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The influence of some antioxidant molecules and oxygen levels on oxidative aging of wine

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SILVIA MOTTA

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"Where shall I begin please, your Majestry?" he asked.
"Begin at the beginning", the king said, very gravely,
"and go on till you come to the end: then stop".(Lewis Caroll)

This thesis is dedicated to Claudio, Zoe and my parents, for their support

Abstract

The oxygen has a key role during all the winemaking process: the oxygen uptake before, during and after the alcoholic fermentation influences wine characteristics and evolution both in positive and negative way.

Generally, if red wines can benefit from a certain degree of oxidation to reduce astringency or enhance/stabilize color, white wines are usually damaged upon exposure to oxygen.

To promote the beneficial effects of oxygen exposure, while avoiding spoilage risks, there is a need to better understand the mechanisms controlling oxygen dissolution and consumption in wine. Several studies have shown the key role of iron and the enhancing effect of copper in catalyizing wine oxidation. Nowadays, the content of iron in wine is lower compared to the past, for the use of stainless steel facilities. On the other hand, many are the winemaking steps, from the vineyard to the bottle, that can influence the final content of copper in wine. Therefore, further studies on the influence of copper content on wine oxidation could be useful for winemakers in particular to evaluate the amount of SO_2 at bottling to prevent wine oxidation.

The attested antioxidant, antioxidasic and antimicrobial properties of SO_2 makes sulfure dioxide the most common additive for the protection of wine against oxidative spoilage.

However, it has been widely proven that a prolonged absorption of SO_2 can cause health problems and an allergenic effect in sensitive subjects.

In the future, a decrease in the SO_2 concentration limits for wine is expected, and, in some cases, the prospective is a completely SO_2-free wine.

Other molecules are now being studied for their antioxidant and antiradical properties, such as reduced glutathione (GSH) and enological tannins (ellagitannins and gallotannins).

Recently, several works have been published that deeply address the mechanisms that regulate the oxidation reactions, with the aim of defining the theoretical basis for the reduction in the use of SO_2 in wines. These experiments were mostly performed on the laboratory scale with model solutions and only in few cases with real wines.

The aim of this PhD thesis, was to study the influence of some antioxidant compounds either naturally present in grapes (polyphenols and glutathione) or exogenous (SO_2 and hydrolysable tannins) on the oxygen consumption kinetc in wine and model solution.

The first part of the thesis regarded the study of the effect of both SO_2 and copper on the oxidation kinetics, that is on the rate of oxygen consumption and on the oxidative reactions that lead to browning.

The results confirmed the key role played by metals, and in particular by copper, in the oxidation of polyphenols. The O_2 cannot react directly with the reducing substances of wine, but with transition metals and free radicals. The important role of SO_2 in protecting wine against the consequences of oxidation was confirmed.

The effect of some antioxidant molecules (GSH, ellagitannins, or gallotannins) as partial substituents of SO_2 was investigated in second part of the study. The different molecules were added to three different white wines from the Cortese cultivar before bottling. The evolution of chemical and sensory characteristics of the wines were then monitored during bottle aging. Moreover, the effect of two different level of oxygen at bottling, medium and high (different bottling conditions) was studied.

The results of both chemical and sensory analysis highlighted the positive and irreplaceable role of SO_2 in protecting white wines from oxidative aging during storage in bottles. No effect on the natural oxidative evolution, and in particular on the color browning and on the appearance of olfactory notes of oxidized was observed either for GSH or for tannins in any trial. The chemical analysis were in agreement with the results of sensory evaluation.

On a practical point of view, to date the only way to extend the shelf life of wines without adding too high an amount of SO_2 at bottling, is to control the oxygen uptake in bottled wine. Furthermore, since the free SO_2 takes part directly to the oxidative process, it is important to limit during winemaking the production of molecules that can combine free SO_2, in order to reduce the doses of total SO_2.

In the last part of thesis, a comparison between the efficiency of the SO_2 and the GSH used at the same molarity on controlling the Fenton reaction was investigated in a model solution. The results confirmed a low efficiency of GSH, also at the high amounts studied. Furthermore, the efficiency of GSH resulted hightly influenced by the composition of the mean, in particular by the content of ferrous ion and of dissolved oxygen.

Riassunto

L'ossigeno ha un ruolo fondamentale durante tutto il processo di vinificazione: l'apporto di ossigeno, prima, durante e dopo la fermentazione alcolica può influenzare le caratteristiche del vino e la sua evoluzione sia in modo positivo che negativo.

In generale i vini rossi possono beneficiare di un certo grado di ossidazione per ridurre l'astringenza e stabilizzare il colore, mentre i vini bianchi di solito sono danneggiati dall'ossigeno.

Per favorire i benefici di esposizione all'ossigeno ed evitare i rischi di deterioramento, è necessario capire a fondo i meccanismi di ossidazione nel vino. Molti studi hanno messo in luce il ruolo chiave dei metalli ferro e rame come catalizzatori delle ossidazioni.

Al giorno d'oggi, l'utilizzo di strutture in acciaio inossidabile in cantina ha portato a un contenuto minore di ferro nei vini. Il contenuto in rame è invece tuttora influenzato dai vari passaggi che portano dal vigneto alla vinificazione. Pertanto ulteriori studi sull' influenza del contenuto di rame nelle ossidazioni del vino potrebbero essere utili per i produttori; in particolare per valutare la quantità di SO_2 necessaria all'imbottigliamento.

L'antiossidante più utilizzato in enologia è infatti la SO_2, grazie alle sue note proprietà antiossidasiche ntimicrobiche e antiossidanti. E' stato tuttavia ampiamente dimostrato che una prolungato assorbimento di solfiti può causare problemi di salute e può avere un effetto allergizzante in soggetti sensibili. La prospettiva futura è quindi una riduzione dei solfiti nei vini fino eventualmente a una completa eliminazione.

Per questo motivo si stanno studiando altre molecole con proprietà antiossidanti e antiradicaliche come il glutatione ridotto (GSH) i e tannini enologici (ellagitannini e gallotannini).

Recentemente sono stati pubblicati molti lavori riguardanti lo studio dei meccanismi di ossidazione per capire meglio le basi teoriche per ridurre i solfiti nei vini. Tuttavia, questi studi sono stati eseguiti per lo più su soluzioni modello e in pochi casi su vino.

Lo scopo di questa tesi di dottorato è stato quello di studiare l'influenza di alcuni composti antiossidanti, sia naturalmente presenti nelle uve (polifenoli e glutatione) o esogeni (SO_2 e tannini idrolizzabili) sulla cinetica del consumo di ossigeno sia in vino che in soluzione modello.

La prima parte della tesi ha riguardato lo studio dell'effetto di SO_2 e rame sulla cinetica di ossidazione, in particolare sul consumo dell'ossigeno e sull'imbrunimento.

I risultati hanno confermato il ruolo chiave svolto da metalli, ed in particolare del rame, sull'ossidazione dei polifenoli e l'importante effetto della solforosa nel proteggere il vino contro le ossidazioni.

L'effetto di alcune molecole antiossidanti (GSH, ellagitannini o gallotannini) come sostituenti parziali della solforosa SO_2 è stato studiato nella seconda parte della tesi. Le diverse molecole sono state aggiunte a tre diversi vini bianchi della cultivar Cortese prima dell'imbottigliamento. L'evoluzione delle caratteristiche chimiche e sensoriali dei vini è stata seguita durante la conservazione in bottiglia. Inoltre, è stato studiato l'effetto di due livelli di ossigeno (medio e alto) a simulare due diverse condizioni di imbottigliamento.

Il positivo e insostituibile ruolo della solforosa nel proteggere i vini bianchi dall'invecchiamento ossidativo è stato confermato sia dall'analisi chimica che sensoriale. Per quanto riguarda gli altri additivi studiati, non è stato osservato alcun effetto protettivo durante la conservazione, né dal punto di vista chimico e né dal punto di vista sensoriale. L'unica possibilità al giorno d'oggi per prolungare la shelf life dei vini senza aggiungere eccessive dosi di solforosa all'imbottigliamento rimane limitare l'apporto di ossigeno all'imbottigliamento e ridurre durante la vinificazione la produzione di molecole che possano combinare la solforosa libera riducendone la sua efficacia.

L'ultima parte della tesi ha riguardato il confronto tra solforosa e glutathione, aggiunti alla stessa dose molare, sulla reazione di Fenton. L'efficacia del GSH è risultata scarsa anche per le alte dosi teastate e altamente influenzata dalla composizione del mezzo, in particolare dal contenuto di ferro ferroso e di ossigeno disciolto.

