, then to 240 °C (held for 10 min) at 5 °C/min. Injection was performed at 250 °C in splitless mode and the injection volume was 1 µL. Detection was carried out by electron ionization (EI) mass spectrometry in full scan mode, using ionization energy of 70 eV. Transfer line interface was set at 220 °C and ion source at 260 °C. Mass acquisition range was *m/z* 30-400 and the scanning rate 1 scan s^{-1}.

Compounds were identified by a triple criterion: i) by comparing their mass spectra and retention time with those of authentic standards, ii) compounds lacking of standards were identified after matching their respective mass spectra with those present in the commercial libraries NIST 08 and Wiley 7, iii) matching the linear retention index (LRI) obtained under our conditions, with already published LRI on comparable polar columns (**Table 3.4.1**).

Quantification of compounds was carried out via the respective total ion current peak areas after normalization with the area of the internal standard. Calibration curves were obtained by duplicate injections of standard solutions, subjected to the above cited extraction procedure, containing a mixture of commercial standard compounds at concentrations between 0.01 to 200 mg L^{-1}, and internal standard at the same concentration as in the samples. The calibration equations for each compound were obtained by plotting the peak area response ratio (target compound/internal standard) versus the corresponding concentration.

For compounds lacking reference standards, the calibration curves of standards with similar chemical structure were used.

Analyses were done in duplicate and data were collected by means of Xcalibur software (Thermo Fisher Scientific, Milano, Italy)

tR (min)	Compound	LRI	Identification[a]
5,04	ethyl 2-methylbutyrate	1078	Std, MS, LRI
5,39	ethyl isovalerate	1090	Std, MS, LRI
5,78	isobutyl alcohol	1104	Std, MS, LRI
6,74	isoamyl acetate	1127	Std, MS, LRI
7,19	n-butanol	1138	Std, MS, LRI
9,44	3-methyl-1-butanol	1194	Std, MS, LRI
10,28	ethyl n-caproate	1221	Std, MS, LRI
11,63	ethyl pyruvate	1265	Std, MS, LRI
12,00	methyl lactate	1281	MS, LRI
12,82	2-hexanol	1304	Std, MS, LRI
13,03	4-methyl-1-pentanol	1309	Std, MS, LRI
13,44	3-methyl-2-buten-1-ol	1319	Std, MS, LRI
13,51	3-methyl-1-pentanol	1321	Std, MS, LRI
14,19	ethyl lactate	1339	Std, MS, LRI
14,52	n-hexanol	1348	Std, MS, LRI
14,84	4-hydroxy-4-methyl-2-pentanone	1357	Std, MS, LRI
14,92	4-methyl-1,3-oxathiolane	1359	MS
15,35	3-ethoxy-1-propanol	1370	Std, MS, LRI
15,72	3-hexen-1-ol	1380	Std, MS, LRI
16,14	nonanal	1391	Std, MS, LRI
17,30	ethyl 2-hydroxy-isovalerate	1421	Std, MS, LRI
17,74	ethyl octanoate	1432	Std, MS, LRI
18,05	5-methyltetrahydro-2-furanyl-methanol	1440	MS, LRI
18,11	2-ethyl-2-methylbutanoic acid	1441	MS
19,03	Furfural	1464	Std, MS, LRI
20,19	cis-5-hydroxy-2-methyl-1,3-dioxane	1493	MS, LRI
20,36	2-mercaptoethanol	1498	Std, MS, LRI
21,05	ethyl-3-hydroxybutyrate	1514	Std, MS, LRI
21,36	2-methyl-3-thiolannone	1522	MS, LRI

tR (min)	Compound	LRI	Identification[a]
21,47	2-(methylthio)ethanol	1524	Std, MS, LRI
22,89	1,3-Dioxolan-2-one	1558	MS
23,07	isobutyric acid	1563	Std, MS, LRI
23,80	propylene glycol	1580	Std, MS, LRI
23,93	ethyl 3-hydroxypropionate	1583	MS
24,35	trans-4-hydroxymethyl-2-methyl-1,3 dioxolane	1593	MS
24,94	γ-butyrolactone	1616	Std, MS, LRI
25,08	n-butyric acid	1623	Std, MS, LRI
25,23	ethyl decanoate	1631	Std, MS, LRI
25,35	N-ethyl acetamide	1637	MS, LRI
26,03	2-furanmethanol (furfuryl alcohol)	1672	Std, MS, LRI
26,25	pentanoic acid	1683	MS, LRI
26,44	diethyl succinate	1693	Std, MS, LRI
27,48	3-methylthio-1-propanol	1733	Std, MS, LRI
28,08	4-hydroxy-2-butanone	1754	MS
28,99	2-hydroxy-methyl ester benzoic acid = methyl salicylate	1787	MS, LRI
29,19	2,7-dimethyl-4,5 octandiol	1794	MS
29,24	ethylphenyl acetate	1796	Std, MS, LRI
29,79	ethyl 4-hydroxybutanoate	1822	Std, MS, LRI
30,01	2-phenylethyl-acetate	1833	Std, MS, LRI
30,11	trans-5-hydroxy-2-methyl-1,3-dioxane	1837	MS, LRI
30,16	4-methyl-2-pentanoic acid	1840	MS
30,76	hexanoic acid	1869	Std, MS, LRI
31,36	N-(3-methylbutyl)acetamide	1899	MS, LRI
31,45	benzyl alcohol	1902	Std, MS, LRI
31,98	ethyl 3-methylbutyl butanedioate	1921	MS, LRI
32,33	2-phenylethanol	1933	Std, MS, LRI
32,86	cinnamyl nitrile	1951	MS
33,35	benzyl oxytridecanoic acid	1967	MS, LRI
34,07	2H-piran-2,6 (3H)-dione	1992	MS

tR (min)	Compound	LRI	Identification[a]
34,63	1H-Pyrrole-2-carboxaldehyde	2017	Std, MS, LRI
34,85	pantolactone	2029	Std, MS, LRI
34,97	diethyl malate	2035	Std, MS, LRI
35,32	octanoic acid	2053	Std, MS, LRI
37,30	N-acetylglycine ethyl ester	2170	MS
37,32	diethyle 2-hydroxypentanedioate	2172	MS
38,03	4-vinyl-2-methoxy-phenol	2213	Std, MS, LRI
38,82	ethyl 5-oxotetrahydrofuran-2-furancarboxylate	2250	MS, LRI
39,17	3-hydroxy-4-phenyl-2-butanone	2267	MS, LRI
39,31	decanoic acid	2274	Std, MS, LRI
39,39	ethyl 2-hydroxy-3-phenylpropanoate	2278	Std, MS, LRI
39,76	3,5-dihydroxy-2-methyl-4H-pyran-4-one	2295	MS, LRI
40,20	glycerine	2313	Std, MS, LRI
40,33	diethyl tartrate	2318	Std, MS, LRI
41,33	ethyl hydrogen succinate	2355	Std, MS, LRI
41,55	4-vinyl phenol	2364	Std, MS, LRI
42,53	2-furancarboxylic acid	2401	Std, MS, LRI
42,92	dodecanoic acid	2427	Std, MS, LRI
43,19	ethyl hydrogen fumarate	2445	MS, LRI
43,50	α-(phenylmethyl) benzeneethanol	2466	Std, MS
44,17	5-(hydroxymethyl)-2-furancarboxaldehyde	2514	Std, MS, LRI
44,25	benzenacetic acid	2521	Std, MS, LRI
46,20	tetradecanoic acid	2673	Std, MS, LRI
48,22	3,4-dimethoxyphenylalanine	2759	MS, LRI
49,39	n-hexadecanoic acid	2803	Std, MS, LRI
50,16	N-acetyltyramine	2840	Std, MS, LRI
50,73	1-H-indole-3-ethanol	2867	Std, MS, LRI
51,77	4-hydroxy-benzenethanol	2944	Std, MS, LRI

Table 3.4.1. List of identified compounds. a identification assignement: Std = comparing mass spectra, LRI and retention times with pure compounds, MS = by comparing mass spectra with NIST 08 and, Wiley 7 spectral database, LRI = matching LRI on comparable polar columns (taken from the following publicly available databases: https://pubchem.ncbi.nlm.nih.gov/; https://www.nist.gov/srd; http://www.flavornet.org/flavornet.html)

3.4.2.6. Statistical analysis

Statistical analysis of the entire dataset was performed using the XLSTAT Software package (Version 2013.2, France). One-way analysis of variance (ANOVA) followed by a post hoc comparison (Tukey's HSD test) and Principal Component Analysis (PCA) were carried out.

3.4.3. Results and discussion

3.4.3.1. Fermentation and oenological parameters

The evolution of fermentation was monitored checking the weight loss of fermentors. All the fermentations were completed in 10 days, even if the presence of chitosan resulted in initially slower fermentation rates (**Figure 3.4.1**). This was somehow expected since chitosan has already been reported to interfere variably on *Saccharomyces* ssp. growth kinetics (Allan & Hadwiger, 1979; Roller & Covill, 1999). In particular, Roller and Covill (Roller & Covill, 1999) found that the effects on *Saccharomyces* spp. cells growth of 0.4 g L^{-1} soluble chitosan spanned from complete inactivation to a three days delayed lag phase, depending on the strain considered. These differences in fungi responses have been suggested to be linked to the polyunsaturated

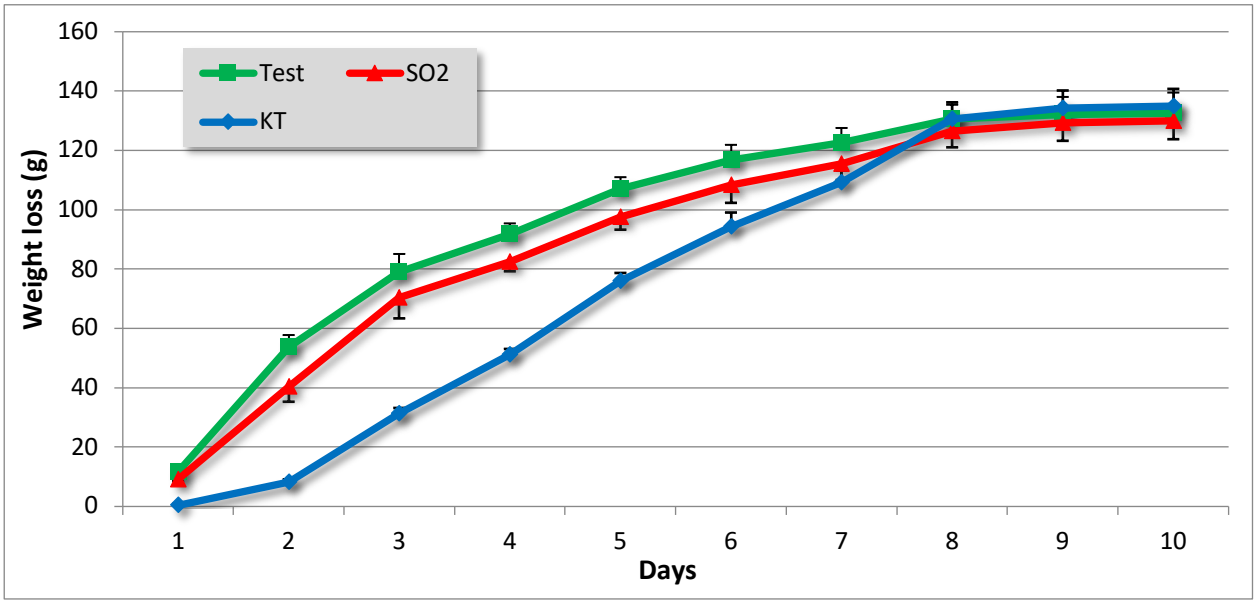

Figure 3.4.1. Weight loss of fermentors during fermentation

free fatty acids content of cells plasma membrane. In sensitive fungi, such as *Neurospora crassa* and *Saccharomyces cerevisiae*, the high content of polyunsaturated free fatty acids enhances membrane fluidity and permeabilization leading to augmented intracellular oxidative stress because of the chitosan entrance in the plasma (Lopez-Moya & Lopez-Llorca, 2016; Zakrzewska et al., 2007; Zakrzewska, Boorsma, Brul, Hellingwerf, & Klis, 2005). In our case, the fermentation of samples added with 1g L^{-1} of chitosan showed a 24 hour extended lag phase but, from day 8 and thereafter, their weight loss was similar to SO_2 or control samples (**figure 3.4.1**). This suggests that the strain used in this experiment was able to resume growth to levels comparable to those observed in untreated musts.

At the end of fermentation, samples treated with chitosan had a decreased content of organic acids, with consequent higher pH values (augmented by 0.08 units) and lower titratable acidity (lessened by 1.1 g L^{-1}) (**Table 3.4.2**). In particular the grape-derived tartaric and malic acids were reduced by about 0.30 g L^{-1} and 0.50 g L^{-1} respectively while, in the same wines, succinic acid amount was 0.25 g L^{-1} lesser.

	Control	SO_2	KT
Alcohol (% v/v)	12,07 a	11,99 a	11,97 a
Titratable Acidity (g L^{-1})	6,52 a	6,23 ab	5,25 b
Volatile Acidity (g L^{-1})	0,39 a	0,36 b	0,42 a
pH	3,11 b	3,11 b	3,19 a
Total SO_2 (mg L^{-1})	1,92 a	48,7 b	2,56 a
Reducing sugars (g L^{-1})	< 2.0 a	< 2.0 a	< 2.0 a
Total phenolics (mg L^{-1})	42,3 a	42,3 a	40,7 a
O. D. 420 nm	0,092 a	0,082 b	0,085 ab
Citric acid (g L^{-1})	0,20 a	0,19 a	0,18 a
Tartaric acid (g L^{-1})	2,94 a	3,03 a	2,67 b
Malic acid (g L^{-1})	2,23 a	2,14 a	1,68 b
Lactic acid (g L^{-1})	0,18 a	0,23 a	0,18 a
Succinic acid (g L^{-1})	0,95 a	0,93 a	0,69 b
Acetic acid (g L^{-1})	0,36 a	0,39 a	0,41 a
Glycerol (g L^{-1})	9,37 a	9,74 a	9,30 a

Table 3.4.2. Oenological parameters of wines at the end of alcoholic fermentation. In the same row, different letters indicate significant differences according to Tukey's test ($p<0.05$). n=3.

The acid binding properties of chitosan had been already claimed and proposed for the treatment of coffee beverages, vegetable or fruit juices (Imeri & Knorr, 1988; Scheruhn, Wille, & Knorr, 1999). This feature is due to the electrostatic interaction between the positively charged amino groups of glucosamine and the anions coming from dissociated acids, whose pKa and hydroxyl content may also play a role (Mitani, Yamashita, Okumura, & Ishii, 1995).

Hence, this would be the reason for the diminution in native organic acids during the 10 days of fermentation. Succinic acid, however, does not come from grapes being produced by yeasts during alcoholic fermentation. Its low amount in KT wines could be the result of reduced fermentative excretion and/or the adsorption by chitosan. It still remains unclear whether one or both the phenomena occurred in our samples.

Alcohol content, volatile acidity and total phenolics index were not affected by the treatments while, as expected, the bleaching and antioxidant capacities of sulphite resulted in lighter yellow nuances of final wines if compared with control sample (see tab. 2, at O.D. 420 nm parameter). In this respect, Kt and SO_2 samples were not significantly different in color, suggesting that chitosan may have controlled the browning development, as already reported by other authors (Chinnici, Natali, & Riponi, 2014; Spagna, Barbagallo, & Pifferi, 2000).

3.4.3.2. Volatile compositions of wines

A list of volatile compounds found in wines before or after storage is reported in **table 3.4.1.** A total of 74 volatiles were elucidated while 12 further compounds lacking of standard and published LRI, were tentatively identified based on their mass spectrum (these compounds are flagged with "MS" in the last column of **Table 3.4.1**). **Table 3.4.3** reports the amounts of the most significant compounds found in wines at the beginning and at the end of bottle storage, grouped as chemical families, which will be separately discussed.

	Wines					
	End of fermentation			12 months of storage		
	Control	SO_2	KT	Control	SO_2	KT
Acids						
isobutyric acid	4,04 a	3,70 a	1,94 b	3,42 a	2,93 a	1,49 b
n-butyric acid	0,28 b	0,31 b	0,35 a	0,18 c	0,25 b	0,30 a
pentanoic acid	3,55 a	3,53 a	2,03 b	3,47 a	3,44 a	1,67 b
hexanoic acid	3,58 b	3,67 b	6,19 a	3,52 b	3,62 b	6,54 a
octanoic acid	3,84 b	3,85 b	7,08 a	3,27 b	3,32 b	6,80 a
decanoic acid	1,49 b	1,26 b	5,33 a	1,16 b	1,02 b	3,77 a
dodecanoic acid	0,20 a	0,21 a	0,18 a	0,05 b	0,05 b	0,10 a
benzenacetic acid	0,13 b	0,22 a	0,06 c	0,03 b	0,09 a	0,05 b
Total acids	*17,11 b*	*16,75 b*	*23,15 a*	*15,09 b*	*14,72 b*	*20,72 a*
Esters						
isoamyl acetate	1,16 b	1,04 b	1,64 a	0,34 a	0,36 a	0,33 a
ethyl hexanoate	0,25 b	0,29 b	0,65 a	0,40 b	0,36 b	0,75 a
ethyl pyruvate	0,06 a	0,06 a	0,06 a	0,13 b	0,19 a	0,10 b
methyl lactate	0,05 b	0,03 b	0,08 a	n.d.	n.d.	n.d.
ethyl lactate	1,08 b	1,30 a	0,86 c	3,92 a	3,39 b	3,44 b
ethyl octanoate	0,10 b	0,20 b	0,44 a	0,70 b	0,54 b	1,33 a

ethyl-3-hydroxybutyrate	0,12 b	0,07 b	0,17 a	0,12 b	0,16 a	0,16 a
ethyl decanoate	0,00 b	0,05 b	0,16 a	0,10 b	0,07 b	0,42 a
diethyl succinate	0,18 a	0,20 a	0,14 b	6,39 a,b	7,45 a	4,48 b
methyl salicylate	0,04 a	0,02 a	0,04 a	n.d.	n.d.	n.d.
ethyl 4-hydroxybutanoate	2,64 b	3,33 a	1,09 c	0,05 a,b	0,12 a	0,01 b
2-phenylethyl acetate	0,87 b	0,93 b	2,10 a	0,12 b	0,14 b	0,36 a
diethyl malate	0,40 a	0,41 a	0,28 b	6,89 b	11,43 a	7,15 b
diethyl tartrate	n.d.	n.d.	n.d.	0,67 b	1,17 a	0,40 b
ethyl hydrogen succinate	2,77 a	2,85 a	2,11 a	11,73 a	13,57 a	14,71 a
Total esters	*9,71 a*	*10,79 a*	*9,83 a*	*31,59 a*	*38,98 a*	*33,64 a*
Alcohols						
Isobutyl alcohol	20,27 b	28,23 a	13,46 c	27,54 a	20,46 b	14,83 c
2-hexanol	0,02 c	0,08 a	0,05 b	0,08 a	0,07 a	0,08 a
3-methyl-1-butanol	30,64 b	40,12 a	30,59 b	55,27 a	45,43 a	55,81 a
2-hexanol	0,05 a	0,05 a	0,05 a	0,03 a	0,03 a	0,03 a
4-methyl-1-pentanol	0,00 c	0,01 b	0,02 a	0,00 b	0,01 a	0,01 a
n-hexanol	0,09 a	0,10 a	0,07 b	0,08 a	0,09 a	0,06 b
3-ethoxy-1-propanol	0,19 a	0,11 b	0,17 a	0,18 a	0,09 c	0,15 b
3-hexen-1-ol	0,03 b	0,03 a	0,03 a,b	0,03 a	0,03 a	n.d.
3-methylthio-1-propanol	1,06 a	1,17 a	0,41 b	0,63 a	0,65 a	0,27 b
Benzyl alcohol	0,20 a,b	0,29 a	0,11 b	0,10 a	0,12 a	0,09 a

2-mercaptoethanol	n.d.	0,01 [a]	n.d.	n.d.	n.d.	n.d.
Phenethyl alcohol	38,97 [a]	38,36 [a]	37,40 [a]	63,50 [a]	72,55 [a]	76,03 [a]
4-hydroxy-benzenethanol	20,54 [a]	20,63 [a]	22,88 [a]	14,26 [a]	19,38 [a]	20,91 [a]
Total alcohols	*112,08 [a]*	*127,20 [a]*	*108,25 [a]*	*161,69 [a]*	*158,89 [a]*	*168,26 [a]*
Others						
cis-5-hydroxy-2-methyl-1,3-dioxane	0,03 [b]	0,05 [a]	0,04 [a]	1,75 [b]	0,87 [c]	3,19 [a]
trans-4-hydroxymethyl-2-methyl-1,3 dioxolane	0,02 [b]	0,10 [a]	0,04 [b]	0,76 [b]	0,44 [c]	1,26 [a]
trans-5-hydroxy-2-methyl-1,3-dioxane	0,04 [a]	0,05 [a]	0,04 [a]	1,64 [b]	1,02 [c]	2,59 [a]
γ-butyrolactone	0,28 [b]	0,37 [a]	0,12 [c]	0,19 [b]	0,26 [a]	0,09 [c]
Furfural	0,07 [a]	0,07 [a]	0,08 [a]	0,12 [c]	0,44 [a]	0,25 [b]
Furfuryl alcohol	0,10 [a]	0,13 [a]	0,12 [a]	0,06 [a]	0,03 [b]	0,07 [a]
4-hydroxy-2-butanone	0,88 [a]	0,76 [b]	0,55 [c]	-0,01 [b]	0,04 [a]	0,05 [a]
ethyl 5-oxotetrahydrofuran-2-furancarboxylate	0,79 [a]	0,86 [a]	0,31 [b]	1,01 [b]	1,61 [a]	0,93 [b]
2-furancarboxylic acid	0,08 [b]	0,17 [a]	0,08 [b]	0,19 [a,b]	0,23 [a]	0,13 [b]
5-(hydroxymethyl) 2-furancarboxaldehyde	n.d.	n.d.	n.d.	0,73 [b]	0,95 [a,b]	1,32 [a]
N-acetyltyramine	0,10 [b]	0,14 [a]	0,13 [a]	n.d.	n.d.	n.d.
Total others	*2,40 [a]*	*2,69 [a]*	*1,51 [b]*	*6,43 [b]*	*5,90 [b]*	*9,87 [a]*

Table 3.4.3. Concentration of the quantified volatile compounds (mg L-1) in wines at the end of the alcoholic fermentation and after 1 year of bottle storage. In the same row, different letters indicate significant differences according to Tukey's test ($p<0.05$). n=3.

- ***Fatty acids***

Our results indicate that treatments with chitosan enhanced the synthesis of three of the main medium chain fatty acids (MCFA), hexanoic, octanoic and decanoic acid (**Table 3.4.3**) that, according to sensory studies, can contribute to the aroma of white wines (Ferreira & Juan, n.d.). During winemaking, a mixture of fatty acids are produced, normally classified as short chain (C2-C4), medium chain (C6-C10), long chain (C12-C18) and branched-chain fatty acids. Metabolism of saturated fatty acids produces straight-chain fatty acids (C4-C12) as intermediate products. (Lambrechts & Pretorius, 2000). The final products, mainly C16 and C18 are incorporated into phospholipids, the backbone of cell membranes. The increased contents of MCFA in wines fermented with chitosan may be due to an augmented permeability of yeast membranes caused by the polysaccharide. As already commented, in fact, at wine pH most of the glucosamine units of chitosan are positively charged due to the protonation of amino groups which allows them to interact with the negatively charged components of cell surface (Zakrzewska et al., 2005).

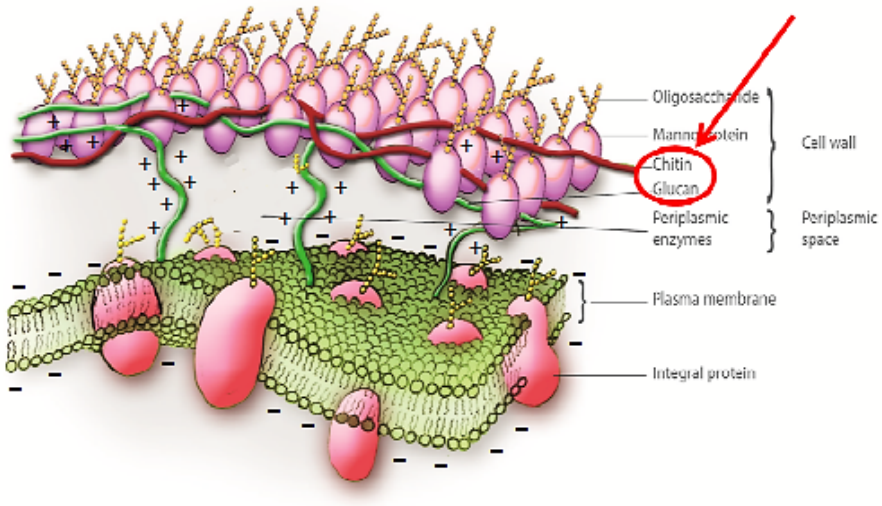

Figure 3.4.2. Electrostatic interaction of chitosan with cell membrane leading to an increase of permeability (Modified from LAFFORT, 2013)

This electrostatic interaction induces changes in the properties of membrane (**Figure 3.4.2**) thus modifying, among other, the cell permeability (Hadwiger, Kendra, Fristensky, & Wagoner, 1986).

Evidences have been given that growing limiting factors, such as an increased membrane permeability, may cause an augmentation in the production of MCFA by the fatty acid synthase complex (Wakil, Stoops, & Joshi, 1983). These C6 to C10 fatty acids at concentrations < 10 mg L^{-1} impart mild and complex aroma to wine. However, at levels above 20 mg L^{-1}, their impact on wines becomes negative (Shinohara, 1985). At the end of fermentation, MCFA concentration in all the samples did not exceed that limit, as reported in **table 3.4.3**. Fermentation conducted in the presence of chitosan showed a decrease in isobutyric and pentanoic acid

amounts. The former acid is not produced by the fatty acid synthetic pathway, being derived from oxidation of the aldehydes formed during amino acid metabolism (Ugliano & Henschke, 2009).