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1. Oxygen in enology

The first knowledge on the influence of oxygen on wine are dated back to Pasteur's early studies in 1866. In his *Etude sur le vin* (Pasteur, 1866), he stated that if on one hand oxygen is the worst enemy of wine, on the other hand oxygen makes wine, which ages under its influence. The next important studies were undertaken by Ribereau-Gayon in 1931.

Since that time, many researches have studied the relationship between oxygen and wine.

Generally, oxygen is detrimental for food since it may have as consequences degradation of vitamins or lipids, loss of nutritional value, development of off-flavours and browning (Lindsay, 1996; Bradshaw *et al.*, 2001). Some wines can be an exception for this rule: maderized wines (e,g. Maderiras or Jerez) wherein the aging under oxidative conditions is essential for the quality of these products (Wildenradt *et al*., 1974). Many red wines also benefit from a certain degree of oxidation to reduce astringency or enhance/stabilize color (Atanasova *et al*., 2002; Castellari *et al*., 1998). White wines are usually damaged upon exposure to oxygen (Danilewicz, 2003; Singleton, 1987; Singleton, 2000).

To promote the beneficial effects of oxygen exposure, while avoiding spoilage risks, there is a need to better understand the mechanisms controlling oxygen dissolution and consumption in wine.

In general, wine can consume a large quantity of oxygen, red more than white, for their higher amount of polyphenols.

The oxygen has a key role during all the winemaking process: oxygen uptake before, during and after the alcoholic fermentation influences wine characteristics and evolution both in positive and negative way.

During crushing, pressing and other processing steps, oxygen comes into contact with grape musts. Oxygen uptake in musts is related to many factors: polyphenols content, polyphenoloxydases (PPO) content and their activity, pH, temperature, etc.

The rate of oxygen consumption in must is related to temperature: it is three times faster at 30°C than at 10°C and decreases at temperature above 40°C because of polyphenoloxidase enzymes inactivation (Dubernet & Ribéreau-Gayon, 1974). The consumption rate of oxygen by grape PPO ranges from 30 to 200 mg/L/hr (Dubernet *et al*., 1974; Dubourdieu *et al*., 1990; Cheynier *et al*., 1993). The uptake is faster initially, but decreases as the phenolic substrates are depleted. Laccase, PPO present in grapes infected with *Botrytis cinerea*, increases the total oxygen intake (Cheynier *et al*., 1993). In the absence of sulfiting, the depletion of oxygen by

PPO occurs rapidly (4 to 20 min) (Ribéreau-Gayon *et al*., 2000). Addition of SO_2 in musts stops oxygen consumption, mainly for the antioxydasic effect of SO_2 (Dubernet and Ribéreau-Gayon *et al*., 1974).

Traditional white winemaking can lead to high oxygen exposure of musts. Moreover, there is a technique, called hyper-oxygenation, which consists in exposing must to high oxygen concentration. It can be performed by various technical processes, for example by insuflating oxygen when must is pumping from tank to tank, or from press to tank, by using oxygen or air instead of nitrogen during flotation for must clarification (Shneider, 1998). For this technique, it can be used both pure oxygen and compressed air. Since the latter contains only 21% oxygen, the gas flow rate has to be five fold faster than when oxygen is used. Hyper-oxygenation is a tool to reestablish the flavonoid-oxygen balance when flavonoid content is too high to be removed by naturally available oxygen. This technique can lead to a reduction of polyphenolic concentration in white wines and is thought to lead to an improvement in wine color stability (Schneider, 1998).

On the other hand, reductive winemaking is a white wine production technique that avoids oxygen contact during the whole vinification process, from pressing to bottling. This technique combines the use of high levels of anti-oxidants molecules and inert gas with practices aimed at limiting the yeasts'oxygen demand during alcoholic fermentation. Reductive winemaking has been widely studied and applied to the grape varieties rich in aromatic precursors (Sauvignon blanc, in particular), which can develop varietal thiol aromas during alcoholic fermentation: the use of reductive winemaking protects these wine aromas from oxidation, thus extending the shelf life of the wines after bottling.

During alcoholic fermentation oxygen has a key role. Even if *Saccharomyces cerevisiae* is optional anaerobic, the addition of oxygen during alcoholic fermentation is necessary for a correct fermentation. Oxygen is essential for yeast to synthesize sterols (David & Kirsop, 1973; Aries & Kirsop, 1977-78) and to desaturate fatty acids to produce unsaturated fatty acids (Ratledge & Evans, 1989; Klein & Volkmann, 1975). These lipids are incorporated into cell wall enhancing membrane permeability and ethanol tolerance of yeasts.

When a stuck of fermentation occurs, an oxygen addition to the must is required to improve the lipids synthesis that increases the fermentation rate (Salmon, 2005).

In anaerobic conditions the role of oxygen in cellular activity can be replaced by the addition of compounds such as ergosterol that the cell is not able to syntethize and that permit the cellular performance as in oxygenated mean (Cyzewski & Wilke, 1976).

Regarding to the amount and the schedule of oxygen addition, Sablayrolles *et al.* (1986) report the amount of oxygen consumed by yeasts during alcoholic fermentation is between 10 and 20 mg/L. To improve the kinetic of fermentation and reduce the risk of sluggish fermentation, the addition of oxygen is often coupled with the addition of nitrogen (Sablayrolles, 1990).

Oxygen addition is an important tool for enologists to control must fermentation.

During fermentative maceration in red winemaking, normally, the oxygen is added by pumpovers and/or délestages. It is difficult to evaluate the oxygen uptake consequent to a pumping or a délestages, because an amount of oxygen is stripped by the CO_2 produced during alcoholic fermentation (Boulton *et al.*, 1996).

Once alcoholic fermentation has finished, dead yeast cells (lees) keep on consuming oxygen during wine aging for at least 6 months when wines are stored at 14°C and in the absence of air. The amount of oxygen consumed by lees varies with the different yeast strains and it does not seem to be influenced by wine composition. Oxygen consumption decreases during the time, expecially within the first two months after the end of alcoholic fermentation and it stabilizes at values ranging from 3 and 11 mg/L/h/10^{-9} cells between the second and the sixth month of aging. A yest lees population of 10^{11} yeast cells is supposed to consume an amount of oxygen ranging from 9.5 to 10.5 mg/L in 10-20 hours. This amount is higher than the amount of oxygen dissolved in wine after a saturation with air (Fornairon *et al*., 1999). Therefore, wines aged in presence of the yeast cells that have fermented the musts (aging *sur lies*), are protected from oxidation during aging, but, on the other hand, these wines might develop reduced notes.

Limited exposure to air during malolactic fermentation seems to enhance diacetyl production (Nielsen & Richelieu, 2000; Bartowsky & Henshke, 2004), which has an important influence on aroma of some wine, for instance in Chardonnay, where diacetyl contributes to the typical buttery aroma of this wine.

Acetic acid bacteria are aerobic micro-organisms that occur in wine. They are known to be responsible for the acetic spoilage of wine due to the conversion of ethanol to acetic acid with the consequent acidification of wine and an increase of volatile acidity.

Acid bacteria go into a viable but not culturable state in wine under limited O_2 conditions (Du Toit *et al*., 2005); the O_2 permeating through oak staves during aging probably supports the survival of acetic acid bacteria in wine.

Oxygen has also been found to contribute to the rapid growth of *Brettanomyces* in wine. *Brettanomyces* are yeasts able to spoil wine by producing, with enzyme cinnamate decarboxylase, 4-vinylphenol and 4-vinylguaiacol respectively from p-coumaric and p-ferulic

acids. These phenols are known for their medicine-like, farmyard and horse sweat off –flavors. Though their low amounts could positively contribute to wine flavor complexity, high amounts impair wine quality.

Many enological operations, such as crushing, pressing, pumping, racking, filtration, centrifugation, refrigeration, filling up, bottling and barrel aging, can cause an increase in dissolved oxygen in must and wine, whose content varies from few mg/L, working in optimal conditions, to saturation, when these operations are performed with vigorous agitation in open air. The amount of oxygen uptake during some cellar operations was determined (Vidal *et al*., 2001, 2003, 2004; Vivas & Glories 1993).

During the storage in wooden barrel, the oxygenation occurs not only during barrel filling and topping up, but also during barrel aging. The entrance of oxygen through the staves is promoted by the depression created in sealed barrels during aging by water evaporation (Moutounet *et al*., 1998). During barrel aging both the humidity of the wood and the thickness and grain of the staves play an important role on the amount of oxygen that can dissolve in wine. Lower humidity, tight grain and thinner staves all allow more oxygen to permeate into wine; but wine is seldom saturated with oxygen for the insufficient contact (Vivas *et al*., 2003).

Micro-oxygenation is a technique developed in France in the mid 1990s aimed at replicate barrel conditions for wine matured in large stainless steel and concrete tank (Ducournau & Laplace, 1995; Lemaire, 1995). It consists in introducing small and controlled amounts of oxygen into wines in order to improve wine colour, aroma and texture (Pontailler & Ribereau-Gayon, 1983; Moutounet & Mazauric 2001; Ribereau-Gayon *et al*., 1998). This technique is performed by using specialised facilities that can dose the oxygen additions (Parish *et al*., 2000; Paul, 2002). The value of micro-oxygenation is that the oxygen is introduced at a rate equal to, or less than the oxygen consumption rate of the wine (Paul, 2002), thus, avoiding accumulation of dissolved oxygen.

The amount of oxygen given to wine during micro-oxygenation can vary from 2 to 90 mg/L/month, depending on both the type of wine (polyphenols content) and the moment of the addition (soon after the alcoholic fermentation or after some months of aging). (Dykes, 2007).

Through barrel aging and micro-oxygenation improve red wine quality, they are generally detrimental for the sensorial characteristics of white and rosè wines (Moutounet & Mazauric, 2001; Ribereau-Gayon *et al*., 1998).

The level of dissolved oxygen and the redox potential are two critical parameters to take into account during winemaking process and during micro-oxygenation (Vivas *et al*., 1992; Vivas & Glories, 1997).

Regarding bottling operations, Gibson and O'Brien (2006) proved that the air retained in the bottle headspace after sealing is the main source of oxygen exposure for bottled wine. In fact, bottle flushing with inert gas prior to filling cannot guarantee alone an oxygen-free headspace: both the bottle headspace and, when using screw-caps, the underside of the cap itself must be flushed with inert gas before sealing.

Regarding the oxygen that permeates through the closures during bottle aging, it is well known how only a flame-sealed glass bulb is completely impermeable to oxygen. On the contrary, the different kinds of commercial closures are permeable to oxygen to an extent varying with the composition. The permeability of each kind of closure is measured as oxygen transfer rate (OTR: µg/day) (Lopes *et al*., 2007).

Studies were performed to verify how different oxygen intakes through the closures (different OTR values) could influence the color evolution and the phenolic composition (Wirth *et al*., 2010), as well as the sensory characteristics (Caille *et al.*, 2010). Higher oxygen intakes (higher OTR) increased both the consumption rate of SO_2 and the degradation of anthocyanins and flavan-3-ols: the wines aged with higher OTR closures had a lower SO_2 content and a higher color intensity than the wines with lower OTR closures (Wirth *et al*., 2010); moreover, they had stronger olfactory notes of fruit and caramel, and lower animal notes.

1.1. Solubility of oxygen in wine

Oxygen represents 20.9% of the atmospheric air, where it mainly occurs as molecular oxygen, O_2. The presence of oxygen in wine is related to the properties of all gases to dissolve into liquids.

A gas dissolved in a liquid is just a physic phenomenon: when a gas and a liquid are in contact, the gas molecules dissolve into the liquid till the equilibrium is reached. At the equilibrium the amount of molecules of the gas dissolved in the liquid is proportional to its partial pressure.

The quantity of oxygen dissolved in a liquid can be calculated both by its concentration in the liquid and by its partial pressure.

More commonly, concentration values are used. In wines concentrations are expressed in ppm (mg/L) or, for lower concentrations, in ppb (µg/L)

Partial pressure is a key parameter and can be defined as the pressure to be applied on the liquid surface to keep a given concentration of the gas into the liquid. Partial pressure is normally expresses as Pascal (P).

Concentration and partial pressure are related through the Henry's law:

"At a constant temperature, the amount of a given gas, that dissolves in a given type and volume of liquid, is directly proportional to the partial pressure of that gas in equilibrium with that liquid":

$$\mathbf{P = kC}$$

Where **P** is the partial pressure of the gas, **C** the concentration and **K** is the solubility constant, the Henry's constant. **K** parameter is influenced by the nature of the gas considered, by the temperature and by the composition of the liquid medium. The higher is the Henry's constant, the lower is the solubility of the gas.

Hence, the amount of oxygen that can be find in a liquid, at a given pressure, changes with the temperature and with the composition of the liquid.

In wine, the concentration of oxygen that can be reached at saturation at 20°C and at atmospheric pressure of 1 atm, is 8.4 mg/L. Temperature has a strong influence on oxygen solubility in wine: cold temperatures increase oxygen solubility in wine, while warm temperatures have the opposite effect.

Approximatly a decrease in temperature of 5°C causes the 10% increase in oxygen solubility.

Ethanol content also influences oxygen solubility: the solubility slightly decreases for low increase in alcohol (as the case of wine), and markedly increases for alcohol higher than 30% v/v (as the case of spirits) (Moutounet & Mazauric., 2001).

Furthermore, oxygen solubility can also be slightly influenced by dry extract: wines with higher dry extract generally have a little lower value of oxygen solubility.

The rate of the oxygen dissolution is settled by Fick's law:

$$\mathbf{dC/dt = k1 * a(C-C1)}$$

where **C** is the concentration of the gas at equilibrium (related to partial pression in Henry's law), **C1** is the initial concentration of the dissolved gas, **k1** a constant, a the surface of the liquid in contact with air.

Therefore, the rate of oxygen dissolution is directly proportional to both the surface contact liquid-gas and the starting concentration of the gas in the liquid.
In particular, oxygen dissolution will be faster as the surface of the liquid increases and the starting oxygen level decreases.

1.2. Oxygen consumption in wine

Wine has a good capacity to consume oxygen dissolved in it.
Oxygen dissolved in wine is consumed with time. In red wine after saturation, oxygen is reduced to below 1 mg/L in *ca* six days at 30°C and in longer length of time at lower temperatures. This reduction is mainly due to reactions with phenolics (Singleton, 1987). Therefore, a certain period after oxygen uptake, no traces of oxygen can be found in wine.
If the oxygen is replenished in the wine by diffusion from headspace or renewed contact with air, the consumption reaction will continue or repeat. The overall amount of O_2 a wine can take up is large, ranging from about 60 to over 600 mL/L from light white to heavy red. The reaction, except in must, or sometimes in very early stages of wine storage, is not enzymatic and is basically the same whether or not the wine has been pasteurized or depleted of proteins.
Oxygen rarely improves white wines, but usually improves red wines.
Phenols are the major substrates for oxygen in wines and wine's capacity to consume oxygen is related to its phenolic concentration (Rossi & Singleton, 1966): while individual phenols can react very differently, the total content of phenols in a wine is a rough measure of its capacity to take up oxygen, its ability to withstand oxidation, and its capacity to change when exposed to oxygen (Singleton 1987). However, it is important to take into account that some today's wine could have very higher phenolic levels as the result of the enological practices of maceration before and after fermentation (Ritchey and Waterhouse 1999) and iron levels lower than in the past (Sauvage *et al*. 2002), principally for the introduction of stainless steel facilities in the cellar. This could increase the resistence of wines to oxidation.

1.3. Phenolic compounds in wines: primary substrates for oxidations

Phenolic compounds are the main substrate for oxygen reaction in wine, especially 1,2-dihydroxyphenols, which can be easily oxidized to quinones.

Controlled oxidation can improve sensory quality of red wines by enhancing and stabilizing color and reducing astringency, on the contrary white wines are generally damaged by exposure to oxygen (Singleton, 1987; Singleton, 2000).

Phenolic molecules originating from grapes can be divided essentially into flavonoid and non-flavonoid compounds.

The flavonoids are characterized by a flavane nucleus, consisting of two benzene rings (A and B) linked by an oxygenated heterocycle (C). Differences in the degree of oxidation of the heterocycling ring (C) and hydroxylation/methoxylation of the three rings lead to different families of flavonoids with essential chemical differences. The oxidation reactions can contribute to produce polimers between flavanols (du Toit, 2006).

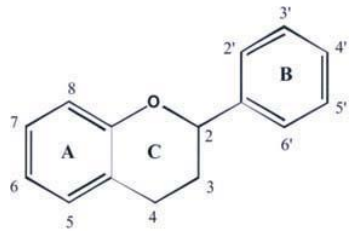


Figure 1.3.1.: Flavonoid molecule

The most important flavonoids in wine are anthocyanins (malvidin-3-glucoside, cyanidin-3-glucoside, peonidin-3-glucoside, petunidin-3-glucoside, delphinidin-3-glucoside), flavan-3-ols (catechin, epicatechin, tannins) and flavonol (kaempferol, quercetin, myricetin).