Unpaired acids though, are derived from propionyl-CoA likely formed via α-ketobutyric acid, a metabolite in threonine degradation (Guitart, Orte, Ferreira, Peña, & Cacho, 1999). Their reduced contents in KT wines could be, hence, apparently related to a modification of the amino acid metabolism in yeasts.

Fatty acids in wines did not change substantially during the 12 months of bottle storage, confirming the relative stability of this class of compounds when stored at room temperature (Garde-Cerdán, Marsellés-Fontanet, Arias-Gil, Ancín-Azpilicueta, & Martín-Belloso, 2008).

- ***Esters***

Volatile esters produced during alcoholic fermentation are of great interest, because of their key role in the sensorial quality of wines, being responsible of fruitiness, candy and perfume-like aroma but also of negative notes like “glue-like” aroma (Lambrechts & Pretorius, 2000; S M G Saerens et al., 2008).

Chitosan seemed to enhance the esters production, particularly isoamyl acetate, phenylethyl acetate and medium chain fatty acids (MCFA) ethyl esters, ethyl hexanoate, ethyl octanoate, ethyl decanoate and ethyl 3-hydroxybutanoate (**Table 3.4.3**). For ethyl esters, this done is in direct relationship with MCFA amounts in respective wines as the latter are the substrates and limiting factors for the syntheses of the former (S M G Saerens et al., 2008).

Acetate esters are formed through the condensation of higher alcohols with acetyl-CoA catalysed in the cell by alcohol acetyltransferase (ATF) enzymes (Mason & Dufour, 2000). However, in KT samples, results did not show any relationship between higher alcohols and acetate esters production (**table 3.4.3**). The reason for the higher amounts of acetates in KT wines is, thus, not clear but it is worth mentioning that ATF enzymes are regulated by the levels of unsaturated fatty acids (UFA) in the medium and that low concentrations in UFA correspond to higher quantities of acetate esters (Sofie M G Saerens, Delvaux, Verstrepen, & Thevelein, 2010).

After alcoholic fermentation, a lesser amount of ethyl lactate, ethyl malate, mono and diethyl succinate was found in KT wines. These compounds come from the esterification of the respective organic acids, whose lower amount in chitosan-treated wines (**table 3.4.2**) may well justify our results.

The presence of sulphites led to enhanced production of ethyl-4-hydroxybutanoate, which could be directly related to higher amounts of γ-butyrolactone in SO_2 added wines (Carrau et al., 2008)

As expected, during storage, acetate esters drastically decreased while ethyl esters increased to various extents (**table 3.4.3**) in accordance with previous findings (Saerens et al., 2008).

In particular, ethyl esters of organic acids significantly raised in concentration after 12 months of storage, and the presence of SO_2 further contributed in promoting their generation as already stated by other authors (Garde-Cerdán et al., 2008).

- ***Alcohols***

Together with acids and esters, alcohols are a further important class of yeast-derived volatile compounds in wines, since they play a considerable role in wine aroma (Nykänen, 1986). At the end of fermentation, there were no significant differences in total alcohols content among samples even if differences for some volatiles were found.

Isobutyl alcohol and 3-methyl-1-butanol amounts were higher in SO_2 added wines, confirming previous results that postulated that the presence of SO_2 during fermentation favours a prompt consumption of amino acids (Herraiz, Martin-Alvarez, Reglero, Herraiz, & Cabezudo, 1989; Sonni et al., 2009).

Quite surprisingly, however, other alcohols deriving from amino acids, such as 2-phenylethanol and 4-hydroxybenzenethanol, were not affected by the presence of SO_2, the reason for this behaviour remaining unclear.

Sulphites affected the amount of 3-ethoxy-1-propanol which, as already consistently reported (Herraiz et al., 1989; Sonni et al., 2009), is produced in lower quantities in the presence of SO_2.

For what concern chitosan, its pre-fermentative addition seemed not to have a considerable influence on alcohols contents, except for the lower levels of isobutyl alcohol and 3-methylthio-1-propanol, the both deriving from amino acid metabolism. This finding may be related to a reduced amino acid availability in musts due to the protein binding features of chitosan (Chatterjee, Chatterjee, Chatterjee, & Guha, 2004).

After 12 months of storage, total amount of alcohols in wines increased mostly due to 3-methyl-1-butanol and 2-phenetyl alcohol, without notable differences among samples. Most of the volatile compounds remained unchanged in quantity except 3-methylthio-1-propanol, benzyl alcohol and 4-hydroxy benzenethanol that decreased similarly to what has been already observed in previous works (Garde-Cerdán et al., 2008).

- ***Other compounds***

In wine, acetylation occurring between acetaldehyde and glycerol gives raise to heterocyclic compounds such as 1,3-dioxane and 1,3-dioxolane isomers. These compounds, with herbaceous or green olfactory nuances, have been reported to increase in content during wine conservation and aging and have been proposed as markers of Madeira wine ages (Câmara, Marques, Alves, & Silva Ferreira, 2003). Results showed that the amounts of 1,3-dioxanes and 1,3–dioxolane increased drastically during the conservation in bottle but, in

sulphite added wines this phenomenon was observed to a significantly lesser extent. This is due to the quenching of acetaldehyde by SO_2 that prevent the reaction with glycerol (da Silva Ferreira, Barbe, & Bertrand, 2002).

Furans are another class of heterocyclic compounds in wine. They mainly originate from monosaccharides that, in acidic medium, degrade via enolization and subsequent dehydration or react with amino acids following the Maillard chemistry (Belitz, Grosch, & Schieberle, 2009).

Their presence usually increases with time and is related to sugars level in wine. **Table 3.4.3** confirms the general augmentation of furanic compounds during storage, in particular for furfural, ethyl 5-oxotetrahydrofuran-2-furancarboxylate and hydroxymethylfurfural that, complessively, tended to be higher in SO_2 samples.

3.4.3.3. PCA Analysis of volatile compounds

Figure 3.4.3 shows the results of the application of PCA (Principal Component Analysis) to the entire dataset of wines volatile compounds. In that figure, for the sake of clarity, only the variables with the highest contribution to the total variance have been plotted.

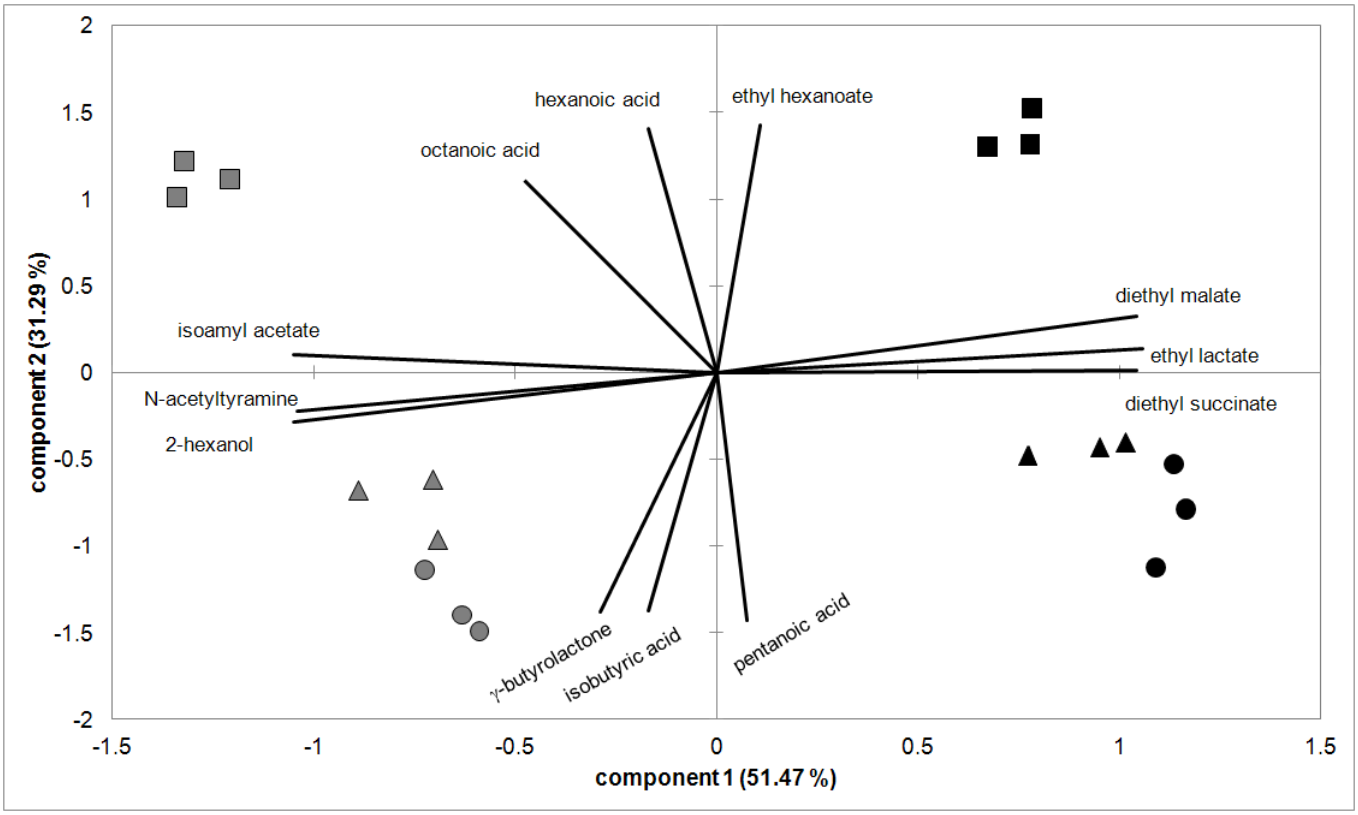

Figure 3.4.3. Principal component analysis. Plot of the samples in the plane defined by the first two principal components, at the end of fermentation (Time 0, grey labels) and at 12 months of storage (Time 12, black labels). Sample labels: ΔControl; ◯SO_2; ☐KT

The first component, which explains 51.47% of variance, clearly discriminates the samples based on the storage time. On this component, samples at bottling are located in the left quadrants, where the highest variance is due to N-acetyltyramine, isoamyl acetate and 2-hexanol. On the right side, the wines stored for 12 months are distinguishable for their content in ethyl esters of succinic, malic and lactic acids. Principal component 2 (31.29% of explained variance) produced a clear separation between KT and the other samples (Control and SO_2) due to the contribution of hexanoic and octanoic acids and ethyl hexanoate higher in KT wines, and γ-butyrolactone, isobutyric and pentanoic acids which characterized all the samples not containing chitosan.

3.4.4. Conclusions

The overall results demonstrated chitosan may affect the fermentation and composition of sulphite-free musts. When present all along the fermentation, chitosan may interact with yeasts, delaying the lag phase, and with organic acids, producing a decrease in total acidity. This fact should be taken into consideration even in the case of its use for musts clarification or during the stabilization steps of wines.

Concerning the volatile compounds, KT wines had higher concentrations of medium chain fatty acids and related ethyl esters, probably due to the alteration of cell permeability and subsequent perturbation of the fatty acid synthase complex.

Except some compounds deriving from amino acids metabolism, alcohols were less affected by the addition of the polysaccharide. Furthermore, differences in volatile composition were maintained over a 12 months storage time. Further investigations are currently being carried out at a semi-industrial scale, which may permit, together with the phenolic characterization, the sensory evaluation of sulphite-free wines fermented in the presence of chitosan.

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Chapter 3.5

"Preliminary studies of winemaking process at laboratory-scale: Evaluation of the influence of chitosan application prior to bottling"

Chapter 3.5

Preliminary studies of winemaking process at laboratory-scale: Evaluation of the influence of chitosan application prior to bottling

3.5.1. Introduction

Stabilization is a procedure carried out in oenology to ensure the quality of the wine over the time avoiding the production of cloudiness, sedimentation or the formation of tartrate crystals. This practice involves either treatments at low temperature where the wine is cooled to provoke crystallization before bottling, and fining. Fining is a winemaking process consisting of the addition of a substance (fining agent) to the wine which interacts with suspended particles, leading to the formation of larger molecules and bigger particles that will precipitate. After fining, soluble substances such as polymerized tannins, coloured phenols or proteins could be removed.

During this study, influence of the presence of chitosan during stabilization of white wines was evaluated. The aim of this work was to deepen into the fining and antioxidant properties of chitosan approaching the real conditions in winemaking. To this purpose, laboratory scale winemaking processes were carried out, and in this case, chitosan was added in wines obtained after fermentation of sulphite-free musts. In order to test the stability over the time of the final product, fixed and volatile composition of resulting wines were analysed after 6 months of bottling and compared to samples treated with sulphur dioxide in the same winemaking step.

3.5.2. Materials and methods

3.5.2.1. Chemicals

HPLC-grade acetonitrile, acetic acid and phosphoric acid were obtained from Merck (Darmstadt, Germany). Water was of Milli-Q quality. Pure standards of organic acids, volatile compounds, internal standard (2-octanol), potassium metabisulphite and ascorbic acid were purchased from Sigma-Aldrich. 75-85% deacetylated chitosan No[Ox] was purchased form the Institut Oenologique de Champagne (France).

3.5.2.2. Microvinifications

Frozen sulphite-free white musts were provided by CAVIRO wineries. Microvinifications were carried out in 2L laboratory glass fermentors (**Figure 3.5.1**)., at room temperature, to start the fermentation. Trials were arranged in triplicate, setting up six fermentors. Before yeast inoculation, three of the must samples (SO_2) were added with potassium metabisulphite at a dosage of 40 $mg \cdot L^{-1}$ while no addition was carried out in the three remaining samples. To avoid microbiological contamination and oxygen entrance during fermentation, each fermentor was provided of a glass tap filled with 37% H_2SO_4. A *Saccharomyces cerevisiae* strain already characterized for its low SO_2 production (Aleaferm Arom, Alea Evolution, Molinella (BO), Italy) (0.6 g/L) was inoculated after rehydration into each 2L of sterilized must. Fermentations were monitored by following the weight loss of samples. Once the weight loss stopped, yeast lees were left to settle down and clarified wines were transferred into new laboratory glass fermentors. Once transferred, wines obtained after SO_2-free fermentations were added with 0.5 $g \cdot L^{-1}$ of chitosan (KT).

Figure 3.5.1. Laboratory glass fermentors containing sulphite-free must before alcoholic fermentation.

All the fermentors were subjected to stabilization for 10 days at 4°C. KT samples were shaken one time a day to favour the contact with chitosan. Once the stabilization finished, clarified wines were transferred by means of a peristaltic pump (VWR international, Milano, Italy) into 125 mL glass bottles, without headspace, and stored for 5 months at room temperatures. Furthermore, to study the influence of SO_2 or KT in combination with other antioxidants, half of the bottles were added with ascorbic acid (AA) up to a concentration of 120 $mg \cdot L^{-1}$.

3.5.2.3. Oenological parameters

All the oenological parameters were determined according the analysis OIV methods (International Organisation of Vine and Wine (OIV), 2015). The pH was determined by using a pH-meter (Mettler Toledo, Spain). Alcoholic graduation of wine was determined by using an oenochemical distilling unit (Gibertini, Italy). Total polyphenols were determined (after wine filtration at 0.45 µm, with regenerated cellulose filters) at 280 nm using an Uvidec 610 spectrophotometer (Jasco, Tokyo- Japan). All the analyses were carried out in triplicate.

3.5.2.4. Organic acids

The HPLC used was a Jasco PU-1580 pump (Tokyio, Japan), a chromatography column model 7981 (Jones Chromatography) and two detectors: a Jasco UV-970 UV/Vis Detector and a Jasco 830-RI refraction index detector. HPLC conditions were the following: mobile phase: H_3PO_4 0.02N; pH: 2.6; flow: 0.6 mL/min; temperature: 45°C; detection wavelength: 215 nm. Chromatographic separation was performed using an isocratic method developed in the laboratory with two columns: a resin based HPLC Organic Acid Analysis Column (BIO-RAD, Aminex HPX-87H Ion Exclusion Column, 300 mm x 7.8 mm) and a column C18 Atlantis (Waters) T3, 5 µm, 100 Å, 6 x 250 mm. For the analysis a calibration curve for concentrations ranging from 0.001 to 2g/L using standard compounds was constructed. Standards for organic acids were: tartaric, malic, pyruvic, lactic, shikimic, citric, acetic and succinic. Analysis were carried out by triplicate.

3.5.2.5. Phenolic acids

Phenolic acid analysis was performed in a HPLC instrument equipped with a quaternary gradient pump Jasco PU-2089, an autosampler Jasco AS-2057 Plus Intelligent Sampler and two detectors: A Jasco UV/Vis MD-910 PDA detector and a Jasco FP-2020 Plus Fluorescence detector. The column was a C18 Poroshell 120 (Agilent technologies), 2.7µm, (4.6 x 150 mm), operating at 35° C with a flow of 0.8 mL/min. Elution solvents were 2% acetic acid in HPLC grade water (Eluent A) and 2% acetic acid in HPLC grade acetonitrile (Eluent B). Gradient elution was as follow: from 98%→95% A in 10 min, 95%→90% A in 7 min, 90→82% A in 6 min, 82%→80% A in 3 min, 80%→70% A in 3 min, 70%→50% A in 3 min, 50%→0% A in 4 min, 98% A in 1 min.

3.5.2.6. Wine volatile compounds

Volatile compounds were extracted according to the method described and validated by Lopez et al. (López, Aznar, Cacho, & Ferreira, 2002) which has been fully described in materials and methods of chapter 4.

3.5.2.7. Statistical analysis

Statistical analysis of the entire dataset was performed using the XLSTAT Software package (Version 2013.2, France). One-way analysis of variance (ANOVA) followed by a post hoc comparison (Tukey's HSD) were carried out.

3.5.3. Results and discussion

3.5.3.1. General Parameters

As shown in **table 3.5.1**, during alcoholic fermentation the presence of SO_2 set some significant differences compared to the wine obtained in the absence of sulphites. Fermentative kinetics of yeast was influenced by the addition of 40 $mg \cdot L^{-1}$, leading to a slightly lower alcohol content. However, despite the differences, the parameters remained within the normal range of the course of a winemaking.

After stabilization of wines, treatments with chitosan induced a reduction of 0,13 $g \cdot L^{-1}$ in total acidity, significantly higher than those samples added with SO_2, which suffer a reduction of 0.03 g/L. Slightly decrease of titrable acidity in SO_2 samples after stabilization could be due to the precipitation of potassium bitartrate during the cold treatment, while the significant reduction of total acidity after treatments with KT could be attributed to the acid biding properties of chitosan, reported by Scheruhn et al. (Scheruhn, Wille, & Knorr, 1999). To deepen this phenomenon, analysis of organic acid after each step of the winemaking process was carried out.

	Must	Alcoholic fermentation		After stabilization	
		SO_2	No SO_2	SO_2	KT
pH	3,35 ±0,01	3,34 ^{b}	3,37 ^{a}	3,30 ^{b}	3,34 ^{a}
Total acidity (g/L)	5,04 ±0,05	6,58 ^{a}	6,48 ^{b}	6,55 ^{a}	6,35 ^{b}
Volatile acidity (g/L)		0,31 ^{a}	0,24 ^{b}	0,29 ^{a}	0,24 ^{b}
[SO_2] (mg/L)		32	-		
Alcohol (v/v)		10,8 ^{a}	10,8 ^{b}		

Table 3.5.1. Oenological parameters of must and wines after each step of the winemaking process. In the same row, different letters indicate significant differences according to Tukey's test ($p < 0.05$). n=3.

3.5.3.2. Organic acids

Organic acid content during different stages of winemaking is displayed in **table 3.5.2**. Excepting pyruvic and citric acid, there were no significant differences between samples for any other organic acid after alcoholic fermentation. Higher content of pyruvic acid in SO_2 samples could be attributed to the accumulation of this metabolite as sulfonate and its successive and gradual hydrolysis. Further, the greater stress suffered from yeast (because of the presence of SO_2), may lead to the over-production of various metabolites, including pyruvic acid (Pronk, Yde Steensma, & Van Dijken, 1996).

After stabilization, significant decrease in organic acid content was displayed in samples conducted in the presence of chitosan, with consequent lower titrable acidity as outlined above. Furthermore, these results agreed with previous work (Castro-Marín, Buglia, Riponi, & Chinnici, 2018; Colangelo, Torchio, De Faveri, & Lambri, 2018) where chitosan, by electrostatic interactions between positive charged amino groups and dissociated organic acids was able to reduce their content in wines. Slightly reduction of total acidity in SO_2 could probably be due to the decrease of tartaric acid by precipitation of potassium bitartrate crystals formed during cold stabilization of wines.

	Must	Alcoholic fermentation		Stabilization		5 month storage			
		No SO_2	SO_2	KT	SO_2	KT	SO_2	KT+AA	SO_2+AA
Tartaric	3,15	3,12 ^{a}	3,09 ^{a}	2,45 ^{b}	2,80 ^{a}	2,15 ^{ab}	2,23 ^{ab}	1,81 ^{b}	2,24 ^{a}
Piruvic		206,28 ^{b}	306,21 ^{a}	190,75 ^{b}	306,92 ^{a}	276,37 ^{b}	354,73 ^{a}	258,67 ^{b}	357,55 ^{a}
Malic	1,54	1,49 ^{a}	1,54 ^{a}	1,25 ^{b}	1,47 ^{a}	1,08 ^{a}	1,07 ^{a}	1,01 ^{a}	1,11 ^{a}
Shikimic		7,60 ^{a}	8,55 ^{a}	5,95 ^{b}	8,49 ^{a}	6,90 ^{a}	6,56 ^{a}	7,12 ^{a}	5,81 ^{a}
Citric	0,31	0,26 ^{b}	0,28 ^{a}	0,23 ^{b}	0,30 ^{a}	0,24 ^{a}	0,21 ^{a}	0,28 ^{a}	0,21 ^{a}
Lactic		0,26 ^{a}	0,28 ^{a}	0,22 ^{a}	0,27 ^{a}	0,34 ^{a}	0,33 ^{a}	0,38 ^{a}	0,27 ^{a}
Acetic		0,21 ^{a}	0,18 ^{a}	0,15 ^{b}	0,19 ^{a}	0,32 ^{a}	0,22 ^{a}	0,31 ^{a}	0,20 ^{a}
Succinic		0,41 ^{a}	0,45 ^{a}	0,40 ^{a}	0,47 ^{a}	0,36 ^{a}	0,23 ^{b}	0,21 ^{b}	0,20 ^{b}

Table 3.5.2. Content (g/L) in organic acids of samples at four distinct stages of the winemaking process. *: Piruvic acid is given as mg/L. In the same row, different letters indicate significant differences according to Tukey's test at p≥ 0.05 (n=3)

3.5.3.3. Browning development

Monitoring of browning development was carried out by analysing the absorbance at 420 nm, which, as mentioned in chapter 3, corresponds to the maximum absorption of brown polymers. As depicted in **Table 3.5.3.**, after stabilization both samples experienced a slight increase in colour probably as a consequence of the oxygen uptake during the process of transferring the clear wines into glass fermentors to carry out the stabilization (see material and methods), being those treated with KT significantly less coloured.

	Must	Alcoholic fermentation		Stabilization		5 month of storage			
		SO_2	No SO_2	SO_2	KT	SO_2	SO_2 + AA	KT	KT + AA
Abs 420	0,046 ±0,004	0,099 ^{a}	0,104 ^{a}	0,136 a	0,123 ^{b}	0,248 ^{c}	0,334 ^{a}	0,239 ^{c}	0,281 ^{b}
Abs 280	5,070 ±0,053	7,030 ^{a}	6,787 ^{a}	7,330 a	7,180 ^{a}	8,303 ^{ab}	8,937 ^{a}	7,713 ^{b}	8,747 ^{a}

Table 3.5.3. Browning development (D.O. 420nm) and total polyphenol content (D.O. 280 nm) of must and wines after each step of the winemaking process. In the same row and for each sampling time, different letters indicate significant differences according to Tukey's test ($p < 0.05$). n=3.

In addition, after 5 months of storage, a similar trend of oxidation was observed without any significant difference between both tests. It is worth mentioning that while SO_2 was spiked again before bottling (see materials and methods), KT was settled down and removed after stabilization step. Hence sulfites were still present in bottle, protecting wines against oxidation However, due to the absence of KT during storage, its antioxidant effect must be triggered in the preceding stage, during stabilization of wine, remaining still active after bottling. As demonstrated in previous chapters, antioxidant effect of chitosan involves several mechanisms: 1) Chelation capacity, reducing the presence of metal slowing down Fenton reaction (Bornet &

Teissedre, 2008), 2) Scavenging of H_2O_2 as demonstrated in chapter 2, 3) Antiradical activity (chapter 1), inhibiting the generation of hydroxyl or 1-hydroxyethyl radical, and blocking the production of aldehydic intermediates of oxidation, such as glyoxylic acid and acetaldehyde (chapter 2), and 4) Direct absorption of polyphenols and their oxidation products (Chinnici, Natali, & Riponi, 2014; G. Spagna, Barbagallo, & Pifferi, 2000).

Regarding samples added with ascorbic acid during bottling, this compound exhibited a pro-oxidant effect as already demonstrated in previous experiments in model matrix (chapter 3). As can be observed in **table 3.5.3**, the presence of ascorbic acid enhanced the colour development of both treatments. In samples containing SO_2 + AA, this effect could be attributed to an insufficient concentration of SO_2 unable to react with H_2O_2 generated by oxidation of AA, enhancing Fenton reaction instead of inhibiting it. With respect to KT + AA, higher browning than in samples in the absence of ascorbic acid (KT) could be linked to the absence of any other compound in the bottle which could scavenge H_2O_2 produced. Nevertheless, combination of AA with a previous treatment with KT led to a lesser evolution of the colour than those bottled with SO_2 and AA. In this contest, the reduction of metallic content consequent to the activity of chitosan during stabilization indoubtedly may have played a role. As mention by Moreaux et al. (Moreaux, Birlouez-Aragon, & Ducauze, 1996) pro-oxidant activity of AA may be correlated to the presence of metal ions such as Fe^{3+} and Cu^{2+} which may be reduced by AA, enter into the redox cycle and therefore, generate hydroxyl radicals in a Fenton-like chemistry, starting browning spoilage (**Figure 3.5.2**).

Chelation capacity of KT may block the oxidation of AA, decreasing the concentration of dehydroascorbic acid and H_2O_2 (**Figure 3.5.2**). As already discussed in chapter 1, chemical oxidation in wines is mainly catalysed by the presence of metals than of H_2O_2. Those results confirmed what obtained in this work, where scavenging of H_2O_2 by means of SO_2 during its presence in the bottle, resulted less effective against oxidation than removal of metal ions during stabilization of chitosan.

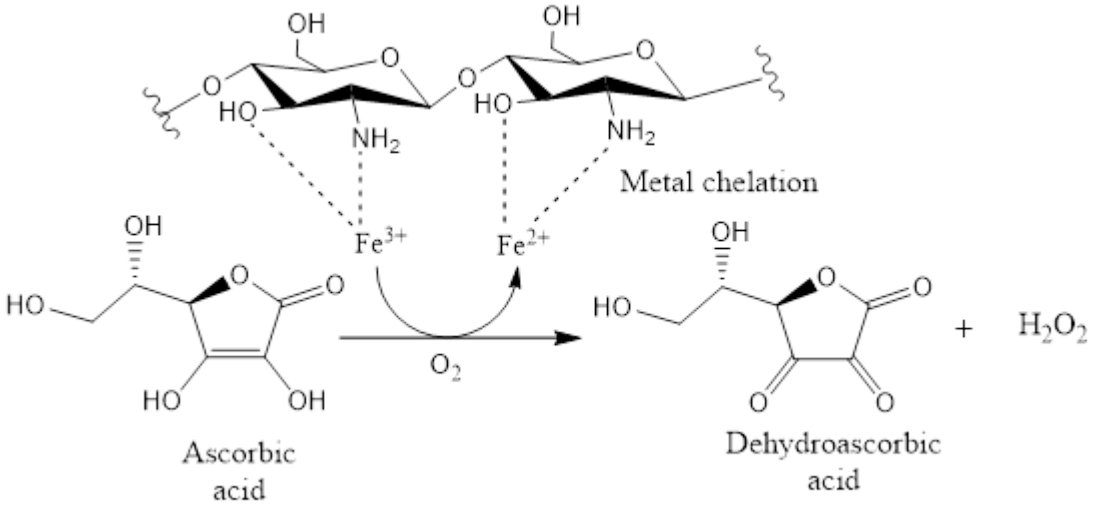

Figure 3.5.2. Mechanism of reduction of pro-oxidant effect of ascorbic acid by means of chitosan

3.5.3.4. Phenolic acids

Phenolic acids evolution over winemaking process is summarised in **Table 3.5.4**. As already reported by Colangelo et al., (Colangelo et al., 2018), almost any significant effect was observed after fining treatments with KT or SO_2. However, catechin, one of the most important polyphenols responsible of wine browning showed a higher decrease after stabilization with KT than those samples added with SO_2. These results, together with those already discussed above where evolution of colour was studied (**table 3.5.3**), may suggest that at least a part of the antibrowning effect of KT could be attributed to the removal of potentially oxidizable polyphenols such as catechin, avoiding future oxidation products. This trend has also been reported by different authors (Chinnici et al., 2014; Giovanni Spagna et al., 1996). Furthermore, Milhome et al., (Milhome et al., 2009) proposed several interaction mechanisms involving amino, acetamido and hydroxy functional groups of chitosan, that are prone to link phenolic acids by ion exchanges, van der Waals interactions and hydrogen bonds.

Interestingly, after 5 months of storage, depletion of catechin (mainly leading to oxidation products) was found to be significantly lower in samples stabilized with chitosan prior to bottling than in those added with SO_2. This trend could be attributed to the different antioxidant strategies carried out by chitosan as already outlined in section 3.3.

	Must	After Fermentation		After stabilization		5 month storage			
	Initial must	SO_2	No SO_2	SO_2	KT	SO_2	SO_2 + AA	KT	KT + AA
Tyrosol	n.d	3,642 ^{a}	3,489 ^{a}	3,468 ^{a}	3,369 ^{a}	3,013 ^{a}	3,043 ^{a}	3,260 ^{a}	2,983 ^{a}
Catechin	1,26 ± 0,013	0,830 ^{a}	0,734 ^{a}	0,624 ^{a}	0,443 ^{b}	0,357 ^{a}	0,310 ^{b}	0,283 ^{c}	0,297 ^{bc}
HMF	1,027 ± 0,003	0,244 ^{a}	0,215 ^{a}	0,455 ^{a}	0,434 ^{a}	1,343 ^{a}	1,397 ^{a}	1,413 ^{a}	1,343 ^{a}
Protocatecuic	0,22 ± 0,01	0,386 ^{a}	0,457 ^{a}	0,422 ^{a}	0,379 ^{b}	0,393 ^{a}	0,477 ^{a}	0,527 ^{a}	0,560 ^{a}
p-OH benzoic acid	0,63 ± 0,027	0,302 ^{a}	0,198 ^{a}	0,251 ^{a}	0,300 ^{a}	0,657 ^{a}	0,363 ^{a}	1,030 ^{a}	0,367 ^{a}
c-coutaric acid	0,88 ± 0,007	0,823 ^{a}	0,886 ^{a}	0,403 ^{b}	0,465 ^{a}	n.d.	n.d.	n.d.	n.d.
p-coumaric acid	n.d	0,451 ^{a}	0,294 ^{b}	0,305 ^{a}	0,188 ^{b}	n.d.	n.d.	n.d.	n.d.
GRP isomer	0,29 ± 0,006	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Caftaric acid	7,44 ± 0,026	6,306 ^{a}	5,937 ^{a}	5,196 ^{a}	5,042 ^{a}	4,880 ^{a}	4,907 ^{a}	4,900 ^{a}	4,520 ^{a}
GRP	1,25 ± 0,014	0,976 ^{a}	0,863 ^{a}	0,747 ^{a}	0,746 ^{a}	0,693 ^{a}	0,807 ^{a}	0,673 ^{a}	0,737 ^{a}
Caffeic acid	n.d	0,746 ^{a}	0,716 ^{a}	0,675 ^{a}	0,641 ^{a}	0,617 ^{a}	0,537 ^{a}	0,623 ^{a}	0,597 ^{a}
Fertaric acid	1,22 ± 0,036	0,534 ^{a}	0,517 ^{a}	0,509 ^{a}	0,489 ^{a}	0,460 ^{a}	0,447 ^{a}	0,470 ^{a}	0,440 ^{a}
Feroulic acid	n.d	0,437 ^{b}	0,478 ^{a}	0,393 ^{a}	0,416 ^{a}	0,413 ^{a}	0,460 ^{a}	0,413 ^{a}	0,413 ^{a}

Table 3.5.4. Phenolic acids content (mg·L-1), of must and wines after each step of the winemaking process. In the same row and for each sampling time, different letters indicate significant differences according to Tukey's test ($p < 0.05$). n=3

3.5.3.5. Volatile composition

The effect of both treatments on the volatile profile of white wine immediately after stabilization with KT or SO_2 and after 5 months of storage was investigated through GS-MS technique. Furthermore, the combination of ascorbic acid with SO_2 and KT was also tested. **Table 3.5.5** contains a list of all the identified compounds together with their corresponding retention time, their linear retention index and the method of identification followed.

tR (min)	Compound	LRI	Identification[a]
5,78	isobutyl alcohol	1104	Std, MS, LRI
6,74	isoamyl acetate	1127	Std, MS, LRI
7,02	3-penten-2-one	1134	Std, MS, LRI
7,19	n-butanol	1138	Std, MS, LRI
9,44	3-methyl-1-butanol	1194	Std, MS, LRI
10,28	ethyl n-caproate	1221	Std, MS, LRI
10,3	Unknown	1222	MS
11,63	ethyl pyruvate	1265	Std, MS, LRI
11,93	3-hydroxy-2-butanone	1275	MS, LRI
12,82	2-hexanol	1304	Std, MS, LRI
13,44	3-methyl-2-buten-1-ol	1319	Std, MS, LRI
14,19	ethyl lactate	1339	Std, MS, LRI
14,52	n-hexanol	1348	Std, MS, LRI
14,84	4-hydroxy-4-methyl-2-pentanone	1357	Std, MS, LRI
15,35	3-ethoxy-1-propanol	1370	Std, MS, LRI
15,72	3-hexen-1-ol	1380	Std, MS, LRI
17,30	ethyl 2-hydroxy-isovalerate	1421	Std, MS, LRI
17,74	ethyl octanoate	1432	Std, MS, LRI
17,82	linalool oxide	1434	MS, LRI
19,03	Furfural	1464	Std, MS, LRI
20,19	cis-5-hydroxy-2-methyl-1,3-dioxane	1493	MS, LRI
21,05	ethyl-3-hydroxybutyrate	1514	Std, MS, LRI
21,94	2-3, butandiol	1536	Std, MS, LRI
22,38	ethyl propanoate	1546	MS, LRI
23,07	isobutyric acid	1563	Std, MS, LRI
23,3	meso 2,3-butanediol	1568	MS, LRI
23,93	ethyl 3-hydroxypropionate	1583	MS
24,35	cis-4-hydroxymethyl-2-methyl-1,3 dioxolane	1593	MS
24,8	ethyl furoate	1608	Std, MS, LRI
24,94	butyrolactone	1616	Std, MS, LRI
25,08	n-butyric acid	1623	Std, MS, LRI
25,23	ethyl decanoate	1631	Std, MS, LRI
25,36	benzeneacetaldehyde	1637	MS, LRI
26,03	2-furanmethanol	1672	Std, MS, LRI
26,25	pentanoic acid	1683	MS, LRI

tR (min)	Compound	LRI	Identification[a]
26,28	Trans-4-hydroxymethyl-2-methyl-1,3-dioxolane	1685	MS
26,44	diethyl succinate	1693	Std, MS, LRI
27,48	3-methyllthio-1-propanol	1733	Std, MS, LRI
27,97	1,2-dihydro-1,1,6-trimethyl naphthalene	1750	MS, LRI
28,08	4-hydroxy-2-butanone	1754	MS
28,15	unk at 28.02	1757	MS
29,19	2,7-dimethyl-4,5 octandiol	1794	MS
29,24	ethylphenyl acetate	1796	Std, MS, LRI
29,79	ethyl 4-hydroxybutanoate	1822	Std, MS, LRI
30,01	2-phenylethyl-acetate	1833	Std, MS, LRI
30,11	cis-5-hydroxy-2-methyl-1,3-dioxane	1837	MS, LRI
30,76	hexanoic acid	1869	Std, MS, LRI
31,36	N-(3-methylbutyl)acetamide	1899	MS, LRI
31,45	benzyl alcohol	1902	Std, MS, LRI
31,98	ethyl 3-methylbutyl butanedioate	1921	MS, LRI
32,33	2-phenylethanol	1933	Std, MS, LRI
34,07	2H-piran-2,6 (3H)-dione	1992	MS
34,25	2-Furyl hydroxymethyl ketone	1998	MS, LRI
34,63	1H-Pyrrole-2-carboxaldehyde	2017	Std, MS, LRI
34,85	pantolactone	2029	Std, MS, LRI
34,97	diethyl hydroxybutanedioate	2035	Std, MS, LRI
35,32	octanoic acid	2053	Std, MS, LRI
37,30	N-acetylglycine ethyl ester	2170	MS
38,82	ethyl 5-oxotetrahydrofuran-2-furancarboxylate	2250	MS, LRI
39,31	decanoic acid	2274	Std, MS, LRI
39,39	ethyl 2-hydroxy-3-phenylpropanoate	2278	Std, MS, LRI
39,91	4-methyl-5-thiazolethanol (hemineurine)	2302	MS, LRI
40,33	diethyl tartrate	2318	Std, MS, LRI
41,33	ethyl hydrogen succinate	2355	Std, MS, LRI
42,53	2-furancarboxylic acid	2401	Std, MS, LRI
42,92	dodecanoic acid	2427	Std, MS, LRI
43,19	ethyl hydrogen fumarate	2445	MS, LRI
43,50	α-(phenylmethyl) benzeneethanol	2466	Std, MS
44,17	5-(hydroxymethyl)-2-furancarboxaldehyde	2514	Std, MS, LRI
45,5	Acetovanillone	2626	MS, LRI
46,20	tetradecanoic acid	2673	Std, MS, LRI
49,39	n-hexadecanoic acid	2803	Std, MS, LRI
50,73	1-H-indole-3-ethanol	2867	Std, MS, LRI
50,76	4-hydroxy benzaldehyde	2875	Std, MS, LRI
51,77	4-hydroxy-benzenethanol	2944	Std, MS, LRI
53,66	octadecanoic acid	3098	Std, MS, LRI

Table 3.5.5. List of identified compounds. [a] identification assignment: Std = comparing mass spectra, LRI and retention times with pure compounds, MS = by comparing mass spectra with NIST 08 and, Wiley 7 spectral database, LRI = matching LRI on comparable polar columns (taken from the following publicly available databases: https://pubchem.ncbi.nlm.nih.gov/; https://www.nist.gov/srd; http://www.flavornet.org/flavornet.html)

- ***Volatile composition of wines prior to bottling***

  Alcohols

Regarding alcohols (**Table 3.5.6**), only little differences were observed prior to bottling (time zero). Statically significant differences were 3-penten-2-ol, n-hexanol and 3-methyltio-1-propanol found in lower concentrations in KT samples while 3-ethoxy-1-propanol resulted in higher content. Concentration of 3-ethoxy-1-propanol was consisted with the results of different authors (Castro-Marin, Gabriela Buglia, Riponi, & Chinnici, 2018; Herraiz, Martin-Alvarez, Reglero, Herraiz, & Cabezudo, 1989; Sonni, Cejudo Bastante, Chinnici, Natali, & Riponi, 2009) been produced in lower quantities in fermentations carried out in the presence of SO_2. Formation of 3-methyltio-1-propanol derives from amino acid metabolism. According to Sonni et al., (Sonni et al., 2009), who found that the generation of this compounds is enhanced by the presence of sulphites during fermentation. However, contrarily to Herraiz et al., (Herraiz et al., 1989), n-hexanol was found in lower concentrations in samples containing SO_2.

Acids

Overall concentration resulted to be tendentially lower for SO_2 samples. However, only significantly differences were observed in octadecanoic acid (**Table 3.5.6**).

Esters

As for esters, similarly to what obtained for volatile acids, SO_2 and KT samples did not differed significantly among them (**Table 3.5.6**).

Others

Significant differences were only observed in 4-hydroxy-benzaldehyde and 2-furancarboxylic acid having a higher concentration in samples treated with KT and 2,5-dihydrothiophene which resulted to increase in SO2 samples (**Table 6**). 4-hydroxybenzaldehyde could be present in the grapes with a glycosidic bond. It could be that the presence of SO_2 somehow inhibited the enzymatic cleavage of this bond resulting in a lower concentration of this volatile phenol in the wine. As expected, the presence of sulfites enhanced the formation of thiol compounds, such as 2,5-dihydrothiophene.

- ***Volatile composition of wines after 5 months of storage***

  Alcohols

After a 5 months period of storage, alcohols content increased mainly due to the higher presence of 3-methyl-1-butanol and 2-phenlylethanol. Concentrations of 3-methylthio-1-propanol and 4-hydroxy-benzeneethanol remained unchanged over the time in samples added with SO_2 while a decrease was observed in KT samples, similarly to what obtained in other works (Castro-Marín et al., 2018; Garde-Cerdán,

Marsellés-Fontanet, Arias-Gil, Ancín-Azpilicueta, & Martín-Belloso, 2008). Furthermore, for both additives, concentration of n-butanol and 3-methyl-1-butanol were found to decrease with the addition of AA. Lastly, content of 4-hydroxy-benzenethanol resulted to increase in the presence of AA in SO_2 samples but decrease in KT ones.

Acids

The small differences found after stabilization have been also confirmed after 5 months of bottle storage. To note the relevant lesser amount of octadecanoic acid in KT samples, reason of which remains not clear.

Esters

As reported in **table 3.5.6**, concentration of esters raised after 5 months of storage in both samples. However, as already stated by Garde-Cerdán et al., (Garde-Cerdán et al., 2008), their production was enhanced by the presence of SO_2. According to what obtained in previous studies by Castro-Marin et al., (Castro-Marin et al., 2018), after storage, lesser content of ethyl pyruvate, diethyl succinate, 2-phenylethyl acetate, diethyl malate and diethyl L-tartrate was observed. Since these compounds are formed via esterification of organic acids, decrease of organic acids after stabilization with KT (**Table 3.5.2**) would lead to lower amounts of these ester compounds. Addition of AA raised the generation of ethyl furoate, a compound provided from the esterification of furoic acid, the latter formed during oxidation of ascorbic acid (Bradshaw, Barril, Clark, Prenzler, & Scollary, 2011). However, the presence of AA exerted similar effect for both SO_2 and KT samples.

Other

Aside from 2-furanmethanol, all furan compounds were found in higher concentrations for SO_2 samples. Interesting results were obtained regarding furfural, which was in a considerably lower concentration in bottles stored after stabilization with KT. However, this effect was completely nullified by the presence of AA. Higher concentrations of N-(3-methylbutyl) acetamide and N-butyl-benzensulfonamide were found for SO_2 samples. Furthermore, only in samples added with AA, two different compounds were detected. One was identified as 3-penten-2-one (a compound already reported to be formed from the degradation of ascorbic acid) while the other remains unknown.

3.5.4. Conclusion

Overall, fining with chitosan showed a significant effect mainly on the fixed composition, by reducing organic acid content through electrostatic interactions. Interestingly, treatments with chitosan seemed to protect against oxidation similarly to the addition of sulphites, suggesting the potentiality of chitosan to reduce the use of sulfur dioxide in winemaking. Furthermore, regarding volatile composition of wines, little differences were observed among SO_2 and KT treatments.

The presence of ascorbic acid did not seem to exert positive results, even in the presence of sulphites and, on the contrary, revealed a tendency to cross-over toward a pro-oxidant behaviour.

Based on our results, stabilization of white wines with chitosan could represent a successful strategy to protect wines from oxidative spoilage or to reduce the applied dose of sulfur dioxide without compromising the overall quality of wine. However, is worth to mention that this work has been carried out at laboratory scale and successive experiments at higher volumes should be performed in order to get closer to real winemaking conditions.

	Wines					
	After stabilization		5 months of storage			
	SO_2	KT	SO_2	SO_2AA	KT	KTAA
Alcohols ($\mu g \cdot L^{-1}$)						
isobutyl alcohol	14764 [a]	13548 [a]	14797 [a]	12381 [a]	15530 [a]	13853 [a]
n-butanol	133 [a]	127 [a]	151 [ab]	125 [b]	210 [a]	124 [b]
3-penten-2ol	728 [b]	621 [a]	n.d.	n.d.	n.d.	n.d.
3-methyl-1-butanol	38460 [a]	35032 [a]	44330 [b]	39861 [b]	55946 [a]	42980 [b]
2-hexanol	237 [a]	221 [a]	n.d.	n.d.	n.d.	n.d.
3-methyl-2-buten-1-ol	41 [a]	38 [a]	n.d.	n.d.	n.d.	n.d.
n-hexanol	153 [b]	133 [a]	171 [a]	170 [a]	173 [a]	146 [b]
3-ethoxy-1-propanol	27 [a]	37 [b]	31 [ab]	27 [b]	35 [a]	32 [ab]
3-hexen-1-ol	24 [a]	21 [a]	32 [a]	31 [a]	34 [a]	28 [a]
2-ethyl hexanol	n.d.	n.d.	33 [ab]	41 [a]	33 [b]	29 [b]
2-3, butandiol	2100 [a]	1991 [a]	1764 [a]	1696 [a]	1555 [a]	1861 [a]
2-3, butandiol (meso)	588 [a]	546 [a]	514 [a]	458 [a]	443 [a]	515 [a]
1-methoxy-2-butanol	82 [a]	95 [a]	n.d.	n.d.	n.d.	n.d.
3-methylthio-1-propanol	545 [b]	460 [a]	610 [ab]	648 [a]	486 [b]	564 [ab]
benzyl alcohol	n.d.	n.d.	138 [a]	138 [a]	108 [a]	131 [a]
2-phenylethanol	15329 [a]	14731 [a]	19559 [a]	20868 [a]	17729 [a]	18990 [a]
1-H-indole-3-ethanol	205 [a]	278 [a]	n.d.	n.d.	n.d.	n.d.
4-hydroxy-benzenethanol	11505 [a]	11189 [a]	11277 [ab]	14575 [a]	9364 [ab]	8138 [b]
Acids ($\mu g \cdot L^{-1}$)						
isobutyric acid	1745 [a]	1821 [a]	1684 [a]	1690 [a]	1809 [a]	1693 [a]
n-butyric acid	451 [a]	506 [a]	485 [ab]	565 [a]	485 [ab]	477 [b]
pentanoic acid	549 [a]	536 [a]	630 [ab]	683 [a]	579 [b]	589 [b]

	Wines					
	After stabilization		5 months of storage			
	SO_2	KT	SO_2	SO_2AA	KT	KTAA
hexanoic acid	2107 [a]	2197 [a]	2323 [a]	2560 [a]	2288 [a]	2569 [a]
octanoic acid	4303 [a]	4714 [a]	4677 [a]	5004 [a]	4017 [a]	5092 [a]
decanoic acid	1257 [a]	1474 [a]	1264 [ab]	1489 [a]	862 [b]	1268 [ab]
dodecanoic acid	54 [a]	64 [a]	n.d.	n.d.	n.d.	n.d.
tetradecanoic acid	83 [a]	75 [a]	79 [a]	77 [a]	81 [a]	78 [a]
n-hexadecanoic acid	495 [a]	400 [a]	345 [a]	328 [a]	369 [a]	377 [a]
octadecanoic acid	814 [b]	457 [a]	1574 [a]	2074 [a]	188 [b]	165 [b]
Esters ($\mu g \cdot L^{-1}$)						
isoamyl acetate	649 [a]	543 [a]	477 [a]	420 [a]	512 [a]	425 [a]
ethyl n-caproate	258 [a]	257 [a]	566 [a]	575 [a]	615 [a]	565 [a]
ethyl pyruvate	147 [a]	115 [a]	268 [a]	256 [a]	115 [c]	174 [b]
ethyl lactate	1091 [a]	1090 [a]	2035 [a]	1989 [a]	2232 [a]	1796 [a]
ethyl 2-hydroxyisovalerate	39 [a]	49 [a]	149 [a]	147 [a]	132 [a]	146 [a]
ethyl octanoate	114 [a]	124 [a]	895 [b]	959 [ab]	1036 [a]	1016 [ab]
ethyl-3-hydroxybutyrate	153 [a]	168 [a]	120 [a]	118 [a]	131 [a]	127 [a]
ethyl 3-hydroxypropionate	n.d.	n.d.	37 [a]	38 [a]	48 [a]	37 [a]
ethyl furoate	n.d.	n.d.	24 [c]	82 [a]	12 [c]	62 [b]
ethyl decanoate	46 [a]	44 [a]	225 [a]	240 [a]	159 [b]	201 [ab]
diethyl succinate	264 [a]	290 [a]	1499 [a]	1691 [a]	1265 [b]	1541 [a]
ethyl 4-hydroxybutanoate	6336 [a]	6312 [a]	2496 [a]	2324 [a]	2091 [a]	2658 [a]
2-phenylethyl-acetate	157 [a]	166 [a]	133 [ab]	142 [a]	103 [c]	110 [bc]
diethyl malate	274 [a]	289 [a]	2397 [a]	2717 [a]	1497 [b]	2000 [ab]
ethyl 2-hydroxy-3-phenylpropanoate	n.d.	n.d.	662 [a]	760 [a]	509 [a]	668 [a]

	Wines					
	After stabilization		5 months of storage			
	SO_2	KT	SO_2	SO_2AA	KT	KTAA
diethyl L-tartrate	n.d.	n.d.	154 ab	191 a	114 b	84 b
ethyl hydrogen succinate	8312 a	8766 a	28445 a	28620 a	23978 a	24338 a
Others ($\mu g \cdot L^{-1}$)						
1H-pyrrole-2-carboxaldehyde	10 a	13 a	n.d.	n.d.	n.d.	n.d.
4-hydroxy-benzaldehyde	347 a	568 b	681 a a	335 b b	126 b b	251 b b
furfural	387 a	446 a	1143 a a	955 a a	187 b b	1128 a a
furfuryl alcohol	104 a	104 a	165 ab ab	161 b b	320 a a	106 b b
2-furyl hydroxymethyl ketone	17 a	19 a	33 ab ab	48 a a	25 b b	32 ab ab
ethyl 5-oxotetrahydrofuran-2-furancarboxylate	467 a	509 a	1278 ab ab	1397 a a	773 b b	970 ab ab
2-furancarboxylic acid	148 a	215 b	468 bc bc	793 a a	255 c c	496 b b
5-hydroxymethyldihydrofuran-2-one	78 a	98 a	n.d.	n.d.	n.d.	n.d.
HMF	125 a	137 a	467 ab ab	633 a a	374 b b	328 b b
3-hydroxy-2-butanone	60 a	49 a	43 a a	53 a a	78 a a	35 bc bc
3-penten-2-one	n.d.	n.d.	0 b b	80 a a	0 b	98 a a
□-butyrolactone	288 a	252 a	296 a a	286 a a	257 a a	256 a a
pantolactone	84 a	76 a	99 ab ab	119 a a	58 c c	77 bc bc
6,7-dihydro-7-hydroxylinalool	n.d.	n.d.	39 ab ab	43 a a	30 b b	35 ab ab
unknown at 10.22	n.d.	n.d.	0 b b	68 a a	0 b b	64 a a
N-(3-methylbutyl)acetamide	137 a	134 a	169 ab ab	172 a a	114 b b	131 ab ab
6,7-dihydro-7-hydroxylinalool	30 a	29 a	n.d.	n.d.	n.d.	n.d.
emineurine	97 a	116 a	113 ab ab	123 a a	106 ab ab	78 b b
ethyl 5-oxo-L-prolinate	n.d.	n.d.	486 ab ab	776 a a	503 ab ab	467 b b

	Wines					
	After stabilization		5 months of storage			
	SO_2	KT	SO_2	SO_2AA	KT	KTAA
N-butyl-benzenesulfonamide	n.d.	n.d.	349 a [a]	452 a [a]	127 a [a]	92 a [a]
2,5-dihydro-thiophene	215 [b]	182 [a]	n.d.	n.d.	n.d.	n.d.