The concentration of flavonoids in wine is strongly influenced by the winemaking practices such as pressing and maceration which influence the degree of extraction from skins and seeds which are rich in flavan-3-ols molecules.

Flavonols are the phenolic group present at lower concentrations both in grapes and in wines, 1-3 mg/L in white wines and about 100 mg/L in red wines. They are differentiated by substitution of the lateral nucleus, producing kampferol (1 OH), quercetin (2 OH) and myricetin (3 OH). All three pigments are present in red wine grapes, whereas white wine grapes only have the first two (Ribereau-Gayon, 1964).

The flavanols include flavan-3-ols (catechins) and flavan-3,4-diols. Different –H and –OH substituents on B ring give different molecules: (+)-gallocatechin, (-)-epigallocatechin, (+)-catechin, (-)-epicatechin; only the last three are present in wine.

These molecules can associate through C4/C6 and C4/C8 bonds to form dimers, trimers and oligomers, the flavans or proanthocyanidins. A further polymerization leads to condensed

tannins or grape tannins. The amount of proanthocyanidins or condensed tannins varies from 1 g/L to 4 g/L in red wines (Ribereau-Gayon *et al*., 2000), while in white wines it is in the range of 100 mg/L and it is influenced by the cultivars composition and by the pressing techniques (Ribereau-Gayon *et al*., 2000). Regarding the sensorial characteristics of these compounds, monomeric catechins are bitter, while polymers are astringent (Peleg *et al*., 1999).

Anthocyanins originate mainly from the skins of red grape cultivar, in wine they are present at concentration ranging from 100 mg/L to more than 1000 mg/L, and are responsible for the color of red wines. The different constituents of the B ring lead to different molecules (Ribereau-Gayon *et al*., 2000; Monagas *et al*., 2005) (Figure 1.3.2.)

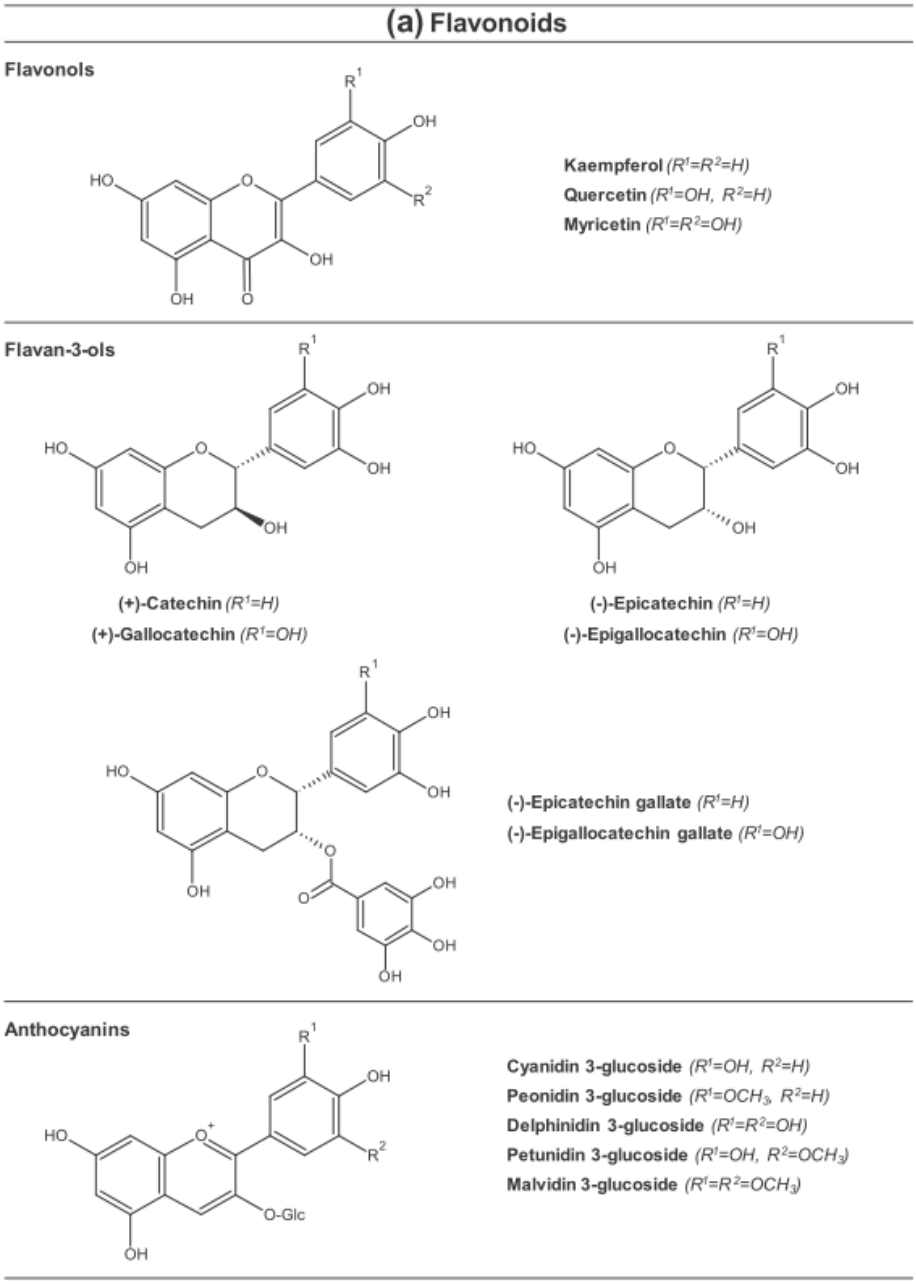


Figure 1.3.2.: Most common flavonoid compounds in wine (Oliveira *et al*., 2011)

Non-flavonoids compounds occur at higher concentration in grape juice, therefore, are the main phenolic molecules in white wines that did not receive long period of skin contact (Margalit, 1997; Mongas *et al*., 2005). Their concentration ranges from 50-250 mg/L, depending on

cultivar and winemaking techniques. The non-flavonoids are mainly hydroxybenzoic and hydroxycinnamic derivatives, the hydroxycinnamyl tartaric acids (HCTA), cis and trans isomers of tartaric esters of caffeic acid, p-coutaric acid and ferulic acids.

Another class of non flavonoids in grape are stilbenes and stilbene glycosides, the most known is *trans*-resveratrol.

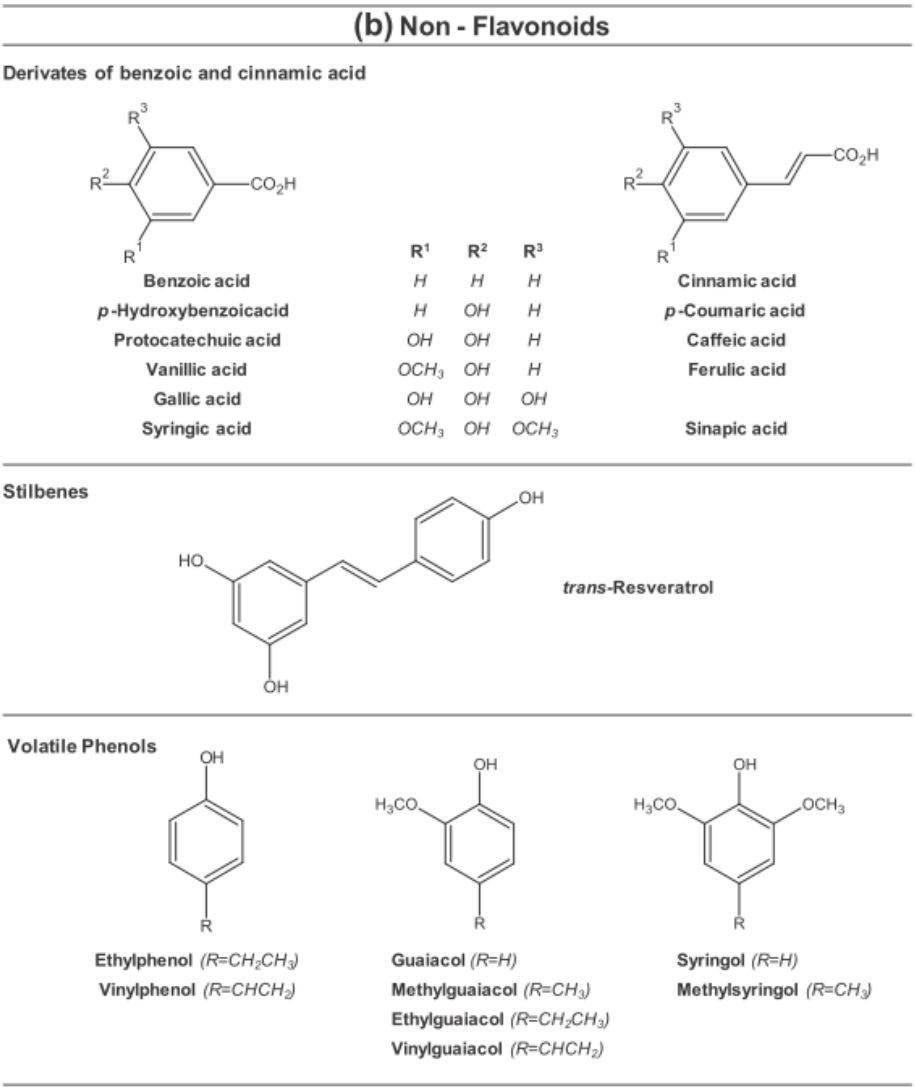


Figure 1.3.3.: Most common non flavonoid compounds in wine (Oliveira *et al*., 2011)

The other major source of phenolics in wine is the wood, when wines come in contact with it, during fermentation and especially during aging. These phenolics are non flavonoids and are

called hydrolysable tannins because they can be hydrolyzed to form gallic or ellagic acids. They are esters of gallic acid (gallotannins) and ellagic acid (ellagitannins) with glucose.

The concentration of these tannins is very low in wines compared to oak, this difference can be related to the oxidations occurring during aging that contribute to their overcome (Vivas & Glories, 1996).

Red wines have higher amount of polyphenols (1 to 5 g/L) than white wines (0.2 to 0.5 g/L). The effect of oxygen addition in red wines include a decrease in some phenolic compounds such as (+)-catechin, (-)-epicatechin, quercetin, caffeic acid and anthocianins and an increase in red polymeric pigments improving the wine color density (Castellari *et al*., 2000)

Catechols are oxidized to quinones by the sequential abstraction of two electrons with the concomitant loss of two protons: in effect the transfer of two hydrogen atoms. Different phenolic molecules have different tendency to oxidize. The reduction potential of each phenolic molecule is determinated mainly by the ring constituents. The lower the reduction potential, the greater the reducing power of the reduced component of the copuple catechol-quinone (Table1.3.1.). The reduction potential of a phenoxyl radical/phenol couple is determined by ring costituents and is due to the effects of substituents on electron density and distribution in aromatic systems. Electro-donating groups, such as vicinal -OH groups, -OMe, -Me, have lower reduction potential; while electron-withdrawing groups such as -COMe or $-CO_2Et$ have the opposite effect (Danilewicz, 2003).

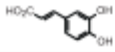
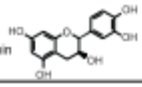
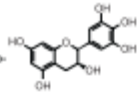
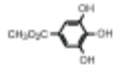
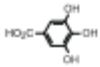
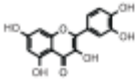
$ArO^{\cdot} + e^{-} + H^{+} \gg ArOH$	pK_r	pK_1	pK_2	E_0^2 V	$E_{3.5}^2$ V	E_7^2 V
Catechol 	5.0	9.45	12.8	1.06[a]	0.85[h]	0.53[a]
3,5-Dihydroxy-anisole 	6.7	9.30	11.3	1.28[h]	1.07[h]	0.84[b] 0.85[e]
Caffeic acid 	4.6	7.6	11.85	1.09[h]	0.88[h]	0.54[c] 0.55[e]
(+)-Catechin 	4.6	A, 9.41 B, 8.64	A, 11.26 B, 13.26	1.12[h]	0.91[h]	0.57[c]
(-)-Epigallo-catechin 	5.5			0.93[d]	0.73[d]	0.42[b] 0.43[e]
Methyl gallate 	4.4 9.2	8.03	11.6	1.12[h]	0.91[h]	0.56[b] 0.56[e]
Gallic acid 	5.0	8.73	12.4	0.98[h]	0.78[h]	0.45[f]
Quercetin 	4.2 9.4	6.74 9.02	11.55			0.33[g]
Ascorbic acid 	-0.45	4.2	11.5	0.99[a]	0.55[h]	0.30[a]

Table 1.3.1.: Dissociation constants and reduction potentials of some semiquinone/phenol systems of relevance to wine at pH 0, 3.5 and 7. (Danilewicz, 2003)

1.4. Oxydation mechanisms in musts and wines

Oxidation in wine can be enzymatic or non-enzymatic (chemical). Normally these 2 pathways are found in sequence during winemaking, but can also coexist in some particular cases.

The enzymatic oxidation occurs in musts and it is very fast, the enzymes involved are the natural grapes tyrosinase and, in grapes infected with *Botrytis cinerea*, laccase (Singleton, 1987).

Laccase has a wider spectrum of substates, mainly 1,4-didroxybenzenes and 1,2-dihydroxybenzenes; as regards tyrosinases the main substrates are the 1,2- dihydroxybenzenes, that is catechol derivates. The most abundant catechol derivates present in must are caftaric and coutaric acids, which are oxidized to the corresponding *o*-quinones by grape tyrosinase (polyphenol oxidase, PPO), released once grapes are crushed (Singleton *et al*., 1985). The further reactions leading to brown pigments and other products are nonenzymatic and are influenced by the redox properties and electronic affinities of *o*-quinones formed (Robards *et al.*, 1999). In particular, since quinones are oxidants, they can oxidize substances with lower potential such as polyphenols, ascorbic acid and SO_2. The quinones are then reduced back to the original phenol. Being electrophiles, they can also react with nucleophiles, such as amino derivatives (Kutyrev *et al.,* 1991). Enzymatic browning in must is then highly correlated with the content of caftaric and coutaric acids (Cheynier *et al.,* 1986) and is supported by flavan-3-ols (Cheynier *et al.,* 1995). PPO activity towards catechol derivates increases when grapes are bruised or damaged in presence of oxygen, but not in presence of nitrogen (Traverso-Rueda *et al,* 1973).

Non enzymatic (chemical) oxidations occur expecially during wine aging.

The cascade of oxidative process starts with the oxidation of polyphenols containing an ortho-dihydroxybenzene (catechol) ring, such as, (+)-catechin/(−)-epicatechin, caffeic acid and its esters; or a 1,2,3-trihydroxybenzene (galloyl) group, such as (+)-gallocatechin, gallic acid and its esters, that are the most readily oxidizable compounds in wine (Singleton, 1987; Singleton, 2000; Kilmartin, Zou, & Waterhouse, 2001; Danilewicz, 2003; Li *et al*., 2008).

These substrates are oxidized to semiquinones and quinones, while oxygen is reduced to hydrogen peroxide. Since oxygen cannot react directly with phenolic compounds, for the limitation of reactivity of triplet oxygen, the reaction is catalized by transition metal ions, in particular by the redox cycle of Fe^{3+}/Fe^{2+} and $Cu^{2+/}Cu^{+}$ (Danilewicz, 2003; Danilewicz, 2008; Waterhouse & Laurie, 2006). Compounds with more isolated phenolic groups, such as

malvidin, *para*-coumaric acid and resveratrol, are oxidized at higher potentials (Kilmartin *et al*., 2001)

Quinones formed from oxidation of polyphenols are unstable and electrofilic, they may react with nucleophilic compounds, such as phenols and thiols, to give dimers or polymers, which have lower reduction potential than the initial phenols and, thus, are much more readily oxidized (Li *et al.*, 2008; Singleton, 1987).

Hydrogen peroxide (H_2O_2), in association with ferrous ions, generates hydroxyl radical ($HO^{\cdot}$) (Fenton reaction). $HO^{\cdot}$ is a strong oxidant, capable of nonspecifically oxidizing virtually all organic constituents, in proportion to their abundance. First of all ethanol and tartaric acid, but also glycerol, sugars and other organic acids (Danilewicz 2003, Danilewicz 2007, Waterhouse & Laurie 2006, Li *et al* 2008).

This PhD thesis was focused on chemical oxidations that occurs during wine aging.

1.5. Chemical oxidation in wine

Oxidation is the chemical process in which an electron is removed from an atom or molecule; on the contrary, reduction is the chemical process in which an electron is added to an atom or molecule.

These processes may or may not involve the oxygen addition or removal and the hydrogen loss or addition.