Table 3.5.6. Volatile composition of wines after stabilization and after 5 months of bottle storage. In the same row and for each sampling time, different letters indicate significant differences according to Tukey's test ($p < 0.05$). n=3.

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Chapter 3.6

"Effects of chitosan on white musts and wines subjected to hyperoxydation and *sur lies* fining: trials at semi-industrial scale"

Chapter 3.6

Effects of chitosan on white musts and wines subjected to hyperoxydation and sur lies fining: trials at semi-industrial scale.

3.6.1. Introduction

After previous experimentations and based on those results a scaling up at the semi-industrial level of the winemaking process was put in place. The aim of the experiment was to study the effect of chitosan during the stages of flotation with air and successive stabilization on yeast lees. Flotation is a dynamic method for must clarification based on the principle of flocculation. By means of a previous addition of a fining agent, such as gelatine or bentonite, must solid particles are coated, producing the formation of floccules. Subsequently, by using a flotation pump, gas bubbles (usually nitrogen) are inserted and adhered to the floccule, rendering it lighter than the must and therefore rising it to the surface to be eliminated.

In our experiments, flotation was conducted by using oxygen as flotating gas, in this way promoting the solubilization of oxygen into the wine in a way similar to what is done during the so-called hyperoxydation.

Hyperoxidation makes use of deliberate oxidation prior to fermentation in order to improve wines' shelf-life thanks to the removal of oxidizable compounds (e.g. flavonoids and phenolic acids) that precipitate as polymers (Schneider, 1998). This technique takes advantage of the enzymic pool of fresh musts in order to early oxidize phenols supposedly without affecting the volatile composition of resulting wines (that, providing from non-aromatic grapes, largely depends on secondary or tertiary aromas) (Schneider, 1998).

Therefore, this works intended to deepen the understanding of how chitosan, characterized by a protein stabilization activity, could limit, during flotation and stabilization, the effect of enzymatic oxidation and, subsequently, the outcomes of the chemical oxidation that occurs during conservation. To this purpose, absorbing effect of the polysaccharide on polyphenols and development of coloured compounds during the whole winemaking process have been evaluated. Furthermore, general parameters, dissolved oxygen, and organic acid content have been assessed as well.

The test, which has been divided into two different experiences one with the addition of chitosan in flotation and stabilization, and a second with sulphur dioxide (SO_2) highlighted significant differences between different samples with particular regard to the fate of phenolic acids and the extent of oxidation.

3.6.2. Materials and methods

Winemaking process has been conducted entirely in the department of research and development of CAVIRO winemakers (Faenza, Italy).

3.6.2.1. Winemaking process

- ***Pressing and flotation***

The variety used in this experiment was the Sangiovese which, without remaining in contact with the skin, has followed a classic white winemaking. The must was obtained with a pneumatic press working at low pressure for a short period of time. The obtained must was introduced in six steel tanks of 30 HL capacity (**Figure 3.6.1**) and two different winemaking processes were carried out: In the first triplicate samples 60 mg/L of SO_2 were added, while other 3 tanks were treated with 80 g/Hl of KT (chitosan No [Ox] -IOC).

Figure 3.6.1. Steel tanks and flotation unit used for the winemaking process

Prior to flotation, 2 g/Hl of pectolytic enzymes (Flottozima, Vason) were added in both tanks to increase the degradation rate of the pectin (responsible for the viscosity of the must) and 5 g/Hl of potato gelatine (Vegecoll, Laffort) to facilitate the flocculation of must.

After the aforementioned addition, flotation was carried out in order to obtain a clear must. Flotation unit was Fattoria 100 (Enomet, Saltara (PU) Italy) with a flow rate of 100 Hl/h and an air flow of 12 l/h for a total duration of 45 minutes, able therefore to float a mass equal to 75 Hl while providing 30 mL/L oxygen, which could be considered a typical value for hourly oxygen consumption in white musts (Schneider, 1998). This mobile unit, which has been designed to allow flotation in all types of wineries, both large and medium-small, permits a small residual volume of part of the product, equal to 2-5% of the initial volume, with a consequent reduction in times and costs of filtration.

- ***Alcoholic fermentation***

At the end of the 45 minutes of flotation and after 1 hour of waiting for flocculation, clear must was transferred and inoculated with 30 g/Hl of a low-sulfur producing yeast (Aleaferm Arom, Alea Evolution, Molinella (BO), Italy) to start the alcoholic fermentation (FA). A 25 g/Hl of Aleavit one, a fermentation activator and 3 g/Hl of gallic tannins were also added. In SO_2 samples, further addition of 40 mg/L of sulfur dioxide was carried out, reaching levels of 70 mg/L of SO_2 during fermentation. All fermentations were carried out in autoclave of 20 Hl

- ***Stabilization and "sur lies" fining***

Once the FA stopped (residual sugars < 2 g/L), 0.5 g/L of chitosan (Microstab, Agrovin) were added to KT samples (and not to SO_2 wines), without removing yeasts lees. Subsequently, samples were stored at 10°C for a duration of 30 days: chitosan-containing tanks were agitated every two days by bubbling CO_2 to enhance the contact of KT with wine components. The aim of this step was to carry out the stay on lees and the stabilization at the same time and inside the same container and to evaluate the effect of KT during the process.

- ***Bottling***

During bottling in volumes of 500 and 750 ml, the entry of oxygen was limited by the use of nitrogen, which, in addition to limiting oxidative phenomena, maintains intact the quality and sensory characteristics of the final product. During this step, each trial was further subdivided into two aliquots one of which added of 100 mg/L of ascorbic acid (AA). At the time of bottling and after 6 months of storage, different determinations were carried out.

3.6.2.2. Chemical Analyses of wines

- ***General parameters, Organic acids and phenolic acids***

These parameters were determined following the methods cited on materials and method section of chapter 3.4.

- ***Sensory analysis***

Sensory analysis of both the thesis was performed. Sensory analyses were performed with a trained panel of 17 judges. A quantitative descriptive analysis (QDA) was carried out using the sheets with the typical descriptors for white wines. Sheets had a graduated scale of 10 cm. Final QDA profile was obtained by reporting the average intensity values for each descriptor in a spider web type graph. (**Figure 3.6.5**), representing the sensorial profile of the product, easily comprehensible, offering an immediate quantitative-descriptive perception.

3.6.3. Results and discussion

3.6.3.1. Development of browning during the winemaking process in the presence of chitosan

Significant effect of KT on colour and browning development was appreciated. As shown in **figure 3.6.2A**, colour of must added with KT before flotation was significantly higher than sample with addition of 60 mg/L of SO_2. However, once flotation was carried out, samples containing SO_2 slight increased its colour due to the oxidation of phenolic compounds during hyperoxygenation, while in KT musts, even if started from a higher coloured must, flotation caused a reduction of coloured pigments responsible of browning in must. This phenomenon could be attributed to three different mechanisms: 1) KT, characterized by a protein stabilization activity as reported by Colangelo et. al, (Colangelo, Torchio, De Faveri, & Lambri, 2018), could limit the effect of enzymatic oxidation, 2) direct absorption of the oxidized compounds, or their precursors, in KT matrix (Chinnici, Natali, & Riponi, 2014; Spagna et al., 1996) 3) the presence of sulphites significantly inhibits oxygen consumption and flotation efficacy (Dubourdieu & Lavigne, 1990).

After flotation, clear must were transferred into tanks where fermentation took place. Once finished, increase of colour was observed in both samples without presenting any significant difference. Rising of colour during fermentation was certainly due to the intake of oxygen during flotation which, differently from the traditional method that uses nitrogen, was carried out in the presence of oxygen, to combine the effect of two techniques of must stabilization, flotation and hyperoxygenation.

Furthermore, after cold stabilization and yeast lees contact for 30 days, the effect of the second addition of KT was appreciated. Samples treated with 0.5 g/L of the polysaccharide showed a reduction of colour, reaching levels significantly lower than in those samples added with SO_2. This result confirmed those obtained in the previous chapters, where oxidised compounds were absorbed in KT matrix, being precipitated like a sediment.

After stabilization, samples were transferred into glass bottles. As outlined in **figure 3.6.2A**, colour level remained constant after 6 months of stabilization in both samples, being still significantly lower in KT samples. It is worth to mention that KT, due to its insolubility in wine, was removed prior to bottling. Hence, the stability presented during storage could be due to a previous absorption of phenolic compounds and reduction of metallic content by means of KT during stabilization as already demonstrated in previous studies.

Regarding total phenolic content **(figure 3.6.2B)**, flotation and stabilization treatments in the presence of KT, exhibited a significant decrease. This result is consistent with browning development described above and supported by many publications which reported the interaction of KT with phenolic compounds where a direct absorption of polyphenols is observed by the formation of a chitosan-polyphenol complex. This phenomenon of absorption confirmed one of the indirect antioxidant mechanisms of chitosan.

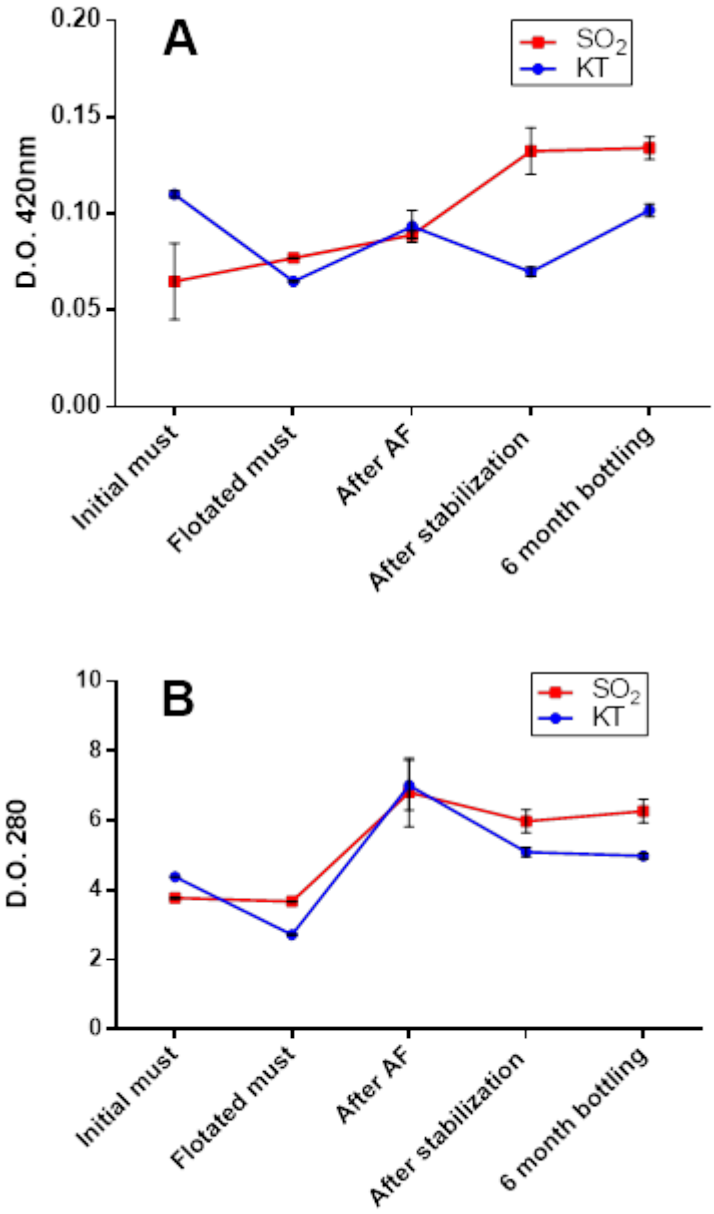

Figure 3.6.2. Browning (**A**) and total polyphenol development (**B**) at different stages of winemaking

3.6.3.2. Evolution of general parameters during winemaking

It is worth to mention that, from the beginning of the winemaking process, initial must was immediately separated into two different fractions. One without any additive and a second one with addition of 60 mg/L of SO_2. Due to this, results were interpreted by focusing on the increment of each sample with respect to its previous step.

As shown in **table 3.6.1**, a similar pattern was observed in the two musts after flotation with KT or SO_2. pH did not change in either case, while total acidity undergone toward a slight decrease in both treatments.

After FA, a decrease in total acidity was noticed in both treatments due to the precipitation of tartaric acid. This trend could also be due to that during FA, samples unexpectedly underwent malolactic fermentation at the same time, with the consequent increase in volatile acidity as well, as outlined in **table 3.6.1**. Furthermore, volatile acidity resulted to be significantly higher in samples floated with KT than in those added with SO_2. This trend could be due to a different strain selectivity of the two additives, that however did not avoid the completion of the MLF.

	Must		After flotation		After fermentation		Pre storage	
	No SO_2	SO_2	KT	SO_2	KT	SO_2	KT	SO_2
pH	3,21 ^{a}	3,22 ^{a}	3,32 ^{a}	3,26 ^{a}	3,36 ^{a}	3,28 ^{b}	3,41 ^{a}	3,28 ^{b}
Total Acidity	5,51 ^{a}	5,72 ^{a}	5,05 ^{a}	5,15 ^{a}	4,65 ^{a}	4,78 ^{a}	4,23 ^{a}	4,45 ^{b}
Volatile acidity	-	-	-	-	0,46 ^{a}	0,39 ^{b}	0,53 ^{a}	0,42 ^{a}
% Alcohol	-	-	-	-	12,1 ^{a}	11,9 ^{a}	-	-

Table 3.6.1. Oenological parameters of must and wines after each step of the winemaking process. In the same row, different letters indicate significant differences according to Tukey's test ($p < 0.05$). n=3.

Regarding alcohol, no significant differences were observed in samples both samples followed the same fermentations kinetics, without significant difference among treatments.

After stabilization and lees contact, decrease in pH was observed in both samples, even if the final pH with KT was 0.04 units higher than SO_2. In the case of total acidity, a slight decrease was appreciated, to a higher extent in samples stabilized with KT when compared with the previous stage: 0.42 g/L with KT and 0.33 g/L with SO_2. This trend was surely due to the precipitation of tartaric acid after cold stabilization, while the greater difference in KT samples could be due to the acid-binding properties of the polysaccharide, as already described in previous experiments.

3.6.3.3. Evolution of organic acids in the presence of chitosan

Organic acid evolution after different stages of winemaking is outlined in **table 3.6.2**. Results demonstrated that 45 minutes of flotation that allowed to float a mass equal to 75 Hl were enough to have a visible effect on colour without influencing the concentration of organic acids of musts. This phenomenon was already observed while studying general parameters, where no differences were found among samples.

Based on the significantly reduction of malic acid and the production of high levels of lactic acid (**table 3.6.2**), development of MLF parallel to FA was confirmed in both samples containing KT and SO_2. This phenomenon was not attributed to a lower protection of chitosan against lactic acid bacteria (LAB), given that MLF also occurred in sulphitated samples, but rather because of the uncontrolled temperature in which FA was conducted. Regarding acetic acid, higher content in wines provided from must floated with KT confirmed the higher volatile acidity discussed above.

During stabilization of wines at 10 ºC, production of lactic acid was stopped in both cases, while a slight increase in acetic acid content of 0.06 g/L in samples stabilized with KT and of 0.05 g/L in SO_2 was observed.

After 6 months of storage, a further increase in the acetic acid and lactic acid content was a noticed, verifying the development of MLF.

	Initial must		Floated must		Alcoholic fermentation		Stabilization		6 month storage	
	No SO_2	SO_2	KT	SO_2	KT	SO_2	KT	SO_2	KT	SO_2
Tartaric	3.51 ^{a}	3.47 ^{a}	3.33	3.43	3.29 ^{a}	3.38 ^{a}	2.88 ^{b}	3.15 ^{a}	2,81 ^{a}	3.02 ^{a}
Malic	1,37 ^{a}	1,24 ^{b}	1,35 ^{a}	1,30 ^{a}	0,17 ^{b}	0,24 ^{a}	0,17 ^{b}	0,27 ^{a}	0,13 ^{b}	0,20 ^{a}
Shikimic	4,11 ^{a}	3,70 ^{b}	4,06 ^{a}	3,70 ^{b}	5,53 ^{a}	4,89 ^{a}	5,38 ^{a}	4,78 ^{b}	5,13 ^{a}	5,07 ^{a}
Citric	0,18 ^{a}	0,15 ^{a}	0,17 ^{a}	0,20 ^{a}	0,18 ^{a}	0,17 ^{a}	0,16 ^{a}	0,19 ^{a}	0,20 ^{a}	0,17 ^{a}
Lactic					1,57 ^{a}	1,55 ^{a}	1,56 ^{a}	1,58 ^{a}	1,98 ^{a}	1,91 ^{a}
Acetic					0,37 ^{a}	0,24 ^{b}	0,43 ^{a}	0,29 ^{b}	0,64 ^{a}	0,5 ^{b}
Succinic					0,39 ^{a}	0,46 ^{a}	0,41 ^{a}	0,45 ^{a}	0,29 ^{a}	0,33 ^{a}
Glicerol					4,93 ^{a}	4,60 ^{a}	4,85 ^{a}	4,60 ^{a}	4,91 ^{a}	4,49 ^{a}

Table 3.6.2. Organic acid content ($g \cdot L^{-1}$) except shikimic ($mg \cdot L^{-1}$) after each winemaking stage. In the same row, different letters indicate significant differences according to Tukey's test ($p < 0.05$). n=3.

3.6.3.4. Evolution of phenolic acids during winemaking process

Analysis of phenolic acids seemed to show that during the present experiment of winemaking at semi-industrial scale, differences among treatments were notable on hydroxycinnamic acids development. Hydroxycinnamic acids are phenolic compounds that belongs to the group of non-flavonoid polyphenols. Normally found in the pulp and in the vacuoles of the skin, they are present in the form of tartaric esters, like caftaric acid (tartaric ester of caffeic acid), coutaric acid (ester of coumaric acid) and fertaric acid (ester of ferulic acid). Furthermore, these compounds can be found on their free form by means of hydrolysis of the link with tartaric acid, releasing their corresponding cinnamic acids: caffeic, coumaric and ferulic acid.

This family of phenolic acids are characterised by different properties: 1) stabilization of colour through co-pigmentation with anthocyanins, 2) antioxidant, by neutralizing free radicals responsible for oxidative processes, 3) aromatic precursors, being converted by *Brettanomyces* and *Dekkera* into volatile phenols such as 4-ethylphenol and 4-ethylguaiacol, associated with the so-called “Brett” aromatic descriptor.

Hydrolysis can take place by means of two different mechanisms: 1) chemical hydrolysis due to wine acidity, a slow process that progresses gradually through winemaking and storage, 2) Enzymatic hydrolysis, through enzymes added or through microorganisms endowed with a specific enzyme, “cinnamoyl esterase”. Some strains of lactic bacteria, such as *Oenoccocus* and *Lactobacillus* are characterized by this enzymatic property.

As mentioned in the previous section, during the AF, parallel MLF took place. In fact, as observed in **figure 3.6.3**, hydrolysis of fertaric acid leading to the production of ferulic acid started only during AF, which confirms the cinnamoyl esterase activity carried out by LAB. However, hydrolysis of caftaric and coutaric acid

were not observed during this stage. This phenomenon could be attributed to the lower concentration with respect to fertaric acid.

Furthermore, after stabilization and yeast lees contact, releasing of caffeic acid with the consequent decrease of caftaric acid was observed. Cinnamoyl esterase activity further continued after 6 months of storage, with an overall increase of the free hydroxycinnamic acids.

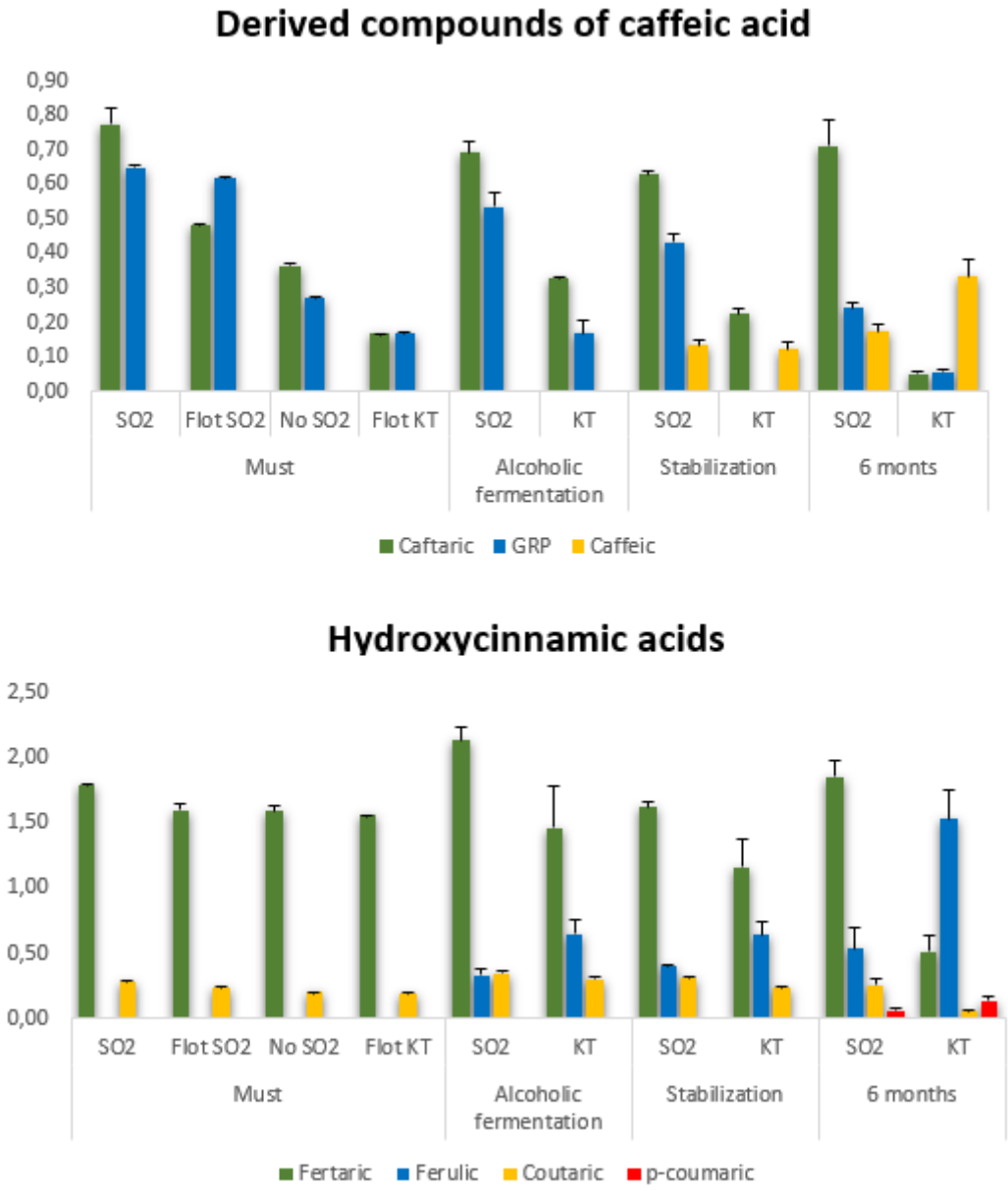

Figure 3.6.3. Hydroxycinnamic acid content ($mg \cdot L^{-1}$) at different stages of the winemaking process. Upper: Derived compounds of caffeic acid. Bottom: Hydroxycinnamic acids

Regarding different treatments, in **figure 3.6.3** can be observed a different development of hydroxycinnamic acids in the presence of KT with respect to SO_2. Ferulic, caffeic and p-coumaric acid generation was enhanced in the presence of KT. Based on the obtained results, it has been hypothesized a microbiologic selection by means of KT, selecting LAB strains with higher cinnamoyl esterase activity. Effectively, as reported by some researchs (Kong, Chen, Xing, & Park, 2010) , LAB activity is influenced by the presence of chitosan.

Furthermore, in **figure 3.6.3** it can be noticed a progressive decrease of GRP content over time with SO_2 whereas samples treated with KT resulted in a greater decrease. Based on results discussed on section 3.1, this trend could be attributed to the combination of two different mechanisms: 1) Oxidation of GRP during hyperoxygenation of must, 2) Direct absorption of GRP by means of KT, as already demonstrated in the previous chapters.

3.6.3.5. Sensory analysis

Sensory analysis after one year of storage is shown in **figure 3.6.4**. As outlined in the graph, samples treated with KT presented lower coloration than those added with SO_2. This result confirmed the browning trend discussed in section 3.1. Regarding aroma, the presence of KT exerted a positive influence, by reducing the oxidised character and increasing aromatic pleasantness and equilibrium. However, in consonance with what was previously mentioned in section 3.3, reduction of organic acids by KT had an impact on the taste, reducing significantly the acidity. Therefore, a preliminary study of chitosan properties and the dose to be applied becomes crucial in a winemaking process in the presence of chitosan. Furthermore, winemaking process in the presence of KT exerted a greater overall complexity, confirming that, excepting acidity, none of the sensory characteristics of the final product was compromised by the presence of KT.