The first molecules oxidized in presence of oxygen are the phenolic compounds, this process starts a cascade of chemical transformations, which need to be taken under control during the production of wine. Rossi and Singleton (1966) observed that, the resistance to oxidation of a wine, was related to its phenolic concentrations. Since red wines are characterized by higher phenolics content than white wines, they can better stand large amounts of oxygen.

Wine phenols exist in either phenol or phenolate forms, it depends on the pH of the wine.

Two are the possible pathways of phenolic oxidation: the auto oxidation of phenols to phenolates, that occurs at high pH, and the oxidation chain reaction catalyzed by metals.

The final product of both the processes is the hydrogen peroxide.

The first pathway occurs under high pH conditions, where the feeble acidic character of phenols (pKa 9 to 10) allows them to form phenolate anion that can react directly with oxygen. The removal of an electron from phenolate leads to the formation of a semiquinone, which is further oxidized to the corresponding quinone, or, to be more precise, disproportionates to yield a

quinone and a phenol. At pH 9-10, 50% or more of the phenol exists as phenolate ion, autoxidation is very quick and it finishes in 30 minutes at room temperature (Rossi *et al*., 1966).

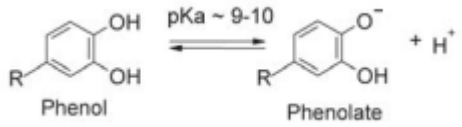


Figure 1.5.1.: Phenol-phenolate anion equilibrium (Waterhouse, 2006)

Oxidation is easier from a phenolate than from the protonated phenol (Singleton, 1987). However, since wine has low pH (3-4) and phenolics have high pKa, only a so small fraction of phenolics is deprotonated following this oxidation pathway, that cannot be considered (Singleton, 1987; Danilewicz, 2003).

Regarding the second pathway, the most quickly oxidized constituents of wine are catechol derivatives with vicinal phenolic functions, such as caffeic acid, (-)- epicatechin, (+)-catechin, (+)-gallocatechin, gallic acid and its esters (Singleton *et al*., 1987; Singleton *et al*., 2000).

The oxygen in its normal triplet state, with two unpaired radical electrons in different orbitals, cannot react directly with polyphenols. Oxygen in triplet state can be activated by accepting single electrons from transitional metals or free radicals to became in singlet form with no unpaired electrons (Danilewicz, 2003).

The oxidative process starts, then, with the oxidation of Fe(II) to Fe(III) by oxygen, a process which is accelerated by copper (Danilewicz, 2011; Danilewicz & Wallbridge 2010).

It is proposed the one-electron reduction of oxygen produces hydroperoxyl radical (figure 5, reaction A) and a further Fe(II) ion takes part to the oxidative process of this radical to hydrogen peroxide (reaction B).

The ferric ions obtained from these reactions react with catechols, oxidizing them first to semiquinones and then to quinones (reactions C and D).

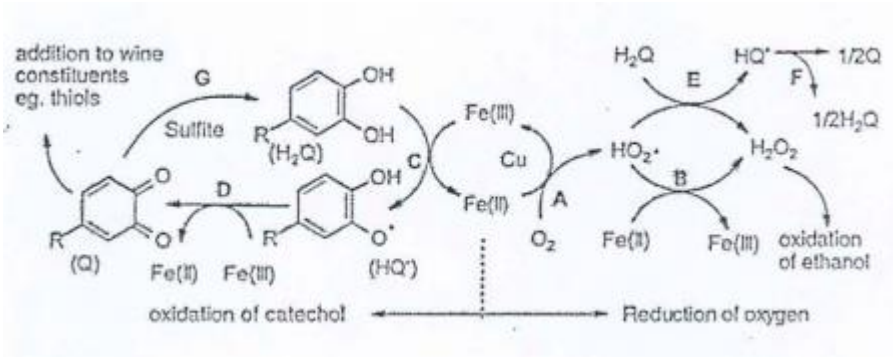


Figure 1.5.2.: Proposed mechanism for the reduction of oxygen to hydrogen peroxide coupled to the oxidation of a catechol (H_2Q) stepwise to a semiquinone ($HQ^{\cdot}$) and quinone (Q) mediated by redox cycling of Fe (Danilewicz, 2013)

Danilewicz, in a latest work, shows that if the oxidation of ferrous ions is quick and is likely to reach the equilibrium, on the contrary, the oxidation of catechols, such as (+)-catechin, by ferric ions does not take place naturally at a significant pace. It needs the presence of molecules that can react quickly with quinones and promote an equilibrium otherwise difficult to reach. These substances have been named as "oxidation promoting nucleophiles" and include sulfite. (Danilewicz, 2011).

Catechols are the main substances which react with hydroperoxyl radical producing semiquinone (reaction E), which may disproportionate (reaction F) (Waterhouse & Laurie, 2006), and hydrogen peroxide.

The second part of the oxidative process regards the reduction of hydrogen peroxide by ferrous ions to produce hydroxyl radical ($^{\cdot}OH$) (Fenton reaction) (Green & Hill, 1984; Boulton, 2003; Danilewicz, 2003), a potent oxidant, capable of nonspecifically oxidizing virtually all organic constituents in proportion to their abundance. Therefore, prime substrates are ethanol and tartaric acid, which produce respectively acetaldehyde and glyoxylic acid.

The cascade of oxidative process starts a complex sequence of transformations, which change wine compositions and characteristics.

The result of the whole oxidation process is the reduction of oxygen to water, by accepting four electrons and the oxidation of two ferrous ions for each reacting oxygen. The reduction is completed with the donation of two electrons. At the same time two ferric ions are reduced back to ferrous ions, oxidizing catechol and completing the recycling process (Danilewicz, 2013).

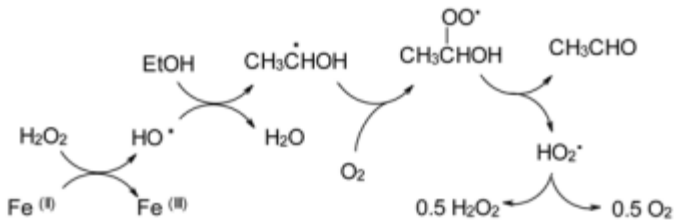


Figure 1.5.3.: Proposed mechanism for the oxidation of ethanol by hydrogen peroxide: the Fenton reaction and involvement of oxygen to regenerate hydrogen peroxide (Danilewicz, 2013)

1.6. The effect of pH

As already reported at the paragraph 1.5., the phenols can exist in either the phenol or phenolate anion forms, and this equilibrium is influenced by the pH. At pH 4 exist 10 times more phenolate than at pH 3 and the autoxidation rate should be 10 times as fast. This can explain why unbalanced high pH wines from hot vintages, even if they have a properly maturation, oxidize readily and quickly become "tired" and "weak". A suitable lowering of pH would improve the shelflife of wines obtained from overripe grapes (Singleton, 1987).

Different phenolic molecules have different behavior at high pH: caffeic acid and gallic acid are less stable towards degradation, than (-)-epicatechin and (+)-catechin. The molecular structures can explain the different behavior: (+)-catechin and (-)-epi-catechin are not planar and the p electrons of the two benzene rings cannot interact with one another due to conjugation. The spatial arrangments of the –OH groups and the p electrons influence the extent of p orbital overlap and, consequently, its susceptibility to chemical change (du Toit, 2006). Care should then be taken, especially when handling white wines with high pH, because these are more susceptible to oxidation, since they contain caffeic acid derivates as the main phenolic molecules (Cilliers & Singleton 1990a; Cilliers & Singleton,1990b; Boulton *et al.,* 1996; Friedman & Jiirgens 2000).

1.7. The role of iron and copper

The average concentration of iron and copper in wine from different regions of the word ranges from 2.7 to 8.8 mg/L and 0.1 to 0.36 mg/L respectively (Ough & Amerine, 1988).

These metals in enology are principally known as sources of wine instability, but they have much wider importance, having a key role in the whole wine chemistry.

The importance of iron (Fe) and copper (Cu) as catalysts of wine oxidation has been known for a long time.

In his doctoral thesis, Jean Ribereau-Gayon reported that when a wine had been long protected from air, the Fe exists in the ferrous state, Fe(II). However, when white wine was saturated with air, O_2 is quickly consumed to oxidize Fe (II) to Fe (III) (Ribereau-Gayon, 1931). He concluded that the concentration of Fe (III) depended on both the rate of formation by reaction of Fe (II) with O_2 and its rate of reduction by wine constituents. Since Fe returned to the ferrous state as O_2 concentrations declined, he proposed that wine oxidation involved the redox cycling of iron acting as an intermediate oxidant.