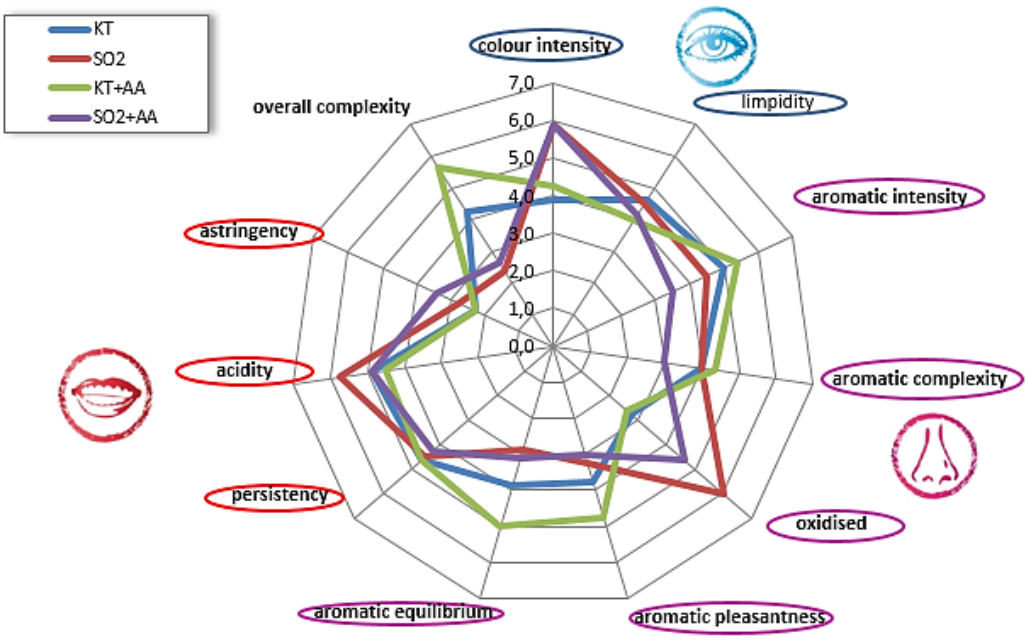

Figure 3.6.4. Sensorial analysis of wines after 12 months of storage

3.6.4. Conclusions

Based on the data discussed above, conclusions obtained were:

- Flotation with chitosan allowed a clarification of the musts without considerably influencing organic acid content.
- Stabilization and yeast lees contact during 30 days in the presence of chitosan was suitable to protect wines against oxidation without affecting sensorial parameters.
- Scaling of laboratory test at semi industrial level was carried out successfully. Chitosan shown a significant effect on colour development both at flotation and stabilization stages, confirming what already obtained in previous chapters. Chitosan by means of several strategies, such as reduction of metal content, hydroxyl radical scavenging, absorption of polyphenols and trapping of coloured compounds, was able to control oxidative browning development. Because of its versatility, it could be possible to consider the hypothesis of using KT as a potential agent to reduce the levels of sulfur dioxide in wines.

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Chapter 3.7

"A Chromatographic-mass spectrometric approach applied to the evolution of phenolic compounds during the storage of white wines in the presence of sulfur dioxide or chitosan"

Chapter 3.7

A Chromatographic-mass spectrometric approach applied to the evolution of phenolic compounds during the storage of white wines in the presence of sulfur dioxide or chitosan

3.7.1. Introduction

Phenolic compounds are the main constituents of wine, since they contribute to wine quality parameters such as colour, astringency, and tendency to oxidation. In addition, many studies described several properties of polyphenols, among them the antioxidant capacity, such as scavenging of reactive oxygen species (ROS) and protection against cardiovascular diseases, anticarcinogen, anti-inflammatory and antivirus, among others (Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999) .

However, phenolic compounds have been demonstrated to be easily oxidised, developing, among others, browning phenomena of wines, causing irreversible damages on the final product (Li, Guo, & Wang, 2008). Since oxygen uptake occurs at every stage of winemaking, from crushing to bottling, management of oxygen becomes challenging for oenologists. Thus, a meticulous control of polyphenolic profile is crucial for the approach of an antioxidant strategy.

The aim of this work is to characterize the phenolic evolution during different stages of a winemaking process in the presence of sulfur dioxide or chitosan, also compared to a control without any addition. To this purpose, metabolomic approaches have been carried out by using HPLC-MS/MS technics, in order to get new insights in the understanding of wine oxidation processes. Specifically, based on the results reported by Pati et. al (Pati et al., 2014) about characterization studies of phenolic compounds, this work will focus on the identification and quantification of flavonols and hydroxycinnamic acids, major components of the grape, as well as their oxidation products.

On the other hand, chemistry of the generation of sulfonated compounds will be discussed in order to deepen about the mechanisms of SO_2 consumption along the winemaking. Furthermore, this work intended to highlight the effect of chitosan on each step of vinification, in order to propose a smarter method of winemaking in the absence of sulfites.

3.7.2. Material & methods

3.7.2.1. Winemaking process

Winemaking process was carried out completely in the experimental cellar of Tebano (Ravenna, Italy). The process is schemed in **figure 3.7.1**:

As depicted in **figure 3.7.1**, 900 L of must was obtained by pressing Sangiovese grapes at low pressures. Then, obtained must was added of gallic tannins (white gold provided by Oenofrance Italia) at 20 g/HL and transferred into 100L capacity tanks giving rise to three different theses: control, chitosan (KT) and sulfur dioxide (SO_2) each carried out by triplicate. Therefore, each test was constituted by 300 L of must. Once transferred, each tank was added with 0.5 g/L of chitosan (KT) or 50 mg/L (SO_2). Three further tanks with no addition represented control fermentations (Control). All musts were left at 4ºC during 3 days in order to carry out the clarification of musts. KT samples were manually agitated each day, to favour the contact with chitosan, while avoiding oxygen entrance.

At the end of stabilization, clear musts were transferred again into 100L capacity tanks and inoculated with 30 g/HL of low-SO_2 production yeast Aleaferm Arom (Alea Evolution, Molinella (BO)) to start the alcoholic fermentation (AF) and 25 g/L of AleavitOne (aminic nitrogen) as an activator. As chitosan remained in the bottom of tanks, KT musts fermented in the absence of polysaccharide.

Once finished alcoholic fermentation (residual sugars < 2 g/L), tests were subdivided into two parallel experiments. Half of the wine was directly bottled (**objective 1**) into bottles of 350 and 750 mL and stored for 12 months. Control samples were divided into: bottled with no addition (control), and with 30 mg/L of GSH (control + GSH). KT samples were divided into: no addition (KT), addition of 0.5 g/L of KT (KT bott), and addition of 30 mg/L of GSH (KT+GSH). Finally, SO_2 test was divided into: addition of 60 mg/L of SO_2 alone (SO_2), and addition of 60 mg/L of SO_2 + 30 mg/L of GSH.

In a parallel way, remaining wine was transferred into 50L hermetic kegs to carry out a second cold stabilization and yeast lees contact (**objective 2**). To this aim, the entire lees settled after fermentation were left at the bottom of the kegs. Treatments were carried out as follows: no addition (control), 0.5 g/L of KT (KT) and SO_2 addition up to 60 mg/L (SO_2). Samples were stored for 20 days at 4 ºC. In order to favour the effect of chitosan, KT test were agitated manually each two days. After stabilization stage, samples were subsequently bottled and divided into the following: control with no addition (control), control with 120 mg/L of ascorbic acid(Control AA), KT with no addition, KT with addition of 0.5 g/L of chitosan, KT with 120 mg/L of ascorbic acid (KT AA), SO_2 with 60 mg/L of SO_2, SO_2 with 120 mg/L ascorbic acid (SO_2 AA)(figure 1).

In order to prevent the entrance of oxygen as much as possible, bottling was carried out in the presence of carbon dioxide (CO_2)

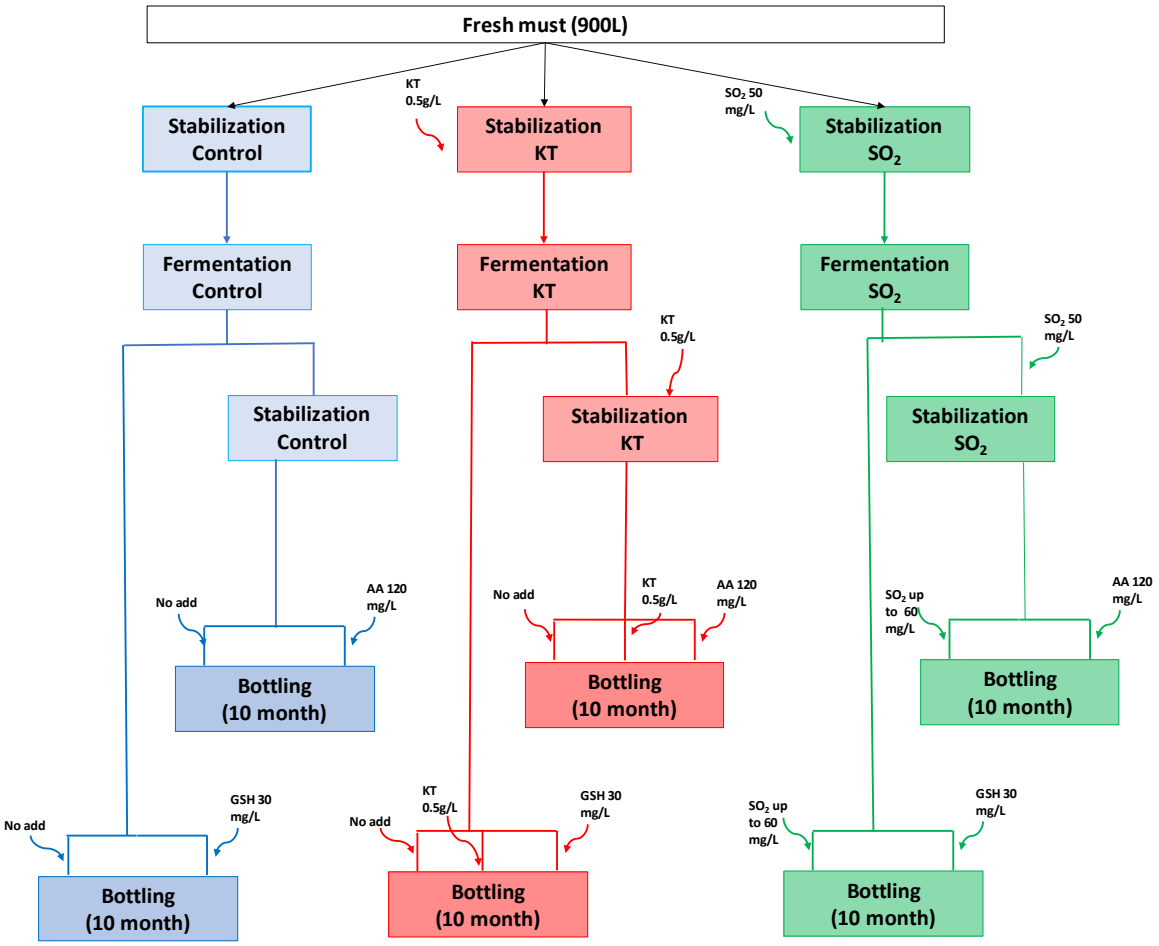

Figure 3.7.1. Diagram of the different winemaking processes.

3.7.2.2. General parameters, Browning, Organic acids and phenolic acids

These parameters were determined following the methods cited in chapter 3.4 in materials and method section.

3.7.2.3. Phenolic analysis by HPLC-ESI-QTOF/MS

For the HPLC-ESI-QTOF analysis of phenolic compounds a RRLC 1200 Series Rapid Resolution LC system (Agilent Technologies, Palo Alto, CA, USA) was used, equipped with a Synergi 4µm Hydro-RP 80A HPLC Column 250 x 4.6 mm (Phenomenex). Solvents were water (solvent A) and acetonitrile (B) the both added of 0.2% acetic acid. Multi-step gradient program was as follows: 0 min, 98% solvent A; 10 min, 95% A; 16 min, 90% A; 21 min, 82% A; 24 min, 80% A; 28 min, 70% A; 31 min, 50% A; 33 min, 0% A; 36 min, 0% A; 37 min, 98% A. Other operating conditions were: flow 0.8 mL/min, temperature 25°C and injection volume 10 µL. Compounds were monitored by means of a time of flight detector microTOF (Bruker Daltonic, Bremen, Germany) coupled with an ESI interface working in negative mode over a mass range of 50-1000 amu. Flow was splitted 1:2 before reaching the mass detector using a “T” type connector.

The source parameters were set as follows: capillary 2.5 kV for negative scan, sampling cone 25 V, extraction cone 3 V, source temperature 210 ◦C, desolvation temperature 500 ◦C, nebulizer gas 9 L/h and nebulizer pressure 2.3 bar. Instrumental external calibration was performed for each injection by direct infusion of a sodium acetate solution, to control the mass accuracy.

All data were collected by using DataAnalysis 4.0 (Bruker Daltonics) software and molecular formulas were selected based on spectral features (mass difference of less than 10 ppm theoretical value, and at least one indicative fragment and isotopic pattern).

Semiquantitative information were obtained with the internal standard method (7-OH coumarin), and expressed as realative areas of molecular ion of each elucidated compound. Before analaysis, samples were filtered with cellulose acetate filters 0.2 μm.

3.7.2.4. Phenolic analysis by HPLC-MS/MS

Fragmentation experiments were performed on an Agilent 1200 series system (Agilent, Palo Alto, CA, USA), equipped with an LC-MSD ion-Trap VL electrospray ionization mass spectrometry system, coupled to an Agilent Chem Station for data processing. After filtration (0.20, cellulose acetate membrane), samples were analysed using the same method indicated in section 2.3. For identification, the ESI-MSn detector was used in positive mode for GRP and GRP-derived compounds and negative mode for hydroxycinnamic and hydroxycinnamoyltartaric acids, setting the following parameters: dry gas, N_2, 11 mL/ min; drying temperature, 350 °C; nebulizer, 65 psi; capillary voltage, -2500 V (positive ionization mode) and +2500 V (negative ionization mode); target mass, m/z 600; compound stability, 40% (negative ionization mode) and 100% (positive ionization mode) and scan range, m/z 50-1000.

3.7.3. Results and discussion.

As already outlined in material and methods section, this experiment was subdivided into two objectives: objective 1, were wines were bottled immediately after fermentation, and objective 2, where cold stabilization and yeast lees contact were carried out simultaneously prior to bottling. In order to study the evolution of polyphenols during the entire process, HPLC-qTOF and HPLC-MS^n studies were carried out on each stage of winemaking. Furthermore, fixed composition was analysed as well.

3.7.3.1. General parameters of wines

Evolution of general parameters during the winemaking process is depicted in **table 3.7.1** and **3.7.2**. It is necessary to point out that both objectives shared the same raw material (must and wine) until the end of the alcoholic fermentation, being divided at this moment in: bottling (**objective 1**), or stabilization and contact with the lees (**objective 2**).

After the first stabilization of musts, a significant increase in pH was appreciated in samples treated with chitosan (KT) when compared to the control and SO_2. This result was also obtained during experiments on wine model solutions (chapter 3, section 3.2) where the rise of pH was attributed to two different mechanisms: 1) acid-binding capacity of KT and 2) generation of OH- by amine groups (NH_2) of chitosan. Slight increase on pH values after alcoholic fermentation could be related to the consumption of malic acid into lactic acid since, unexpectedly, MLF started during fermentation in both the thesis absent of sulfites (data not shown). This phenomenon could explain the lower pH of wines fermented in the presence of SO_2, were samples were microbiologically protected by the presence of sulfites. Furthermore, pH remained stable in almost all samples bottled immediately after fermentation and stored for 10 months, with a decrease in control samples. As expected, significantly higher pH value was obtained in samples bottled in the presence of chitosan (KT bott) confirming the data discussed above.

Regarding objective 2, only samples stabilized in the presence of 0.5 g/L of KT showed a slightly increase in pH (0.05 units) while both control and SO_2 remained unchangeable. Furthermore, excepting samples bottled with KT, where pH was further increased, pH was stable over 10 months of storage.

Regarding total acidity, a significant difference was found since the end of the alcoholic fermentation, due to the development of MLF. This trend was maintained after storage period in both S1 and S2 treatments

Generation of volatile acidity resulted to be significantly lower in samples fermented in the presence of SO_2 (table 1 and 2) than in control or KT where no additive was present (chitosan was removed from the must before fermentation). This result agrees with those discussed above, where SO_2 demonstrated to better protect must from microbial spoilage, thus avoiding the production of volatile acidity. Regarding objective 1, almost no changes were appreciated after 10 months of storage, as depicted in table 1. Interestingly, samples bottled in the presence of KT (KT bott) had a decrease of volatile acidity with respect to samples after fermentation. This trend could be attributed to the acid-binding capacity of chitosan, which was able to remove acetic acid form the media by quenching it, resulting in a decrease on volatile acidity.

Similar trend in volatile acidity was observed in Objective 2 (**table 3.7.2**), remaining unchangeable except for control samples where increased progressively over the time, reaching levels of 0.7 g/L. Moreover, in both experiments (Objective 1 and 2), samples added with SO_2 showed the lowest values of volatile acidity because of the powerful antimicrobial properties of sulfur dioxide.

On the other hand, significantly differences were observed on the development of browning (abs 420 nm) among different treatments (**Table 3.7.1** and 3.7.**2**). Overall, Control samples showed higher values of Abs_{420} than SO_2 and KT, possible due to oxidation processes. After cold stabilization (**Table 3.7.1**), all musts exhibited a decrease in absorbance, mainly due to the precipitation of insoluble coloured particles. SO_2 and KT presented lower Abs_{420} than control, possibly due to higher protection against enzymatic oxidation, bleaching power of SO_2, and absorption properties of chitosan. Unexpectedly, no differences were appreciated after alcoholic fermentation. Moreover, wine stabilization "sur lies" (S2) in the presence of KT and SO_2 induced a further decrease in colour due to the mechanisms listed above. Regarding storage period, in samples bottled after fermentation (S1-10M), Abs_{420} remained unchanged on KT treatments, with no differences among KTbott and KT+GSH. Combination of KT and GSH, as already discussed in chapter 3 (section 3.1, paragraph 5), seemed not to exert any effect. This result has been hypothesized to be related to a depletion of GSH by absorption on chitosan backbone. As expected, addition of SO_2 during bottling resulted in a significant reduction of colour (Abs_{420}), while it was further developed on Control samples, with a significant higher value of Abs_{420} (**Table 3.7.1**). Furthermore, similar trend was observed in samples bottled after stabilization "sur lies" (**Table 3.7.2**). It is worth to mention than chitosan and SO_2 exhibited similar antibrowning behaviour.

	Initial must	Stabilization 1. Must			Alcoholic fermentation			10 months storage						
		SO_2	KT	Control	SO_2	KT	Control	SO_2	SO_2 +GSH	KT	KT bott	KT + GSH	Control	C +GSH
pH	3,21 ±0,02	3,17 ^{b}	3,23 ^{a}	3,17 ^{b}	3,21 ^{c}	3,27 ^{b}	3,34 ^{a}	3,17 ^{e}	3,17 ^{e}	3,29 ^{b}	3,32 ^{a}	3,28 ^{b}	3,22 ^{d}	3,26 ^{c}
Total acidity	5,98 ±0,03	5,56 ^{b}	5,53 ^{b}	5,78 ^{a}	6,43 ^{a}	5,76 ^{b}	5,57 ^{c}	6,06 ^{a}	6,04 ^{a}	5,51 ^{bc}	5,58 ^{b}	5,63 ^{b}	5,44 ^{c}	5,58 ^{b}
Volatile acidity					0,52 ^{b}	0,58 ^{a}	0,62 ^{a}	0,53 ^{b}	0,51 ^{b}	0,61 ^{a}	0,47 ^{c}	0,62 ^{a}	0,64 ^{a}	0,60 ^{a}
% alcohol (v/v)					11,6 ^{a}	11,4 ^{a}	11,3 ^{a}							
Abs 420		0,176 ^{a}	0,200 ^{a}	0,295 ^{a}	0,109 ^{a}	0,119 ^{a}	0,101 ^{a}	0,095 ^{bc}	0,081 ^{c}	0,104 ^{b}	0,103 ^{b}	0,109 ^{b}	0,132 ^{a}	0,133 ^{a}

Table 3.7.1. Objective 1. General parameters after each stage of the winemaking process. Within the same stage, and in the same row, different letters indicate significant differences according to Tukey's test ($p < 0.05$). n=3.

	Stabilization 2. Wine			10 months storage						
	SO_2	KT	Control	SO_2	SO_2 +AA	KT	KT bott	KT + AA	Control	Control +AA
pH	3,23 ^{b}	3,32 ^{a}	3,30 ^{a}	3,20 ^{d}	3,21 ^{d}	3,29 ^{b}	3,37 ^{a}	3,29 ^{b}	3,27 ^{c}	3,27 ^{c}
Total acidity	5,76 ^{a}	5,75 ^{a}	5,58 ^{a}	6,14 ^{a}	6,18 ^{a}	5,48 ^{bc}	5,41 ^{c}	5,56 ^{b}	5,54 ^{bc}	5,48 ^{bc}
Volatile acidity	0,56 ^{c}	0,61 ^{b}	0,65 ^{a}	0,49 ^{c}	0,47 ^{c}	0,59 ^{b}	0,58 ^{b}	0,64 ^{b}	0,71 ^{a}	0,70 ^{a}
Abs 420 nm	0,089 ^{b}	0,080 ^{b}	0,106 ^{a}	0,093 ^{b}	0,091 ^{b}	0,093 ^{b}	0,077 ^{b}	0,105 ^{b}	0,133 ^{a}	0,128 ^{a}

Table 3.7.2. Objective 2. General parameters after each stage of the winemaking process. Data referred to initial must, stabilization of must and alcoholic fermentation are the same as in table 1. Within the same stage, and in the same row, different letters indicate significant differences according to Tukey's test ($p < 0.05$). n=3.

3.7.3.2. Evolution of phenolic compounds during winemaking process

With the aim to characterize the phenolic profile after each step of winemaking process on must and wines added with either chitosan or SO_2, evolution of these compounds was investigated by HPLC-MS/MS and compared to a control with any addition (**table 3.7.4** and **3.7.5**).

- ***Identification of compounds***

Thirty-five compounds have been identified in at least one wine sample by means of HPLC analysis. To confirm the elucidated compounds, a triple criterion was used which included i) comparison with retention time of pure standard compound (when available), ii) high resolution detection for exact mass determination, iii) comparing information coming from MS^2 experiments with already published reports. In addition, elution orders in similar analytical conditions (i.e. RP-HPLC) as reported in other papers, was used as a further validation rule.

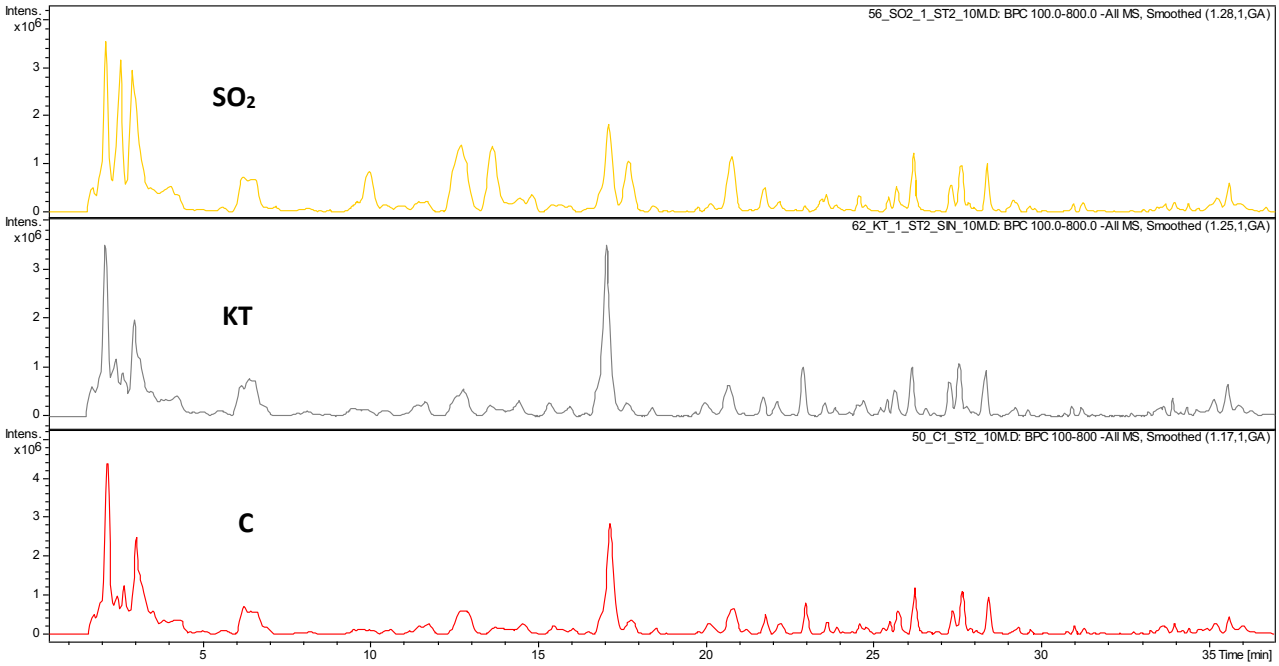

Figure 3.7.2. Representative base peak chromatograms of SO_2; KT and C samples, acquired in negative ionization mode

During a preliminary phase, in order to gain the maximum amount of data useful for identification purposes, both negative and positive ESI modes were investigated. A higher number of compounds was better detected in negative mode (**figure 3.7.2**) even if, in some cases, positive ionization was the polarity of choice (particularly for some glutathionated derivatives whose ionization was appreciably higher when set as positive). The differences between the two ESI modes for a SO_2GSH sample can be appreciated in **figure 3.7.3.** **Table 3.7.3** illustrates the elucidated molecules together with their relevant mass information, retention times and fragments obtained after MS/MS experiment.

Apart from 5 molecules bearing the benzoic or flavanol chemical structure, the largest part of compounds belonged to the group of cinnamates, which comprised 18 analytes, including several glutathionated derivatives (**table 3.7.3**). For most of those compounds, diagnostic losses induced by MS fragmentation were found at 132 and 40 m/z which correspond to the loss of CO_2 and tartaric acid respectively from the acid and the tartaric acid moieties of cinnamates and esters. Indeed, for a number of major peaks, UV spectrum could have provided additional confirmation for their identification but, unfortunately, our MS instrumentation was not equipped with this type of detector.

Peak nr	*rT*	*m/z exp.*	*m/z theoretical*	*Error (ppm)*	*name*	*Identification*	*MS/MS (m/z)**
				Hydroxybenzoic acids and flavanols			
1	6.70	169.0143	169.0142	-0.6	gallic acid	std, HRms, MS/MS	125
2	20.3	577.0000	577.1346	233.2	procyanidin dimer	std, HRms, MS/MS	425, 407
3	21.9	289.0723	289.0718	-1.7	catechin	std, HRms, MS/MS	245, 175
4	24.7	289.0731	289.0718	-4.5	epicatechin	std, HRms, MS/MS	245, 205
5	26.3	197.0453	197.0455	1.0	ethylgallate	std, HRms, MS/MS	169, 126
				Hydroxycinnamic acids			
6	12	311.0401	311.0409	2.6	c-caftaric acid	HRms, MS/MS	179, 149
7	13.09	311.0398	311.0409	3.5	t-caftaric acid	HRms, MS/MS	179, 149
8	14.67	616.1075	616.1090	2.4	t-GRP	HRms, MS/MS	484, 440, 272
9	17.3	295.0459	295.0460	0.3	c-coutaric acid	HRms, MS/MS	163, 149
10	17.4	616.1075	616.1090	2.4	c-GRP	HRms, MS/MS	484, 440, 272
11	17.9	295.0449	295.0460	3.7	t-coutaric acid	HRms, MS/MS	163, 119
12	20.80	325.0555	325.0565	3.1	c-fertaric acid	HRms, MS/MS	193, 149
13	21.57	484.1045	484.1045	0.0	2-S-glutathionyl-*t* -caffeic acid	HRms, MS/MS	$468^{(+)}$, $393^{(+)}$, $264^{(+)}$
14	21.85	325.0555	325.0565	3.1	*t*- fertaric acid	HRms, MS/MS	193, 149
15	22.4	644.1399	644.1475	11.8	*t* -GRP-Et	HRms, MS/MS	$543^{(+)}$, $517^{(+)}$, $264^{(+)}$
16	23.02	179.0353	179.0350	-1.7	caffeic acid	std, HRms, MS/MS	135
17	23.2	484.1031	484.1045	2.9	2-S-glutathionyl-c-caffeic acid	HRms, MS/MS	$357^{(+)}$, $264^{(+)}$
18	23.77	644.1399	644.1398	-0.2	*c* -GRP-Et isomer	HRms, MS/MS	$517^{(+)}$, $264^{(+)}$
19	25.6	165.0553	165.0541	-7.3	dihydro-*p*- coumaric acid	HRms, MS/MS	147
20	25.8	644.1406	644.1398	-1.2	*t* -GRP-Et isomer	HRms, MS/MS	$571^{(+)}$, $517^{(+)}$, $264^{(+)}$
21	26.6	163.0384	163.0401	10.4	p-coumaric acid	std, HRms, MS/MS	119
22	28.4	193.049	193.0506	8.3	ferulic acid	std, HRms, MS/MS	149, 134
23	34.01	207.0663	207.0656	-3.4	ethylcaffeoate	HRms, MS/MS	179, 165, 135
24	35.2	191.0714	191.0708	-3.1	ethylcumarate	HRms, MS/MS	145, 163
				Other compounds			
25	18.5	137.0597	137.0608	8.0	tyrosol	std, HRms, MS/MS	
26	31.1	160.0767	160.0768	0.6	tryptophol	std, HRms, MS/MS	
				Oxidation-related products			
27	2.5	386.0324	386.0333	2.3	s-sulfonate glutathione	HRms, MS/MS	368, 306, 272
28	15.4	373.112			hydroxy-caffeic acid dimer isomer	HRms, MS/MS	327, 175, 129
29	15.9	373.1125			hydroxy-caffeic acid dimer isomer	HRms, MS/MS	193, 178
30	16.4	373.1108			hydroxy-caffeic acid dimer isomer	HRms, MS/MS	193, 178
31	17	373.1118			hydroxy-caffeic acid dimer isomer	HRms, MS/MS	355, 327, 281, 175
32	22.09	446.0788	446.0770	-4.0	Indole lactic acid hexoside sulfonate	HRms, MS/MS	266, 222, 142
33	23.7	355.1012			Caffeic-(o-quinone-caffeic)-ether	HRms, MS/MS	217, 193, 175
34	23.8	240.0347	240.0333	-5.8	Tryptophol sulfonate	HRms, MS/MS	178, 160
35	25.55	366.1187	366.1193	1.6	Indole lactic acid glucoside	HRms, MS/MS	204, 186, 142

Table 3.7.3. List of identified compounds in wine samples. * Identification assignment: std= comparing MS spectrum and retention time with pure compound;
HRms= based on high resolution mass; MS/MS= comparing MS^2 spectra with those reported in literature (see text for details)
(+) denotes identification which were confirmed in positive ion mode

At the same time, however, we were able to reproduce some chromatographic runs on a HPLC-DAD apparatus that allowed us to record and confirm the UV spectrum of the most responsive cinnamates, benzoate and flavanols.

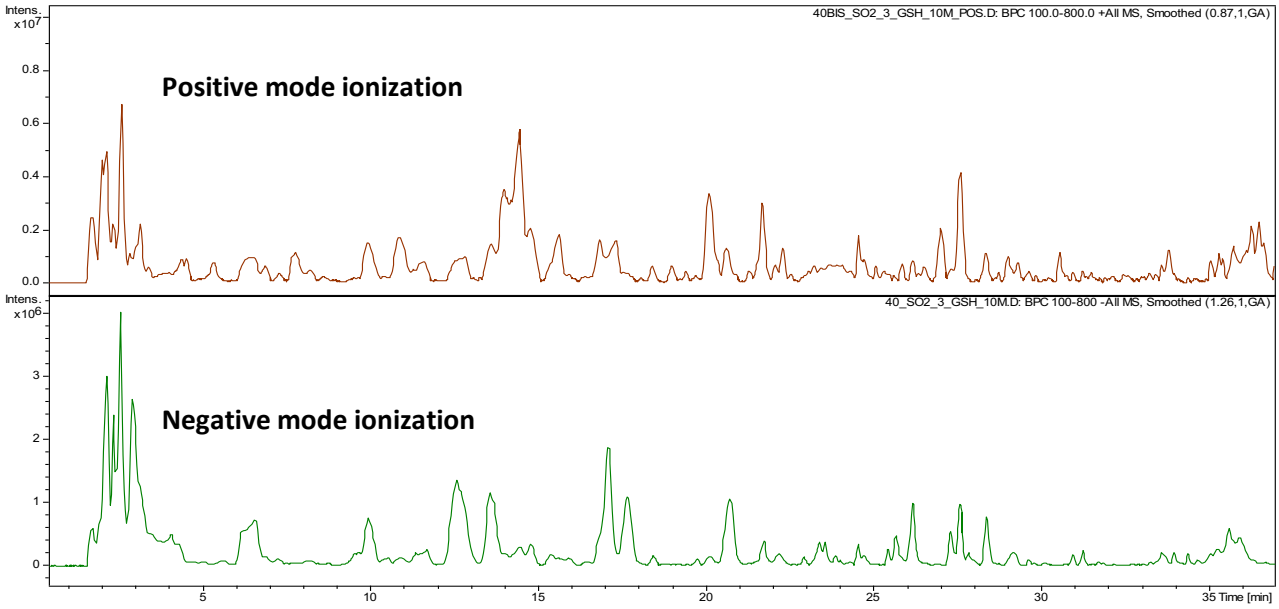

Figure 3.7.3. Comparison of chromatograms of the same sample (SO_2GSH) acquired in both positive and negative ionization modes

Among, others, some unconventional cinammates derivatives were found and identified in almost all the samples. In particular, 2-S-glutathionyl-caffeic acid isomers (*cis* and *trans*) and three GRP ethyl isomers have been elucidated based on positive ionization fragmentation that was consistent with data gathered by Cejudo-Bastante et al. (Cejudo-Bastante, Pérez-Coello, & Hermosín-Gutiérrez, 2010) (**figure 3.7.4 and 3.7.5**).

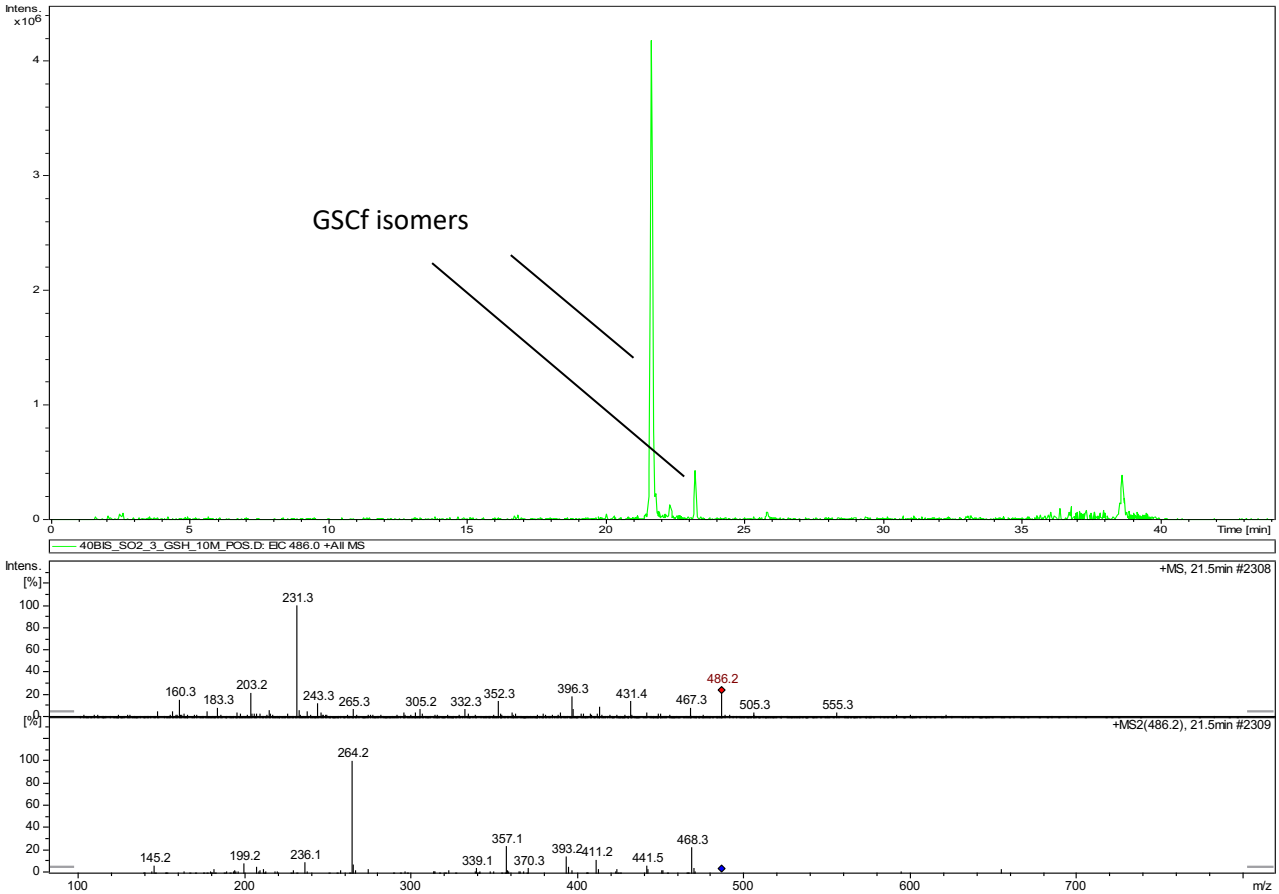

Figure 3.7.4. Extracted ion chromatogram at m/z 486 $(M+H)^+$ and MS^2 fragmentation of peaks corresponding to 2-S-t-glutathionyl-caffeic acid (GSCf) at 21.57 min.

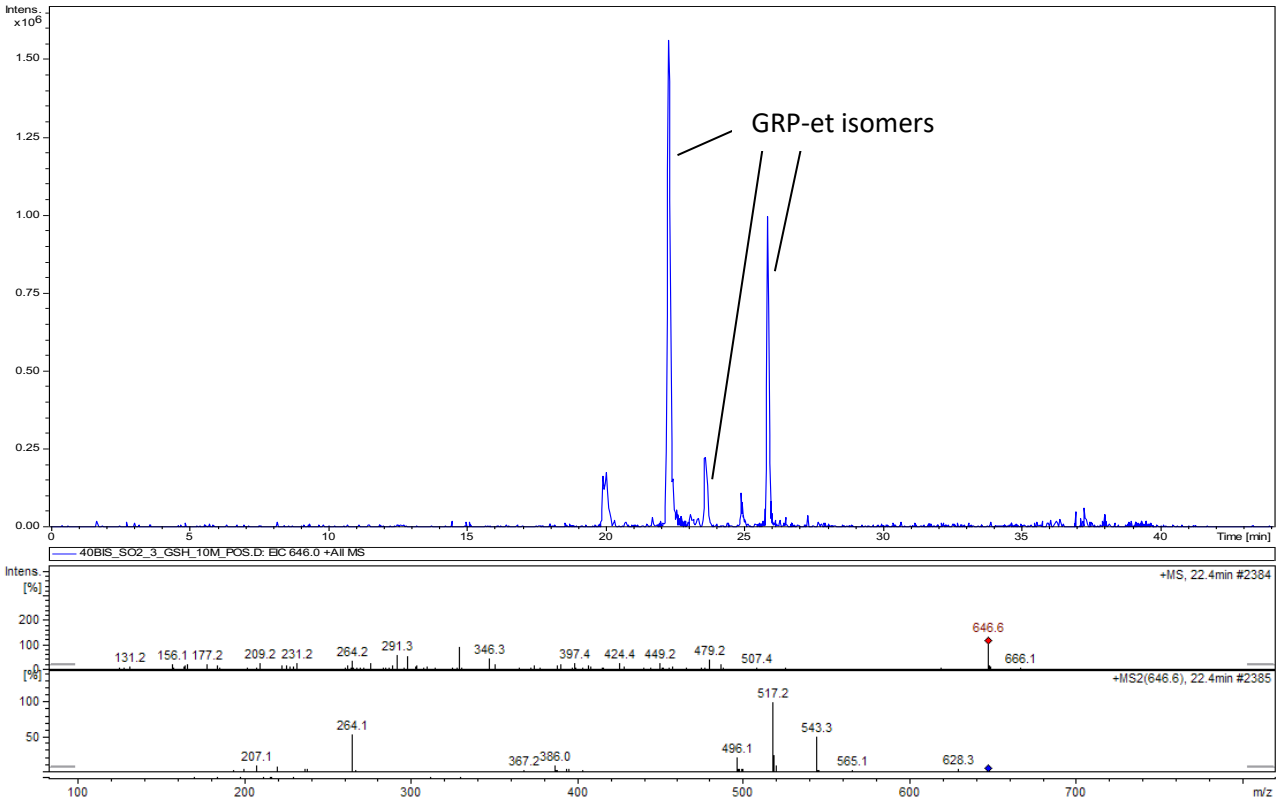

Figure 3.7.5. Extracted ion chromatogram at m/z 646 $(M+H)^+$ and MS^2 fragmentation of peaks corresponding to t-GRP-et at 22.40 min.

Table 3.7.3 also includes other uncommon compounds that have been classified as "oxidation related", based on previous scientific evidences. Under this "family" we grouped certain derivatives essentially coming from two sources: i) oxidative dimerization of caffeic acid (Pati et al., 2014) and ii) sulfonation promoted by the presence of oxygen (Arapitsas et al., 2016; Arapitsas, Guella, & Mattivi, 2018).

In spite oxidative dimers of caffeic acid have been often identified in model solution during mechanistic studies on cinnamates oxidation (Antolovich et al., 2004; H Tazaki, Taguchi, Hayashida, & Nabeta, 2001), their presence in wines was rarely claimed. Quite recently, Pati et al. (Pati et al., 2014) reported a study on the presence of such derivatives in sulfite-free white wines, at the same time giving some details on typical fragmentation under negative ESI mode. As an example, in **figure 3.7.6** are illustrated our results after having carried out MS^2 experiment on 373 m/z molecular ion identified as hydroxy-caffeic acid dimer. The MS^2 spectrum of this and the other caffeic acid oxidation products listed in **table 3.7.3** (caffeic-(o-quinone-caffeic)-ether), agreed pretty well with the ones showed by the cited authors.

A second sub-class of oxidative-related molecules on which we focused our attention are sulfonates. Arapitsas et al. (Arapitsas et al., 2016), in a metabolomic investigation on white wine oxidative markers, reported that sulfonated derivatives of indoles and glutathione were prompted by the simultaneous presence of sulfites and high oxygen level during storage. The presence of these compounds, identified in our samples (**figure 3.7.7** and **3.7.8**), will be, hence, discussed in the following section.

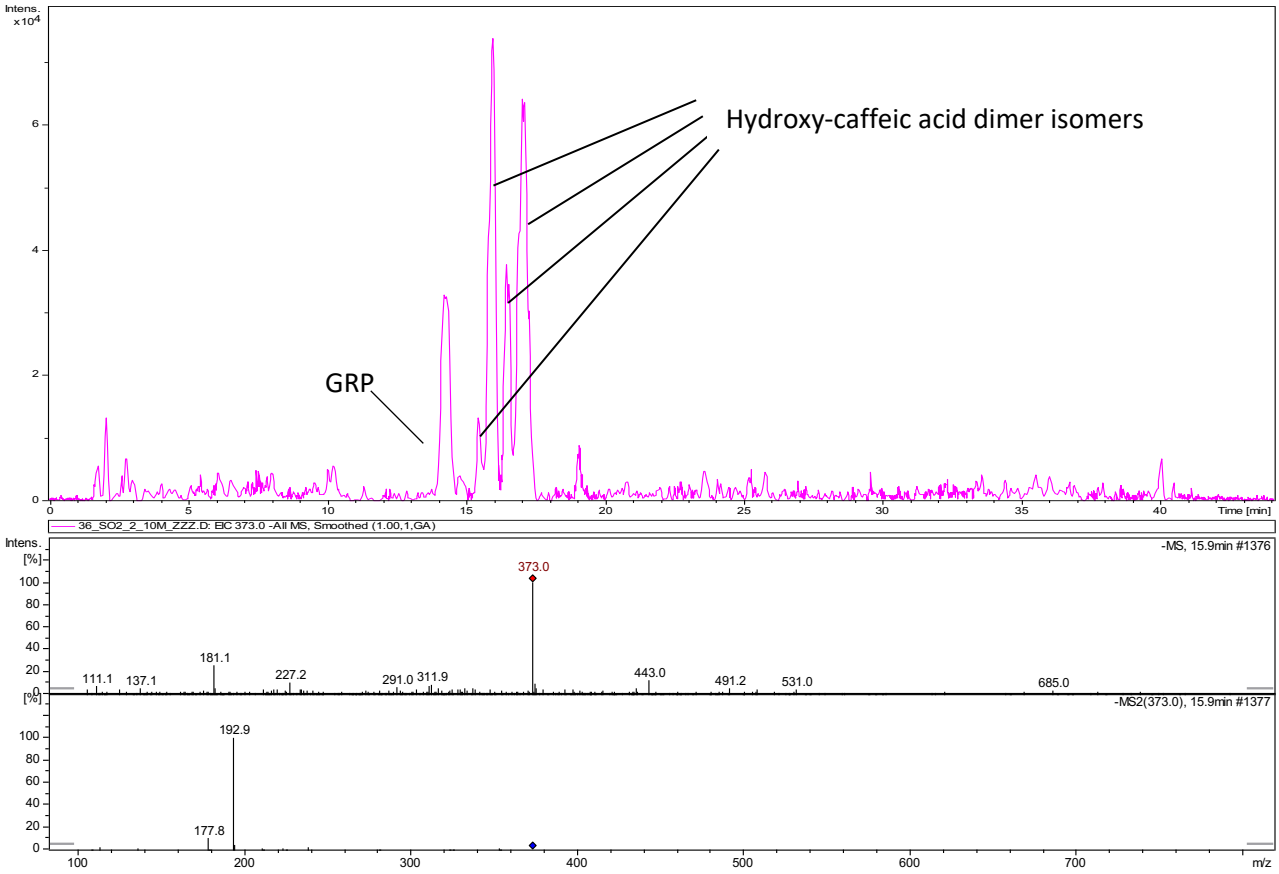

Figure 3.7.6. Extracted ion chromatogram at m/z 373 $(M-H)^-$ and MS^2 fragmentation of peaks corresponding to hydroxy-caffeic acid dimer isomer at 15.90 min.

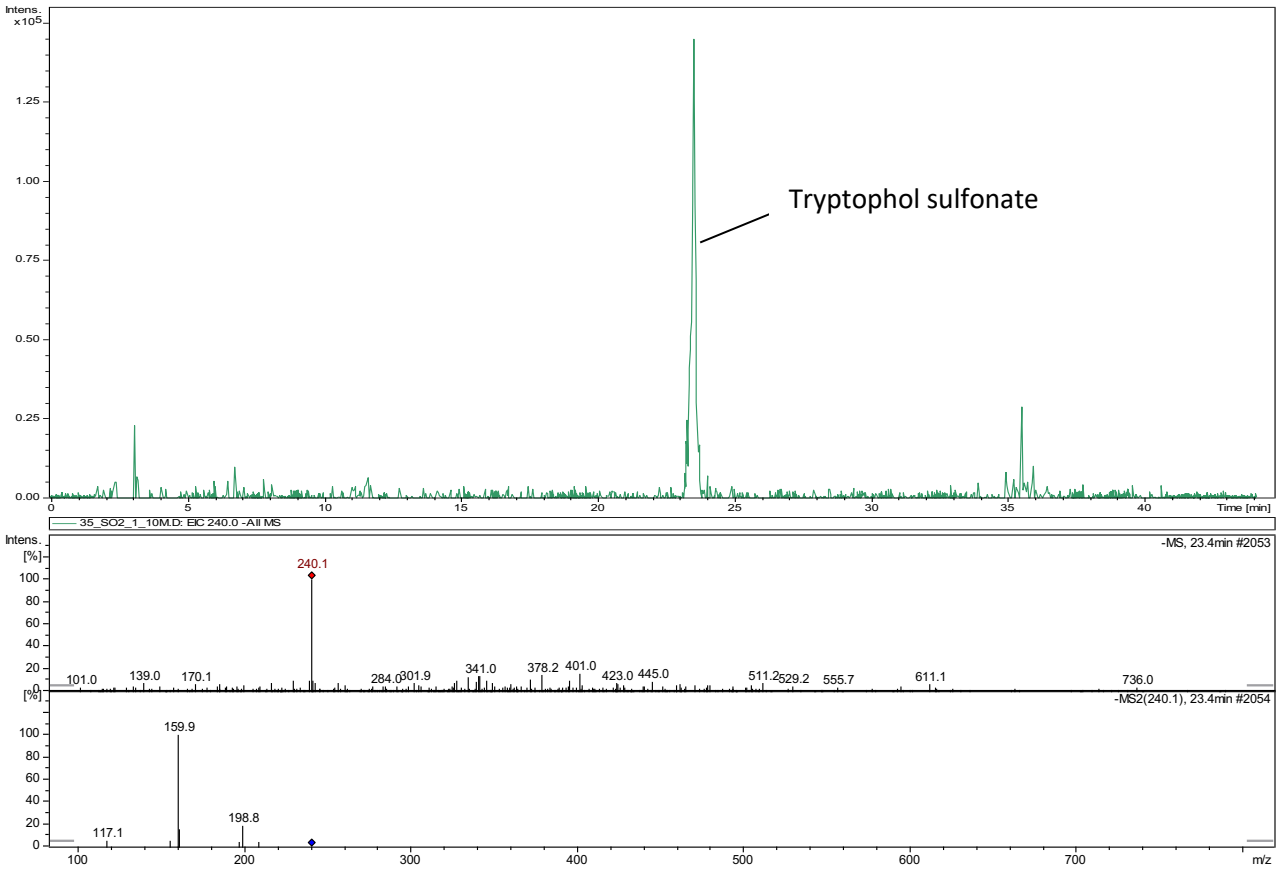

Figure 3.7.7. Extracted ion chromatogram at m/z 240 $(M-H)^-$ and MS^2 fragmentation of peaks corresponding to tryptophol sulfonate at 23.80 min.

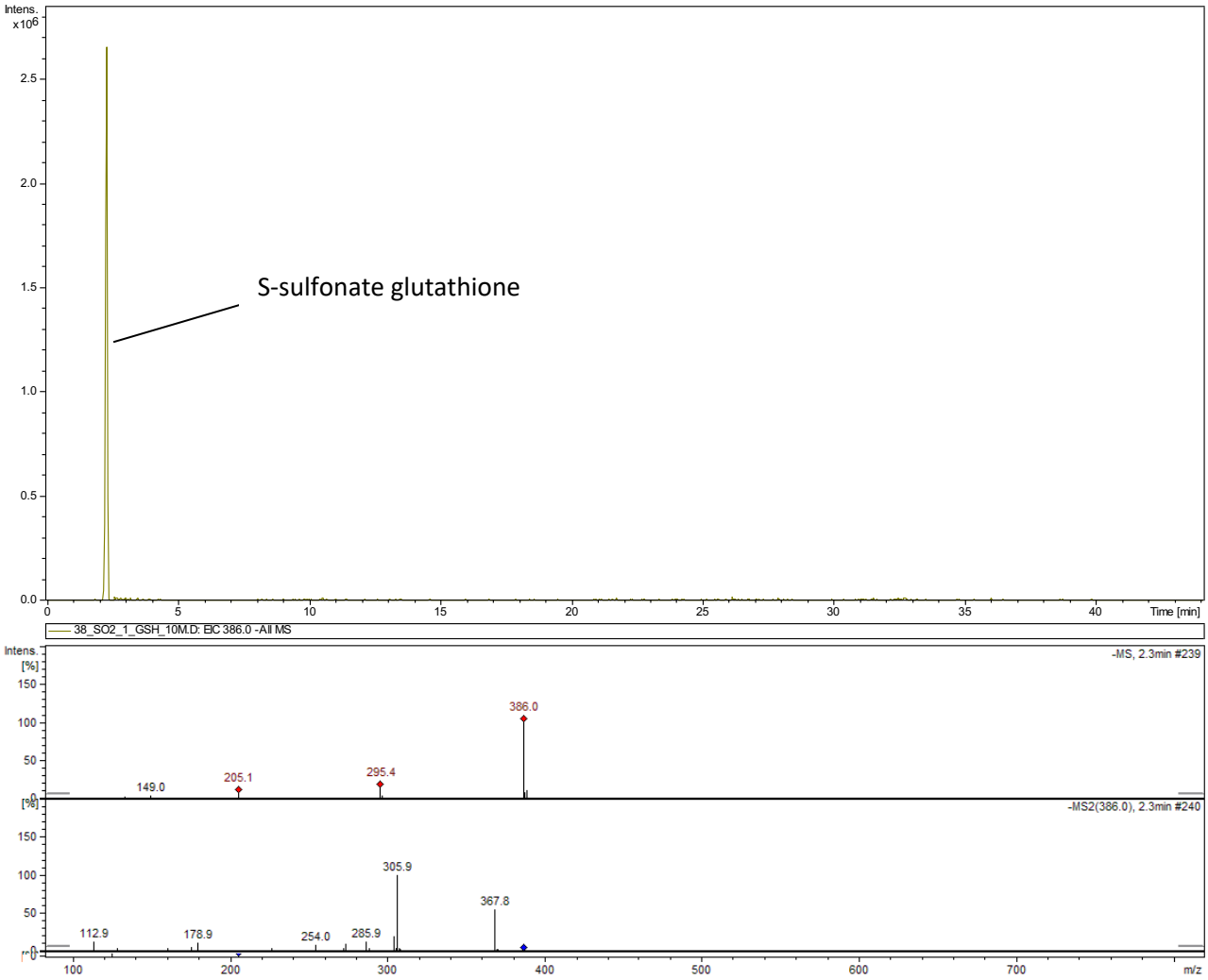

Figure 3.7.8. Extracted ion chromatogram at m/z 386 $(M-H)^-$ and MS^2 fragmentation of peaks corresponding to S-sulfonate glutathione at 2.30 min.