Further studies showed that oxygen cannot react directly with wine constituents without the help of iron salts and traces of copper, which enhance the catalytic role of iron (Peynaud, 1984). The need of catalytic molecules is due to the electronic configuration of oxygen in its triplet ground state. Since oxygen contains unpaired electrons, it cannot accept electron from substances which contain paired electrons, such as polyphenols and sulfite, but it accepts electron by reacting with transition metals and free radicals (Danilewicz, 2003).

Ribereau-Gayon (1931) also showed that sulfite oxidation was markedly accelerated by Fe in model wine and its action was further enhanced with the addition of Cu.

More recent studies of the oxidation of polyphenols in presence of sulfite showed that the oxidation of 4-methylcathecol in a winelike system containing SO_2 starts to be significant after the addition of Fe and increases further after the addition of Cu (Danilewicz, 2007). These studies confirm the key role of Fe as catalyst of oxidation and the enhanced effect of copper.

The accelerating effect of copper on ferrous oxidation suggests that Cu facilitates Fe(III)/Fe(II) redox cycling (Figure 1.7.1.).

The importance of metals is also supported by studies which demonstrate that the rate of oxidation of white wines could be slowed and eventually stopped by the progressive removal of Fe and Cu with potassium ferrocyanide (Ribereau-Gayon, 1931; Danilewicz & Wallbridge, 2010).

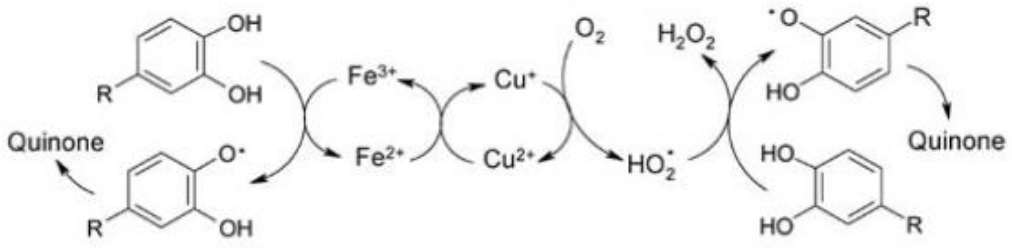


Figure 1.7.1.: Proposed catalytic action of iron and copper in the oxidation of catechols to produce quinones and and hydrogen peroxide (Danilewicz, 2008)

1.8. Primary oxidation products in wine

As already reported in the previous paragraphs, the primary substrates for oxidation are phenolic compounds: the catechol functional groups react with hydroperoxyl radical to form a semiquinone radical and hydrogen peroxide (Figure 1.8.1., reaction 2). The semiquinone radical disproportionates to give one equivalent of quinone (Figure 1.8.2., reaction 3). Quinones in varying degrees of polymerization produce yellow-brown color. This oxidation reaction is influenced by the copper and iron concentrations.

Even if the main phenolic molecules present in white wines are the hydrocynnamic derivatives, the major role in the browning of white wine is played by flavanols, especially (+)-catechin, (-)-epicatechin and dimeric procianidins B1-B4. Hydroxycinnamic acids can contribute to browning because of involving in coupled oxidation reactions with these flavanols (Simpson, 1982; Fernandez-Dubano *et al*., 1995)

Hydrogen peroxide reacts with ferrous iron to give hydroxyl radical (Figure 8, reaction 4) which is very unstable and reacts with all substances present in solution proportionaly to their concentration, first of all alcohol and organic acids (Figure 8, reaction 5). The oxidation products in wine are acetaldehyde from ethanol and keto acids from organic acids, for instance, tartaric acid forms many small aldehydes while malic acid yields pyruvic acid (Fenton, 1984).

This non selective reaction mechanism suggests that many other products are formed from other abundant molecules of wine such as sugars, glycerol and acids.

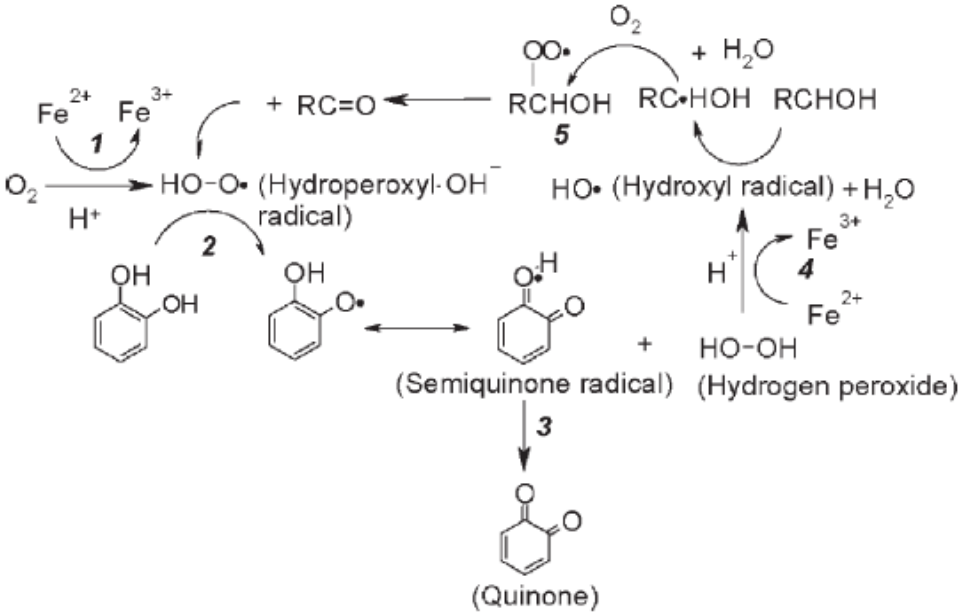


Figure 1.8.1.: Reductive oxidation cascade and primary oxidation products (Waterhouse, 2006)

1.9. Secondary oxidation products in wine

Quinones formed from oxidation of polyphenols can react with nucleophiles, first of all with thiols or phenolics and then with other nucleophiles, depending on their relative concentrations and reactivity. One of the most well known reaction quinone-thiol is the reaction between the quinone of caftaric acid and the tripeptide thiol glutathione to produce the 2-S-glutathionyl caftaric acid, also called grape reaction product (GRP) (Singleton, 1987) (Figure 1.9.1.).

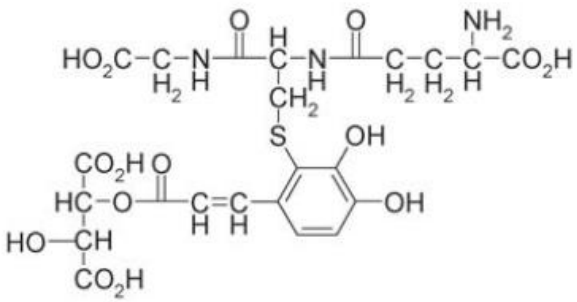


Figure 1.9.1.: Generation of 2-S-glutathionyl caftaric acid (GRP)

Another important reaction quinone-thiol involves catechin and 3-mercaptohexanol that leads to the loss of fruity varietal aroma of Merlot, Cabernet Sauvignon and Cabernet Franc (Blanchard *et al*., 2004).

Regarding the reactions quinones-phenolics, they generally occur with the electron-rich A ring of flavanols, the consequence is the formation of a new bond between two phenolic substances. When those two molecules are on condensed tannin chains, the result is a new, bigger tannin molecule, with bonds that cannot be broken by acid present in wine. These reaction could thus form a polymeric phenolic structure (Singleton, 2001; Cheynier *et al*., 2002; Danilewicz, 2003).

An important pathway of browning is represented by the oxidation of tartaric acid that occurrs in presence of catechin, Fe(III) and Cu(II) ions, and yields glyoxylic acid, which acts as a bridging mechanism between flavanol molecules. Glyoxylic acid reacts, then, with catechin to produce a (+)-catechin/glyoxylic acid adduct, which reacts with a further (+)-catechin molecule to form a carboxymethine linked (+)-catechin dimer. Dehydration of the dimers forms xantenes which can undergo oxidation to form xanthylium salts. These salts have a yellow color and a maximum absorption at 440 and 460 nm for the non-esterified and esterified salts respectively. Copper and iron ions catalyse this reaction.

Acetaldehyde formed by oxidation of ethanol can form ethyl bridges between (+)-catechin and (-)-catechin molecules.