- ***Semi-quantification of phenolic compounds***

  a) Hydroxybenzoic acids and flavanols

Flavonoids are one of the mains constituents of grape must and wines. In our conditions, 5 compounds were identified, namely gallic acid, procyanidin dimer, catechin, epicatechin and ethyl gallate. After cold stabilization of musts (S1) only SO_2 resulted to be significantly different when compared to the other treatments (KT and Control), probably due to a higher inhibition of polyphenol oxidase (PPO), one of the main responsible of enzymatic oxidation of musts (Danilewicz, 2007). It appears, hence, that in these conditions (cold stabilization for 3 days and agitation on daily basis) chitosan did not absorbed a significant amount of benzoic acids being not different from control musts.

After alcoholic fermentation (AF), none of them resulted to be affected by the treatments except gallic acid and epicatechin, this latter being the flavanol most susceptible to oxidation (Labrouche, Clark, Prenzler, & Scollary, 2005) resulting significantly lower in control samples (**Table 3.7.4**).

During stabilization "*sur lies"* (S2), epicatechin and ethylgallate, resulted the only flavanols in a significantly lesser content in KT samples (**Table 3.7.5**). In this case, the presence of chitosan had an impact on phenolic content, whose amount was reduced by absorption, as already reported in previous chapters.

After 10 months of ageing of samples bottled after alcoholic fermentation (S1-10M), all treatments exhibited significant differences each other (**Table 3.7.4**). Overall, reduced flavanol content was obtained in KT samples, with even lower concentrations in wines bottled in the presence of chitosan (KT bott) evidencing the absorptive power of chitosan discussed above. Interestingly, addition of GSH in SO_2 wine (and not in KT or control wine) resulted in a higher concentration of procyanidin dimer and gallic acid, probably due to the antioxidant effect offered by GSH when present in suitable molar concentration with respect sulphites and potentially oxidizable phenolics (Sonni, Clark, Prenzler, Riponi, & Scollary, 2011).

Storage period of samples stabilized "sur lies" prior to bottling (S2-10M) is also summarized in **Table 3.7.5**. Flavanols showed a similar trend than samples bottled after fermentation, with a significantly higher content in those stored in the presence of ascorbic acid and SO_2, mainly due to the combined antioxidant action of both additives (Barril, Clark, & Scollary, 2012). Gallic acid tended to increase during storage in all the sample because of the progressive hydrolysis of gallic tannin added on the must. In addition, this increase is notably higher in those wines stabilized with lees contact (compare S1-10M and S2-10M samples) supposedly as a consequence of some residual enzymatic activity of yeast cell. On KT and Control samples, the presence of ascorbic acid did not seem to influence the evolution of benzoic and flavanol compounds.

b) Hydroxycinnamic acids

Most of the identified compounds belonged to the group of hydroxycinnamate acids, namely cis and trans caftaric acid, cis and trans coutaric acid and cis and trans fertaric acid, and their free form, hydroxycinnamic caffeic, p-coumaric and ferulic acid respectively (**Table 3.7.3**). The latter compounds are released from the hydrolysis of hydroxycinnamates, leading to the leakage of tartaric acid bond.

Caftaric acid and coutaric acid were more influenced by the presence or absence of SO_2 during stabilization of musts (S1) whereas fertaric acid was not affected, since no significant differences were observed in any of the three treatments (**Table 3.7.4**).

Furthermore, hydroxycinnamate derivatives did not show any significantly differences among treatments after alcoholic fermentation (AF). However, alcoholic fermentation of musts treated with chitosan (KT) showed the highest content in hydroxycinnamic acids, such as caffeic acid and p-coumaric acid (**Table 3.7.4**), and therefore on their derivatives compounds, dihydro-p-coumaric acid and ethylcaffeoate. All these compounds were not present in musts and apparently comes from the hydrolysis of GRP and coutaric acid respectively. This result was also commented in the previous chapter, where chitosan seemed to enhance the loss of tartaric acid moiety from hydroxycinnamic ester. Again, there were no differences among samples

regarding fertaric acid. It is worth noting that the accumulation of ethyl ester of GRP and caffeic acid started since the end of fermentation (e.g. when ethanol appeared in the medium). This is in contrast with the finding of Cejudo Bastante et al. (Cejudo-Bastante et al., 2010) who reported these compounds to be present only in 1 years old white wines and give some further details on the fate and decay of glutathionated derivatives.

Most of the hydroxycinnamates (*cis* and *trans* caftaric, coutaric and fertaric acids) showed reduced concentrations after stabilization "*sur lies*" (**Table 3.7.5**) on KT treatments (S2). This trend, as already outlined in previous chapters, could be due to an absorption of these compounds by means of chitosan (Chinnici, Natali, & Riponi, 2014; Spagna et al., 1996) but also to the hydrolysis of tartaric bond, leading to the release of free hydroxycinnamic acids, as depicted in **table 3.7.5** with the increase of caffeic and its derivative ethyl caffeoate, and p-coumaric acid. During this technological step, other cinnamate derivatives such as 2-S-gluthationil-caffeic acid and one isomer of GRP ethyl ester appeared in wines.

As expected, the presence of hydroxycinnamates, as a sum, on samples bottled after alcoholic fermentation (S1-10M) was higher in SO_2 mainly due to the protection effect against their oxidation exerted by of SO_2 since the very first stages of vinification (**Table 3.7.4**). For these samples, the presence of GSH apparently drove to the accumulation of caftaric and coutaric acids, providing indirect evidence that such tartaric esters can be generated during the storage period. Regarding KT and KT+GSH, both treatments presented similar trends than after stabilization "*sur lies*" in the presence of chitosan, with a general reduction of hydroxycinnamates, followed by a significant increase in free hydroxycinnamic acids (e.g. caffeic acid, p-coumaric acid). However, the opposite was observed in bottles added with KT (KT bott), with higher contents of free hydroxycinnamates such as t-caftaric acid, c-fertaric acid or c-coutaric acid and lower amounts of free hydroxycinnamic acids (**table 3.7.4**). This particular behaviour of chitosan in bottle could be of great interest to prevent the development of ethyl phenols by *Brettanomyces*, since free hydroxycinnamic acids are the main precursors of their formation.

Furthermore, the evolution of hydroxycinnamates and hydroxycinnamic acid was identical to samples bottled immediately after fermentation. Interestingly, particular behaviour of chitosan during storage (KT bott) was repeated, supporting the data previously discussed, resulting in higher concentrations of hydroxycinnamates, followed by lower content of hydroxycinnamic acids such as caffeic, and p-coumaric acid (**table 3.7.5**).

c) Grape Reaction Product (GRP) and isomeric forms

Evolution of all GRP-derived species (sum of peaks 8, 10, 13, 15, 17,18, 20, **table 3.7.3**) along the winemaking process is depicted in **Figure 3.7.9**. T*rans*-GRP was the only compound detected on musts (S1) with no differences among treatments. Furthermore, overall reduction of GRP content after alcoholic fermentation (AF) took place, probably related to oxidative phenomena or metabolization by yeasts.

As can be observed in **figure 3.7.9**, a further decrease was appreciated after stabilization "sur lies" (S2), with a significantly lower content on KT samples. This trend could be attributable to the combination of two events, oxidation of GRP species, and absorption capacity of chitosan.

With respect to AF, depletion of GRP was observed in SO_2 and control samples after 10 months of storage of samples bottled immediately after fermentation (S1-10M) while slightly increased with KT. However, lower content of GPR was observed on KT bott, probably due to an absorption by means of the polysaccharide.

However, an opposite effect took place in samples stored after 30 days of stabilization "sur lies" (S2-10M), showing a general increase in all treatments. Once again, less concentration of GRP species was detected on KT bott samples. Interestingly, the presence of AA in KT samples exhibited a greater protection against GRP depletion and even a further production of GRP during storage.

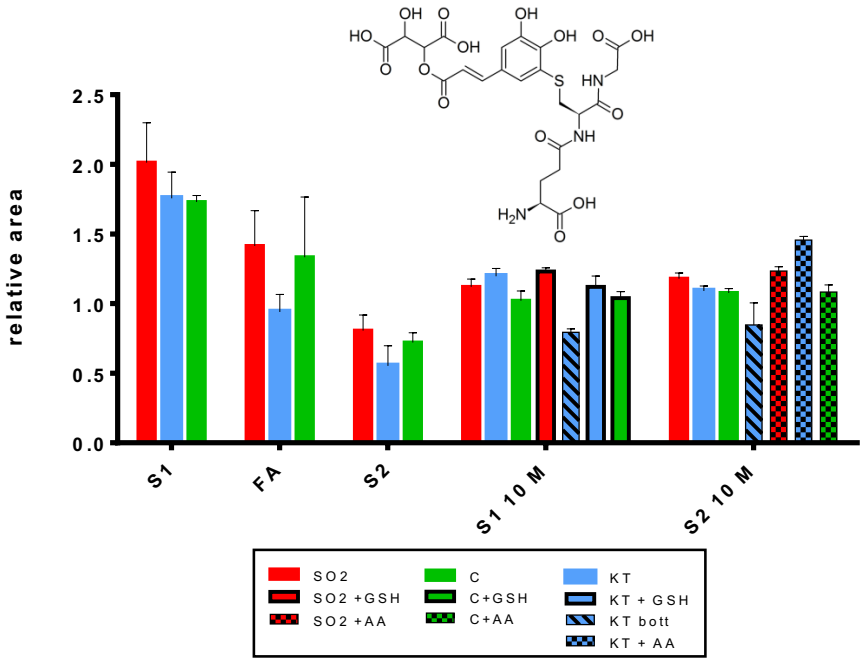

Figure 3.7.9. Evolution of GRP derivatives during distinct stages of vinification. S1= Stabilization of must; FA= Alcoholic fermentation; S2= Stabilization "sur lies" of wine; S1 10M= Samples storage 10 months after fermentation; S2= Samples storage 10 months after stabilization "sur lies".

d) S-sulfonate glutathione

Special attention should be given to the evolution of s-sulfonate glutathione. Glutathione, in aerobic conditions could be oxidised, leading to the formation of its disulfide GSSG (**figure 3.7.10**). However, Arapitsas et at., (Arapitsas et al., 2016) identified s-sulfonate glutathione, as a marker of oxidation of GSH in sulfited wines, resulting from the reaction of GSSG with SO_2.

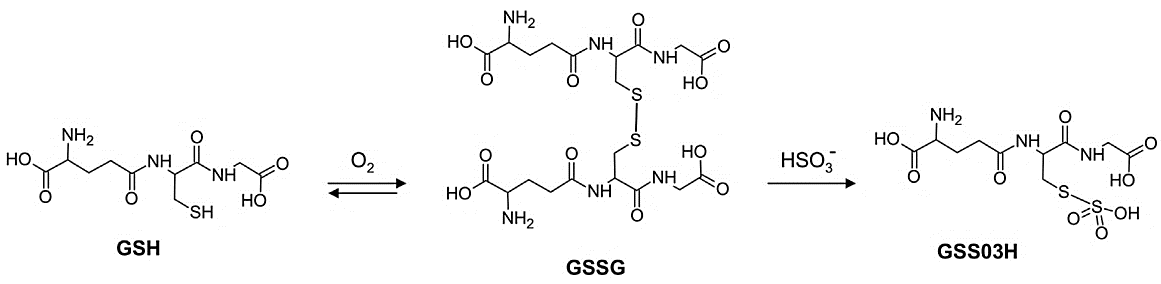

Figure 3.7.10. Oxidation of **GRP**, leading to the formation of glutathione disulfide (**GSSG**) and reaction of GSSG with SO_3H^- to give S-sulfonated glutathione (**GSSO3H**) (Retrieved from (Arapitsas et al., 2016))

In our conditions, as commented in the above section, s-sulfonate glutathione was first detected only after alcoholic fermentation (AF) carried out in the presence of SO_2 (**figure 3.7.11**) probably as a result of the reaction of oxidised GSH produced by yeasts and added sulfites. It is worth noting that no oxidized glutathione (GSSG) was found in our samples (**table 3.7.4** and **3.7.5**) (except traces amounts in SO_2 + GSH wines), confirming what other stated about the fact that GSSG in wines represents less than 5% of initial GSH and that promptly participate in further reactions (Arapitsas et al., 2016; du Toit, Lisjak, Stander, & Prevoo, 2007; Vallverdú-Queralt, Verbaere, Meudec, Cheynier, & Sommerer, 2015)

After the second stabilization "sur lies" (S2), s-sulfonate glutathione remained unchanged. However, after 10 months of storage of samples bottled after fermentation (S1 10M), a successive increase of this compound was observed, mainly due to the oxygen uptake during storage, promoting the further oxidation of GSH into GSSG, that would further react with added SO_2 to produce s-sulfonate glutathione. As expected, addition of GSH in SO_2 wines enhanced the production of s-sulfonate glutathione, as depicted in **figure 3.7.11**. Furthermore, in bottles stored after stabilization "sur lies" (S2-10M) increase of this compound was also appreciated, in a significantly lesser amount in SO2 + AA samples, possibly due to the double reduction of oxygen and SO_2 by means of ascorbic acid via its oxidation. One further done that could be appreciated from **figure 3.7.11** is that even in the absence of GSH addition, sulphites may trap a portion of reduced glutathione, reducing its efficacy as an antioxidant.

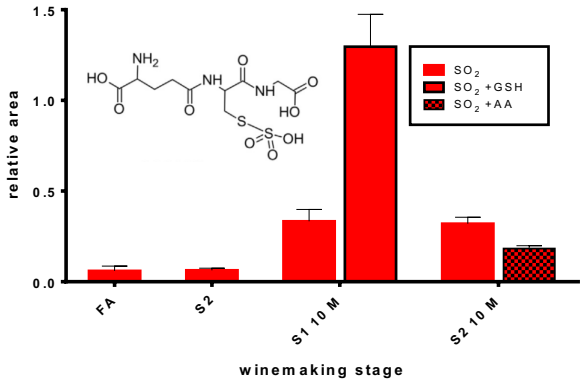

Figure 3.7.11. Evolution of S-sulfonate glutathione during distinct stages of winemaking. S1= Stabilization of must; FA= Alcoholic fermentation; S2= Stabilization "sur lies" of wine; S1 10M= Samples storage 10 months after fermentation; S2= Samples storage 10 months after stabilization "sur lies".

e) Oxidation-related phenolic compounds

e.1) Hydroxy-caffeic acid dimer Isomers

The sum of the products of oxidation of caffeic acid, namely hydroxy-caffeic acid dimers is depicted in **figure 3.7.12**. Although in low concentration, hydroxy-caffeic acid dimers were already observed in the must in all the treatments (S1). Previously, other authors elucidated the structures of these oxidation products in model solutions or in wines (Pati et al., 2014; Rompel et al., 1999; Hiroyuki Tazaki, Taguchi, Hayashida, & Nabeta, 2001) but, to the best of our knowledge, this is the first evidence that the generation of these compounds starts since the very first phase of vinification. As a matter of fact, their formation could derive from both enzymatic and chemical oxidation (Rompel et al., 1999) even if, as vinification proceeds, enzymes are denatured and nonenzymatic route should prevails. As expected, oxidation compounds tended to increase after alcoholic fermentation (AF) and the following technological step "sur lies"

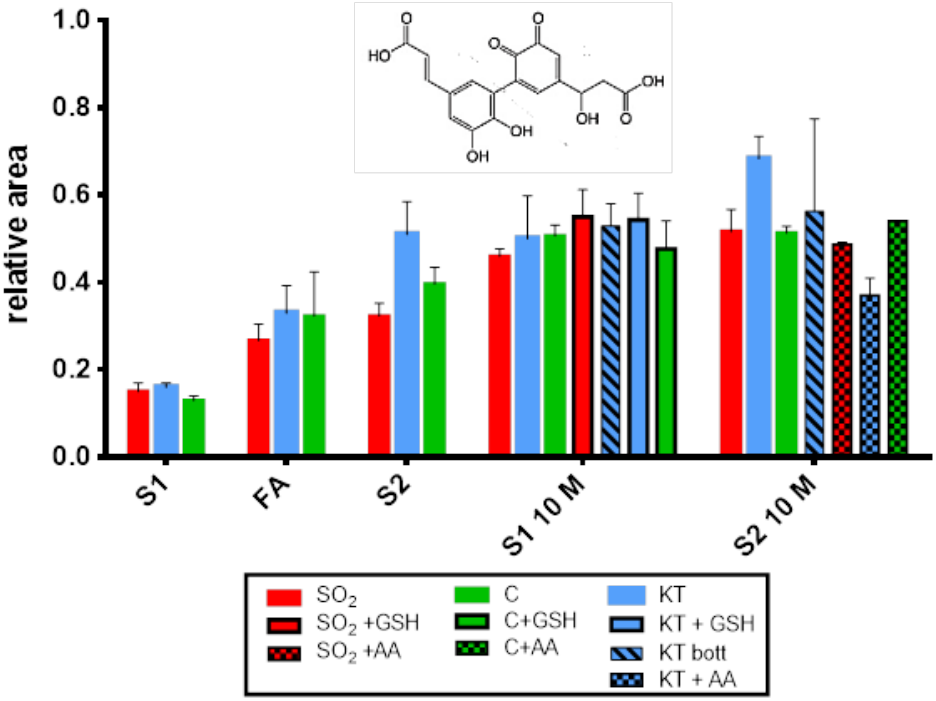

Figure 3.7.12. Evolution of hydroxy caffeic acid dimers during distinct stage of winemaking. S1= Stabilization of must; FA= Alcoholic fermentation; S2= Stabilization "sur lies" of wine; S1 10M= Samples storage 10 months after fermentation; S2= Samples storage 10 months after stabilization "sur lies".

Furthermore, it can be observed a correlation between the formation of caffeic acid and its oxidation products, being generated in to a higher extent on KT samples and at low quantities in SO_2 ones (**table 3.7.4** and **3.7.5**). Interestingly, it is worth to mention that caftaric acid dimers were not detected in our conditions (**table 3.7.4** and **3.7.5**), confirming the results of Arapitsas and co-workers (Arapitsas et al., 2016) who suggested oxidation is less favoured than its hydrolysis to caffeic acid. Therefore, in our experiments, caffeic acid also appeared to be the compound with the greatest tendency to oxidation.

Regarding storage period, similar results were obtained in both objectives (S1-10M and S2-10M) with a progressively increase in these oxidation products, which generation is correlated to the presence of oxygen at the moment of bottling. It could be worth of mention that the simultaneous presence of chitosan and ascorbic acid (samples KT+AA) lead to significant reduction of caffeic dimers (**figure 3.7.12**). The reason of this evidence still remains unclear even if the reducing ability of ascorbic acid against the semiquinones of caffeic acid may play a role. In addition, as already hypothesized in chapter 5 (section 3.3, paragraph 4), since chitosan may reduce the metal content of wine samples, higher amounts of AA could be available to exert its antiradical activity.

In their work Pati et al., (Pati et al., 2014) found that in sulfited wines, hydroxycaffeic acid dimers were not formed because of the reduced oxidative environment. Our data demonstrate that, even with the presence of sulphites, oxidation of caffeic acid occurs to a significant extent.

On the other hand, oxidation products of caffeic acid raised more drastically in KT samples due to the higher content of caffeic acid. However, regarding quinone ether of caffeic acid, treatments with KT resulted in a minor development of this compound.

e.2.) Oxidised sulfur-derived compounds

It is worth noting that the formation tryptophan oxidation derivatives, specially indol lactic acid sulfonate and tryptophol sulfonate was triggered only during stabilization "*sur lies*" (S2) in the presence of SO_2 (**Table 3.7.5**). Figure illustrates the sum of sulphonated compounds (tryptophol and indole-3-lactic sulphonate). Generation of these compounds, which have been previously reported by Arapitsas et al. (Arapitsas et al., 2016, 2018) has been established to be prompted by the presence of oxygen and sulfonation after fermentation, requiring the presence of SO_2 (**Figure 3.7.13**), which is in perfect agreement with our results (**Figure 3.7.14**).

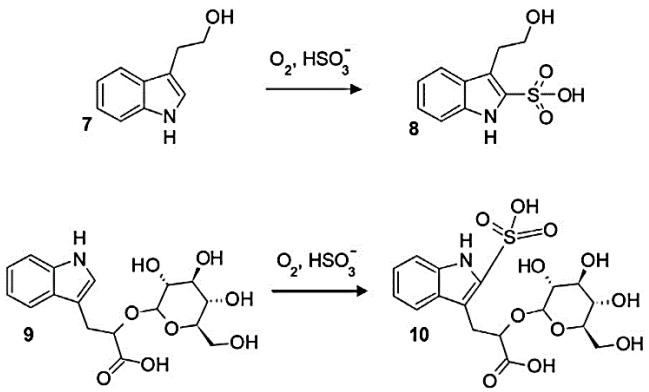

Figure 3.7.13. Sulfonation of tryptophol and indole-3-lactic hexoside to their corresponding derivatives

Furthermore, even if not present after fermentation, further addition of SO_2 before bottling (S1-10M) enhanced the production of indole lactic acid sulfonate and tryptophol sulfonate only in SO_2. Therefore, as a result of its sulfonation, tryptophol content was reduced in SO_2 samples (**Table 3.7.4**).

Regarding samples bottled after stabilization "*sur lies*" (S2-10M), rise of tryptophan derivatives was also observed only in sulfited wines (SO_2) (**Table 3.7.5**). As discussed above, depletion of tryptophol took place in SO_2 as a consequence of the formation of tryptophol sulfonate. However, the latter compound was formed in a much lesser extend in SO_2 + AA. This phenomenon could be related to the absence of available SO_2 to carry out the sulfonation due to the competition of a parallel reaction with hydrogen peroxide generated from the oxidation of ascorbic acid (Bradshaw, Barril, Clark, Prenzler, & Scollary, 2011).

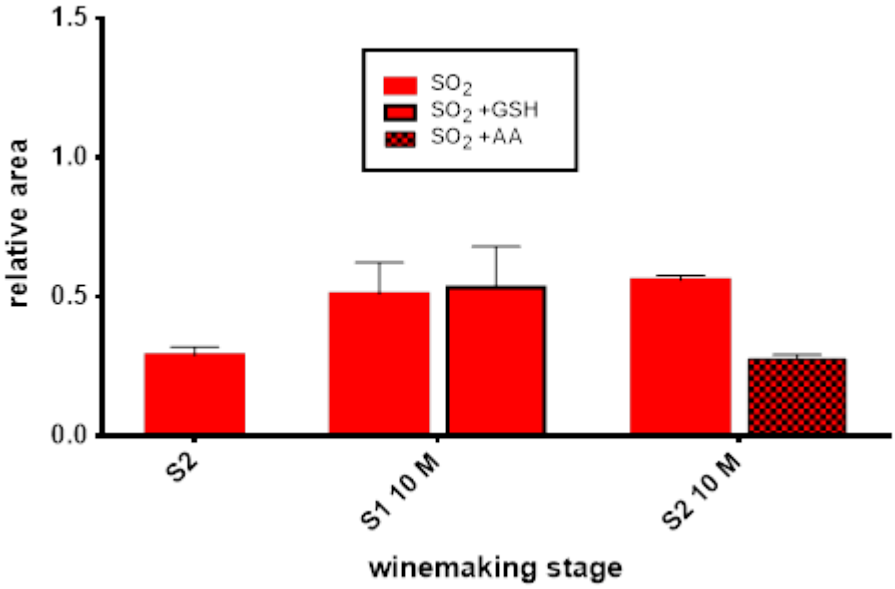

Figure 3.7.14. sum of sulphonated compounds (tryptophol and indole-3-lactic sulphonate) during distinct stage of winemaking. S2= Stabilization "sur lies" of wine; S1 10M= Samples storage 10 months after fermentation; S2= Samples storage 10 months after stabilization "sur lies".

3.7.4. Conclusion

This work gives further details on the evolution of phenolic compounds over a whole process of winemaking of wines obtained by using different antioxidant strategies. Sulphonated compounds related to oxidation were monitored (e.g. S-sulfonated glutathione, and tryptophol and indole-3-lactic sulfonates) being produced after alcoholic fermentation or stabilization "sur lies" and increasing after 10 months of storage. On the other hand, as already appreciated in chapter 6, treatments with chitosan seemed to enhance the hydrolysis of hydroxycinnamates, releasing their corresponding hydroxycinnamics acids. Indeed, the particular behaviour of chitosan when present in bottle after stabilization "sur lies", avoided the phenomenon of hydrolysis and showed higher inhibition against phenolic products of oxidation such as hydroxycaffeic acid dimers. Furthermore, antioxidant capacity seemed to be enhanced after addition of ascorbic acid to KT-treated wines before bottling, reducing the generation of oxidations products and even rising the production of GRP derived phenols, correlated to a better protection against oxygen. Our results confirmed how the absorption properties of chitosan contributed to significantly reduce the total amounts of phenolic compounds and in this way, limiting the oxidative susceptibility of wines. Thanks to this, browning development of KT samples presented a behaviour similar to SO_2 samples, showing a significantly lower development when compared to control samples.

		Stabilization 1. Must (S1)			Alcoholic fermentation (AF)			Storage ST1 10 monts (S1-10M)						
	Must	$S1SO_2$	S1KT	S1C	AF SO_2	AFKT	AFC	SO_2-10M	SO_2-GSH-10M	KT-10M	KT-bott-10M	KT-GSH-10M	C-10M	C-GSH-10M
Hydroxybenzoic acids and flavanols														
Gallic acid	n.d.	0,940 ^{a}	0,760 ^{a}	0,820 ^{a}	1,440 ^{a}	1,010 ^{b}	1,210 ^{ab}	1,212 ^{de}	1,771 ^{a}	1,496 ^{bc}	1,614 ^{ab}	1,402 ^{cd}	1,263 ^{de}	1,141 ^{e}
procyanidin dimer	n.d.	0,116 ^{a}	0,064 ^{b}	0,068 ^{b}	0,066 ^{a}	0,050 ^{a}	0,062 ^{a}	0,058 ^{b}	0,079 ^{a}	0,059 ^{b}	0,025 ^{c}	0,065 ^{ab}	0,049 ^{b}	0,054 ^{b}
(+)-catechin	n.d.	0,365 ^{a}	0,271 ^{ab}	0,250 ^{b}	0,336 ^{a}	0,355 ^{a}	0,333 ^{a}	0,446 ^{a}	0,442 ^{ab}	0,313 ^{cd}	0,215 ^{d}	0,374 ^{abc}	0,340 ^{bc}	0,376 ^{abc}
(-)-epicatechin	n.d.	0,171 ^{a}	0,112 ^{b}	0,128 ^{b}	0,175 ^{a}	0,143 ^{ab}	0,118 ^{b}	0,240 ^{a}	0,213 ^{a}	0,120 ^{de}	0,081 ^{e}	0,153 ^{cd}	0,165 ^{bc}	0,200 ^{ab}
ethylgallate	n.d.	n.d.	n.d.	n.d.	0,369 ^{a}	0,406 ^{a}	0,370 ^{a}	1,060 ^{ab}	1,106 ^{a}	0,902 ^{b}	0,921 ^{b}	0,928 ^{ab}	1,021 ^{ab}	1,019 ^{ab}
Hydroxycinnamic acids														
c-caftaric acid	0,111	0,674 ^{a}	0,468 ^{b}	0,472 ^{b}	0,563 ^{a}	0,439 ^{a}	0,492 ^{a}	0,344 ^{ab}	0,505 ^{a}	0,217 ^{b}	0,272 ^{b}	0,192 ^{b}	0,316 ^{ab}	0,329 ^{ab}
t-caftaric acid	0,481	3,928 ^{a}	2,641 ^{b}	2,717 ^{b}	3,903 ^{a}	2,651 ^{a}	3,132 ^{a}	3,547 ^{b}	4,249 ^{a}	1,867 ^{c}	2,400 ^{c}	1,777 ^{c}	2,367 ^{c}	2,140 ^{c}
t-GRP	0,486	2,014 ^{a}	1,759 ^{a}	1,726 ^{a}	1,362 ^{a}	0,917 ^{a}	1,300 ^{a}	0,973 ^{a}	1,056 ^{a}	1,017 ^{a}	0,690 ^{b}	0,945 ^{a}	0,893 ^{a}	0,926 ^{a}
c-coutaric acid	0,571	1,063 ^{a}	0,886 ^{a}	0,909 ^{a}	0,783 ^{a}	0,569 ^{a}	0,680 ^{a}	0,637 ^{a}	0,754 ^{a}	0,267 ^{c}	0,474 ^{b}	0,255 ^{c}	0,416 ^{b}	0,382 ^{bc}
c-GRP	n.d.	n.d.	n.d.	n.d.	0,033 ^{a}	0,019 ^{a}	0,032 ^{a}	0,066 ^{a}	0,075 ^{a}	0,095 ^{a}	0,064 ^{a}	0,108 ^{a}	0,059 ^{a}	0,036 ^{a}
t-coutaric acid	n.d.	1,888 ^{a}	0,870 ^{b}	1,045 ^{b}	1,658 ^{a}	1,047 ^{a}	1,189 ^{a}	1,723 ^{a}	2,014 ^{a}	0,714 ^{b}	0,996 ^{b}	0,658 ^{b}	0,908 ^{b}	0,813 ^{b}
c-fertaric acid	1,217	1,772 ^{a}	1,613 ^{a}	1,488 ^{a}	1,714 ^{a}	1,448 ^{a}	1,493 ^{a}	1,777 ^{b}	2,052 ^{a}	1,216 ^{d}	1,411 ^{cd}	1,252 ^{cd}	1,479 ^{c}	1,372 ^{cd}
2-S-glutathionyl-t-caffeic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0,045 ^{ab}	0,048 ^{a}	0,033 ^{bc}	0,025 ^{c}	0,034 ^{bc}	0,035 ^{abc}	0,039 ^{ab}
t-fertaric acid	0,108	0,142 ^{a}	0,110 ^{a}	0,109 ^{a}	0,140 ^{a}	0,129 ^{a}	0,135 ^{a}	0,144 ^{ab}	0,170 ^{a}	0,078 ^{bc}	0,108 ^{abc}	0,085 ^{bc}	0,056 ^{c}	0,093 ^{bc}
trans-GRP-Et isomer	n.d.	n.d.	n.d.	n.d.	0,019 ^{a}	0,006 ^{a}	0,008 ^{a}	0,053 ^{ab}	0,066 ^{a}	0,057 ^{ab}	0,029 ^{c}	0,045 ^{bc}	0,051 ^{ab}	0,054 ^{ab}
caffeic acid	n.d.	n.d.	n.d.	n.d.	0,031 ^{b}	0,149 ^{a}	0,111 ^{ab}	0,083 ^{d}	0,107 ^{d}	0,759 ^{ab}	0,279 ^{cd}	0,824 ^{a}	0,516 ^{bc}	0,483 ^{c}
2-S-glutathionyl-c-caffeic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0,002 ^{a}	0,002 ^{a}	0,004 ^{a}	0,003 ^{a}	0,004 ^{a}	0,002 ^{a}	0,001 ^{a}
cis-GRP-Et	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0,005 ^{a}	0,005 ^{a}	0,007 ^{a}	0,004 ^{a}	0,005 ^{a}	0,003 ^{a}	0,002 ^{a}
dihydro-p-coumaric acid	n.d.	n.d.	n.d.	n.d.	0,532 ^{b}	0,623 ^{a}	0,569 ^{b}	0,478 ^{bc}	0,459 ^{c}	0,591 ^{abc}	0,562 ^{abc}	0,605 ^{ab}	0,627 ^{a}	0,634 ^{a}
trans-GRP-Et isomer	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0,022 ^{a}	0,027 ^{a}	0,020 ^{ab}	0,009 ^{b}	0,016 ^{ab}	0,015 ^{ab}	0,019 ^{ab}
p-coumaric acid	n.d.	n.d.	n.d.	n.d.	n.d.	0,015 ^{a}	n.d.	0,029 ^{d}	0,031 ^{d}	0,090 ^{ab}	0,038 ^{cd}	0,107 ^{a}	0,065 ^{bc}	0,062 ^{bc}
Ferulic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0,025 ^{a}	0,024 ^{a}	0,027 ^{a}	0,018 ^{a}	0,039 ^{a}	0,031 ^{a}	0,023 ^{a}
ethylcaffeoate	n.d.	n.d.	n.d.	n.d.	0,054 ^{b}	0,101 ^{a}	0,092 ^{a}	0,156 ^{b}	0,140 ^{b}	0,150 ^{b}	0,212 ^{a}	0,162 ^{b}	0,166 ^{ab}	0,172 ^{ab}
ethylcumarate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0,063 ^{a}	0,031 ^{a}	0,027 ^{a}	0,054 ^{a}	0,031 ^{a}	0,036 ^{a}	0,041 ^{a}
Sum		11,48 ^{a}	8,35 ^{b}	8,47 ^{b}	10,792 ^{a}	8,113 ^{b}	9,233 ^{ab}	10,17 ^{a}	11,82 ^{a}	7,24 ^{b}	7,65 ^{b}	7,14 ^{b}	8,04 ^{b}	7,62 ^{b}
Oxidation products														
s-sulfonate glutathione	n.d.	n.d.	n.d.	n.d.	0,061 ^{a}	n.d.	n.d.	0,332 ^{b}	1.296 ^{a}	0,007 ^{c}	n.d.	n.d.	n.d.	n.d.
Oxidized GSH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0,011 ^{a}	n.d.	n.d.	0,005 ^{a}	n.d.	0,01 ^{a}
hydroxy-caffeic acid Isomer	n.d.	0,103 ^{ab}	0,119 ^{a}	0,086 ^{b}	n.d.	n.d.	n.d.	0,019 ^{b}	0,042 ^{a}	0,036 ^{ab}	0,037 ^{ab}	0,038 ^{ab}	0,021 ^{b}	0,019 ^{b}
hydroxy-caffeic acid Isomer	0,078	0,045 ^{a}	0,043 ^{a}	0,043 ^{a}	0,112 ^{a}	0,114 ^{a}	0,107 ^{a}	0,172 ^{ab}	0,215 ^{a}	0,15 ^{b}	0,165 ^{ab}	0,173 ^{ab}	0,16 ^{ab}	0,153 ^{b}
hydroxy-caffeic acid Isomer	n.d.	n.d.	n.d.	n.d.	0,031 ^{a}	0,050 ^{a}	0,046 ^{a}	0,071 ^{a}	0,084 ^{a}	0,064 ^{a}	0,081 ^{a}	0,068 ^{a}	0,073 ^{a}	0,063 ^{a}
hydroxy-caffeic acid Isomer	0,039	n.d.	n.d.	n.d.	0,125 ^{a}	0,164 ^{a}	0,171 ^{a}	0,199 ^{a}	0,206 ^{a}	0,25 ^{a}	0,247 ^{a}	0,263 ^{a}	0,254 ^{a}	0,242 ^{a}
Indole lactic acid hexoside sulfonate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0,036 ^{a}	0,033 ^{a}	n.d.	n.d.	n.d.	n.d.	n.d.
Caffeic-(o-quinone-caffeic)-ether	0,0494	0,066	0,072 ^{a}	0,058 ^{b}	0,053 ^{a}	0,055 ^{a}	n.d.	0,079 ^{a}	0,073 ^{a}	0,048 ^{a}	0,061 ^{a}	0,038 ^{a}	0,062 ^{a}	0,063 ^{a}
Tryptophol sulfonate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0,308 ^{a}	0,318 ^{a}	n.d.	n.d.	n.d.	n.d.	n.d.
Indole lactic acid hexoside	n.d.	n.d.	n.d.	n.d.	0,250 ^{a}	0,253 ^{a}	0,194 ^{b}	0,201 ^{a}	0,215 ^{a}	0,204 ^{a}	0,200 ^{a}	0,220 ^{a}	0,261 ^{a}	0,259 ^{a}
Other														
tryptophol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0,006 ^{a}	0,010 ^{b}	0,009 ^{b}	0,019 ^{ab}	0,018 ^{ab}	0,023 ^{a}	0,022 ^{ab}	0,020 ^{ab}
tyrosol	n.d.	n.d.	n.d.	n.d.	0,103 ^{a}	0,120 ^{a}	0,111 ^{a}	0,141 ^{bc}	0,173 ^{ab}	0,179 ^{a}	0,180 ^{a}	0,174 ^{ab}	0,133 ^{c}	0,116 ^{c}

Table 3.7.4. Objective 1. Phenolic acids amount (relative peak area) after each stage of the winemaking process. Within the same stage and in the same row, different letters indicate significant differences according to Tukey's test ($p < 0.05$).

	Stabilization 2. Wine (S2)			Storage ST2 10 months (S2-10M)						
	$S2SO_2$	S2KT	S2C	SO_2-10M	SO_2-AA-10M	KT-10M	KT-bott-10M	KT-AA-10M	C-10M	C-AA-10M
Hydroxybenzoic acids and flavanols										
Gallic acid	1,125 ^{a}	1,119 ^{a}	1,286 ^{a}	1,700 ^{abc}	1,719 ^{abc}	1,896 ^{a}	1,811 ^{ab}	1,934 ^{a}	1,491 ^{c}	1,551 ^{bc}
procyanidin dimer	0,072 ^{a}	0,053 ^{a}	0,054 ^{a}	0,089 ^{a}	0,104 ^{a}	0,054 ^{bc}	0,040 ^{c}	0,068 ^{b}	0,055 ^{bc}	0,058 ^{b}
(+)-catechin	0,442 ^{a}	0,349 ^{a}	0,411 ^{a}	0,477 ^{ab}	0,504 ^{a}	0,371 ^{bcd}	0,264 ^{cd}	0,251 ^{d}	0,371 ^{bcd}	0,383 ^{abc}
(-)-epicatechin	0,225 ^{a}	0,159 ^{b}	0,169 ^{ab}	0,221 ^{ab}	0,279 ^{a}	0,120 ^{c}	0,101 ^{c}	0,101 ^{c}	0,161 ^{bc}	0,194 ^{b}
ethylgallate	0,558 ^{ab}	0,437 ^{b}	0,636 ^{a}	1,108 ^{ab}	1,128 ^{a}	0,951 ^{c}	1,003 ^{abc}	0,992 ^{bc}	1,057 ^{abc}	0,103 ^{abc}
Hydroxycinnamic acids										
c-caftaric acid	0,459 ^{a}	0,290 ^{b}	0,430 ^{a}	0,343 ^{a}	0,315 ^{a}	0,248 ^{a}	0,246 ^{a}	0,301 ^{a}	0,265 ^{a}	0,290 ^{a}
t-caftaric acid	3,165 ^{a}	1,589 ^{b}	2,506 ^{a}	3,952 ^{a}	4,114 ^{a}	1,355 ^{c}	1,612 ^{bc}	1,193 ^{c}	2,374 ^{b}	2,317 ^{b}
t-GRP	0,779 ^{a}	0,532 ^{a}	0,691 ^{a}	0,969 ^{b}	1,037 ^{b}	0,968 ^{b}	0,739 ^{c}	1,272 ^{a}	0,892 ^{bc}	0,919 ^{b}
c-coutaric acid	0,603 ^{a}	0,333 ^{b}	0,525 ^{a}	0,629 ^{a}	0,643 ^{a}	0,205 ^{b}	0,284 ^{b}	0,204 ^{b}	0,314 ^{b}	0,349 ^{b}
c-GRP	0,014 ^{a}	0,011 ^{a}	0,013 ^{a}	0,123 ^{a}	0,088 ^{a}	0,035 ^{a}	0,061 ^{a}	0,075 ^{a}	0,108 ^{a}	0,084 ^{a}
t-coutaric acid	1,425 ^{a}	0,568 ^{c}	0,933 ^{b}	2,177 ^{a}	2,099 ^{a}	0,429 ^{c}	0,594 ^{bc}	0,430 ^{c}	0,991 ^{b}	1,010 ^{b}
c-fertaric acid	1,464 ^{a}	1,038 ^{b}	1,374 ^{ab}	1,972 ^{a}	2,091 ^{a}	1,147 ^{bc}	1,139 ^{bc}	0,992 ^{c}	1,419 ^{bc}	1,467 ^{b}
2-S-glutathionyl-t-caffeic acid	0,013 ^{a}	0,008 ^{a}	0,011 ^{a}	0,043 ^{ab}	0,052 ^{a}	0,052 ^{a}	0,036 ^{bc}	0,044 ^{ab}	0,045 ^{abc}	0,040 ^{ab}
t-fertaric acid	0,101 ^{b}	0,101 ^{b}	0,133 ^{a}	0,106 ^{a}	0,120 ^{a}	0,092 ^{a}	0,106 ^{a}	0,092 ^{a}	0,093 ^{a}	0,099 ^{a}
trans-GRP-Et isomer	0,011 ^{a}	0,006 ^{a}	0,008 ^{a}	0,059 ^{bc}	0,074 ^{ab}	0,061 ^{bc}	0,034 ^{d}	0,082 ^{a}	0,050 ^{cd}	0,055 ^{c}
caffeic acid	0,054 ^{b}	0,574 ^{a}	0,374 ^{a}	0,107 ^{d}	0,120 ^{d}	0,936 ^{a}	0,751 ^{bc}	0,919 ^{a}	0,549 ^{c}	0,489 ^{c}
2-S-glutathionyl-c-caffeic acid	n.d.	n.d.	n.d.	0,007 ^{a}	0,004 ^{a}	0,002 ^{a}	0,000 ^{a}	0,003 ^{a}	0,006 ^{a}	0,007 ^{a}
cis-GRP-Et	n.d.	n.d.	n.d.	0,006 ^{a}	0,007 ^{a}	0,005 ^{a}	0,005 ^{a}	0,006 ^{a}	0,007 ^{a}	0,009 ^{a}
dihydro-p-coumaric acid	0,462 ^{b}	0,519 ^{b}	0,640 ^{a}	0,485 ^{bc}	0,463 ^{c}	0,602 ^{a}	0,613 ^{a}	0,601 ^{a}	0,597 ^{abc}	0,601 ^{a}
trans-GRP-Et isomer	0,003 ^{a}	0,004 ^{a}	0,003 ^{a}	0,022 ^{ab}	0,027 ^{a}	0,021 ^{ab}	0,009 ^{c}	0,023 ^{ab}	0,021 ^{abc}	0,017 ^{bc}
p-coumaric acid	0,023 ^{b}	0,065 ^{a}	0,053 ^{a}	0,036 ^{d}	0,037 ^{d}	0,121 ^{a}	0,097 ^{bc}	0,124 ^{a}	0,063 ^{c}	0,057 ^{cd}
Ferulic acid	0,022 ^{a}	0,033 ^{a}	0,029 ^{a}	0,032 ^{a}	0,030 ^{a}	0,041 ^{a}	0,045 ^{a}	0,042 ^{a}	0,022 ^{a}	0,020 ^{a}
ethylcaffeoate	0,052 ^{b}	0,096 ^{a}	0,105 ^{a}	0,153 ^{a}	0,167 ^{a}	0,155 ^{a}	0,154 ^{a}	0,147 ^{a}	0,176 ^{a}	0,169 ^{a}
ethylcumarate	0,013 ^{a}	0,019 ^{a}	0,022 ^{a}	0,048 ^{a}	0,053 ^{a}	0,022 ^{a}	0,021 ^{a}	0,023 ^{a}	0,036 ^{a}	0,044 ^{a}
Sum	8,66 ^{a}	5,79 ^{b}	7,85 ^{a}	11,27 ^{a}	11,54 ^{a}	6,50 ^{c}	6,55 ^{c}	6,57 ^{c}	8,03 ^{a}	8,04 ^{a}
Oxidation products										
s-sulfonate glutathione	0,061 ^{b}	n.d. ^{a}	n.d. ^{a}	0.323 ^{a}	0.182 ^{b}	n.d.	n.d.	n.d.	n.d.	n.d.
Oxidized GSH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
hydroxy-caffeic acid Isomer	0,005 ^{b}	0,015 ^{a}	0,000 ^{c}	0,036 ^{ab}	0,035 ^{b}	0,055 ^{a}	0,037 ^{ab}	0,023 ^{b}	0,036 ^{ab}	0,040 ^{ab}
hydroxy-caffeic acid Isomer	0,147 ^{a}	0,149 ^{a}	0,152 ^{a}	0,211 ^{a}	0,199 ^{ab}	0,192 ^{ab}	0,182 ^{ab}	0,125 ^{b}	0,168 ^{ab}	0,182 ^{ab}
hydroxy-caffeic acid Isomer	0,063 ^{a}	0,061 ^{a}	0,069 ^{a}	0,090 ^{a}	0,078 ^{ab}	0,086 ^{a}	0,076 ^{ab}	0,050 ^{b}	0,074 ^{ab}	0,071 ^{ab}
hydroxy-caffeic acid Isomer	0,107 ^{b}	0,283 ^{a}	0,176 ^{b}	0,178 ^{b}	0,176 ^{b}	0,350 ^{a}	0,265 ^{ab}	0,169 ^{b}	0,234 ^{ab}	0,251 ^{ab}
Indole lactic acid hexoside sulfonate	0,008 ^{a}	n.d.	n.d.	0,042 ^{a}	0,002 ^{b}	n.d.	n.d.	n.d.	n.d.	n.d.
Caffeic-(o-quinone-caffeic)-ether	0,061 ^{a}	0,061 ^{a}	0,060 ^{a}	0,068 ^{a}	0,068 ^{a}	0,056 ^{ab}	0,053 ^{ab}	0,029 ^{b}	0,052 ^{ab}	0,050 ^{ab}
Tryptophol sulfonate	0,053 ^{a}	n.d.	n.d.	0,377 ^{a}	0,020 ^{b}	n.d.	n.d.	n.d.	n.d.	n.d.
Indole lactic acid hexoside	0,239 ^{a}	0,227 ^{a}	0,246 ^{a}	0,184 ^{a}	0,255 ^{a}	0,237 ^{a}	0,205 ^{a}	0,174 ^{a}	0,230 ^{a}	0,236 ^{a}
Other										
tryptophol	0,015 ^{a}	0,024 ^{a}	0,019 ^{a}	0,009 ^{a}	0,013 ^{a}	0,017 ^{a}	0,013 ^{a}	0,016 ^{a}	0,019 ^{a}	0,017 ^{a}
tyrosol	0,132 ^{a}	0,126 ^{a}	0,132 ^{a}	0,161 ^{a}	0,173 ^{a}	0,175 ^{a}	0,160 ^{a}	0,162 ^{a}	0,157 ^{a}	0,163 ^{a}

Table 3.7.5. Objective 2. Phenolic acids amount (relative peak area) after each stage of the winemaking process. Within the same stage, and in the same row different letters indicate significant differences according to Tukey's test ($p < 0.05$).

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4. Conclusions

4. Final conclusions and perspectives

Based on the obtained results, the starting premise, where it was hypothesized that the natural polysaccharide chitosan could present antioxidant activity in wine media, could be confirmed. Electron spin resonance (EPR) experiments in synthetic and real wines demonstrated, in fact, a direct antiradical capacity of that molecule toward hydroxyl radical ($^{\cdot}OH$) and inhibition of the generation of the radical intermediate of ethanol, namely 1-hydroxyethyl radical (1-HER).

Chitosan has been found to further interfere on the Fenton oxidative cascade by a double mechanism: quenching of hydrogen peroxide and strong metal (Fe^{+}; Fe^{++}, Cu^{+}) chelation. A corollary evidence we obtained suggests that the chemical oxidation in wines is much more influenced by the presence of metals than by the concentration of H_2O_2. Therefore, the use of a natural metal chelator such as chitosan would be a suitable choice to inhibit oxidative phenomena in oenology. Indeed, results of EPR analysis showed a strong relationship between the chelating effect of chitosan and the development of oxidative processes. The effect of chitosan on the reduction of metal content was doubly confirmed by EPR and flame atomic absorption spectroscopy analysis.

A consequence of the cited activities was that the presence of **chitosan in wine or wine-like media, inhibited the development of oxidative aldehydic intermediates, such as acetaldehyde and glyoxylic acid,** up to magnitudes of 50 to 70%, depending on the doses. Those aldehydes are the primary product of wine oxidation, impacting its evolution by facilitating the formation of brown polymers and acting as a bridge in nucleophilic reactions.

Winemaking experiments carried out both at laboratory and semi-industrial scale, offered a further confirmation of some of the previous results. Conclusions obtained are summarized as follow:

- Chitosan showed an **inhibitory effect on browning** development both during the winemaking process and during storage period.
- Treatment with chitosan produces a **reduction of polyphenolic content of wines, due to its nature as polyelectrolyte and consequent absorption of dissolved ions**. This contributed to enhance the oxidative stability of wines but, at the same time, lowered its patrimony in physiologically active molecules
- For the same reason, depending on the dose and stage in which it is used, chitosan can significantly **reduce the content of organic acids**, so previous panning of its application becomes crucial to avoid damaging the final product.
- When used during fermentation chitosan may have an **impact on the volatile profile of resulting wines**, increasing pleasant compounds such as isoamyl acetate or β-phenylethyl acetate.

- HPLC-DAD-MS studies showed an **enhanced hydrolysis of hydroxycinnamates** thus increasing the releasing of the corresponding hydroxycinnamic acids.
- During long term storage, chitosan lowered the development of browning and **inhibited the formation of compounds related to oxidation** such as hydroxy caffeic dimers.
- As opposite to sulphites, chitosan did not appear to interact with other antioxidants admitted in oenology. Its simultaneous utilization with ascorbic acid and glutathione did not changed its efficacy.
- Sensory test did not show negative parameters in samples treated with chitosan, **being positively appreciated by the tasters**.

Based on the promising antioxidant activity exerted by chitosan, its use as a potential alternative to the use of sulfites in wines could be confirmed even if additional studies (on red wines or sparkling wines, for example) are certainly needed.

Further subjects of investigation will consider how an increased solubility of the polysaccharide will affect its performances and doses. In principle, in fact, by increasing the specific surface area of the polymer, an increase of absorption and chelation features should be observed. Increased chitosan solubility will raise, in addition, the needs for a second subject of research, which is the development of an analytical method for its determination in wine. Up to now, in fact, due to the insolubility in wine matrix, the necessity of an official or validated method to be applied in wine has been overlooked.

Another field of investigation should involve the exploitment of filming capacities of chitosan. This would be a promising feature to be used in an innovative approach to “active packaging” of wine with antibrowning and antimicrobic purposes.