Nucleophilic addition by electron-rich flavonoids to the protonated aldehyde yield a benzylic alcohol which is prone to protonation producing water and benzylic cation, a charged intermediate easily attacked by other nucleophiles. When the nucleophile is another phenolic ring, the reaction yields an "ethyl-linked" product. Ethyl-linked anthocyanins-flavanols was first reported by Timberlake and Bridle (1976), later acetaldehyde-flavanol condensation products were observed in model solution and in red wine (Fulcrand *et al*., 1996; Saucier *et al*., 1997). Direct reaction between acetaldehyde and malvidin-3-glucoside to produce vitisin B was observed (Bakker *et al*., 1997). Furtheremore, were confirmed the flavanol association cross linked by glyoxylic acids (Es Safi *et al*., 2002) and ethyl bridged anthocyanins (Atanasova *et al.*, 2002).

Pyruvic acid can be formed both by yeast activity and from malic acid oxidation by hydroxyl radical as proposed by Danilewicz (2003). Anyway, it should be deepened if this could happen under wine conditions.

Pyruvic acid reacts with anthocyanins to form vitisins, or pyranoanthocyanins, modified anthocianins with an additional conjugated 6-membered ring that resist SO_2 bleaching as well as color changes with pH shifts (Fulcrand *et al*., 1998).

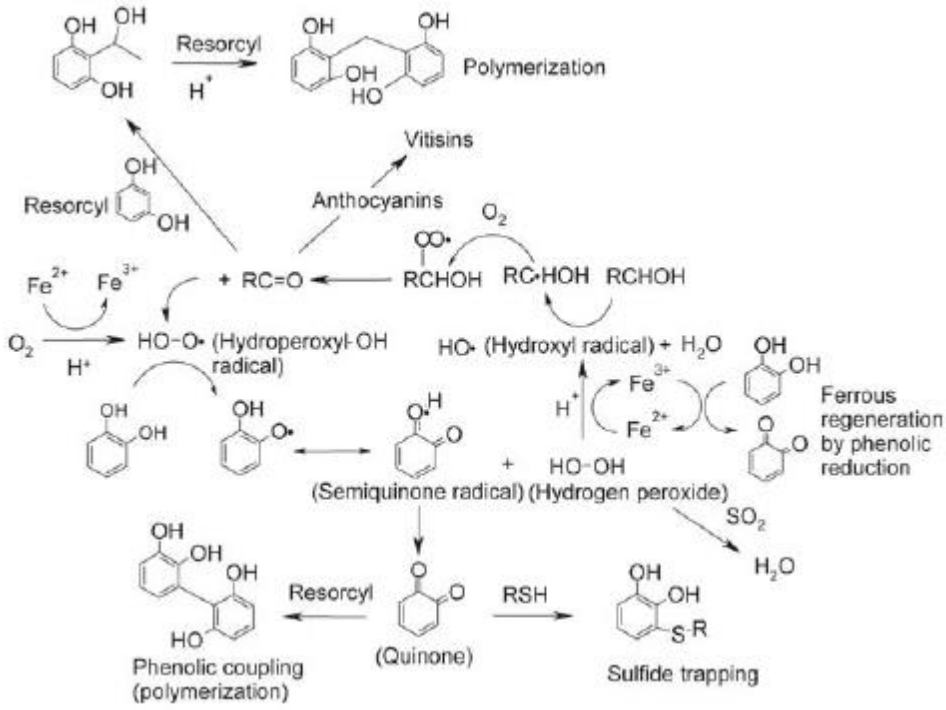


Figure 1.9.1.: Wine phenolic oxidation pathway and subsequent hydroxyl radical oxidation of major wine compounds (Waterhouse *et al.,* 2006)

2. Aim of the study

The influence of some anti-oxidant compounds, either naturally present in grapes (polyphenols and glutathione) or exogenous (SO_2 and hydrolysable tannins), on the oxygen consumption kinetic in wine and model solution is evaluated in this research.

In oenology, SO_2 is the most widely used and the most effective anti-oxidant, but a prolonged absorption of SO_2 can cause health problems: thiamin deficiency, histological modifications of the stomach, and delayed growth (Ribereau-Gayon *et al*., 2004) Furthermore, SO_2 has an allergenic effect for sensible subjects, even at low doses (few milligrams).

Recently, several works have been published (Elias *et al*., 2010; Danilewicz *et al*., 2008; Danilewicz *et al*., 2010; Danilewicz, 2011) that deeply address the mechanisms that regulate the oxidation reactions, with the aim of defining the theoretical basis for the reduction in the use of SO_2 in wines. These experiments were mostly performed on the laboratory scale with model solutions and only in few cases with real wines. Other molecules are now being studied for their antioxidant and antiradical properties, such as reduced glutathione (GSH) and enological tannins (ellagitannins and gallotannins).

The aim of the present work is to study the effect on the oxidative evolution of bottled wines and model solutions of some antioxidant molecules, GSH and hydrolysable tannins in presence of different oxygen levels. The purpose was to better understand the oxidative kinetic and to evaluate the possibility to reduce the amount of SO_2 in wines.

The work is articulated in 3 main parts.

The first part regards the study of the oxidative kinetic and the role of copper in a model solution.

The second part regards the study of the oxidative evolution during bottle aging of three white wines (Cortese) after the addition of some antioxidant additives at bottling in presence of different levels of oxygen (different bottling conditions).

The additives studied were SO_2, GSH, ellagitannins and galliotannins.

The work was focused on the evaluation of the effectiveness of GSH as anti-oxidant: this is a topical subject, since 2 resolutions are being studied at OIV in order to admit the use of GSH as an additive to musts and wines.

The third part of the study regards the study of the Fenton reaction in a model solution, in order to better understand the role of GSH in the this process.

3. Effect of copper content on oxidative kinetics in a model solution

Several studies have shown the key role of iron and the enhancing effect of copper in catalyzing wine oxidation, as reported in the paragraph 1.7. For example, 4-methylcatechol (4-MeC) is not oxidized at a significant rate in winelike system containing sulfite until Fe is added. Addition of copper produces a marked synergic effect (Danilewicz, 2007). Furthermore, when iron and copper were added separately, it was observed only a modest increase in rate of catechol oxidation; however, when combined, marked synergism was observed and the rate is related to copper content. Since the addition of copper markedly increases the rate of Fe(II) oxidation, it was supposed that Cu, by interacting with oxygen, facilitates the Fe(III)/Fe(II) redox cycling. The catalytic role of metals in the oxidative process has also been demonstrated in wine: their removal with potassium ferrocyanide slows down the oxidative process and in white wine it can be completely prevented (Danilewic & Wallbridge, 2010)

Copper, as iron, has a role in the formation of brown pigments and it probably catalizes the degradation of tartaric acid to glyoxilic acid. Some studies showed the relationship between the degradation of (+)-catechin, browning and the concentration of copper (Es Safi *et al*., 2003).

The influence of copper concentration on the rate of catechol oxidation was also demonstrated (Danilewicz 2007).

Since many winemaking steps, from the vineyard to the bottle, can influence the final content of copper in wine, further studies on the influence of copper content on wine oxidation could be useful for winemakers, in particular to evaluate the amount of SO_2 at bottling to prevent wine oxidation.

Effectively, grapes have *per se* low amount of copper, which can increase for the use of fungicides, mainly to fight Peronospora (*Plasmopara viticola*), for the use of cellar facilities containing copper or brass, and for the enological practice of adding copper sulfate ($CuSO_4$) to wines in order to remove the unwanted sulforate compounds responsible of reduced notes.

The use of stainless stell facilties in modern winemaking has reduced the risks of product contamination by metals during wine storage. On the other hand, the development of organic viticulture, has caused an increase in the use of copper sulfate as fungicide.

There are then wines with different levels of copper on the market and an investigation on the effect of different levels of copper at bottling on wine aging should be needed.

3.1. Aim of the study

The aim of this first part of the thesis was to study the effect of both SO_2 and copper on the oxidation kinetics, that is on the rate of oxygen consumption and on the oxidative reactions that lead to browning .

For this purpose, a winelike solution containing (+)-catechin and iron was saturated with oxygen, afterwards different amounts of SO_2 and copper were added to it.

3.2 Materials and methods

A liter of model solution was prepared as follows. A 5 g/L tartaric acid solution was obtained dissolving L(+)-Tartaric acid (25 g) (Sigma-Aldrich St. Louis, MO,USA) in ultrapure water (~ 3.5 L) in a 5 L volumetric flask (5g/L). Ethanol 95% was added to give a 12% v/v final concentration, pH was adjusted to 3.4 with NaOH 1 N. 5 g of (+)-catechin (Sigma-Aldrich St. Louis, MO,USA) was added to the model solution (1 g/L) which was then saturated with oxygen (8.12 mg/L at 24.9°C, measured with Orbisphere Micro O_2 Logger 3650) and transferred into 36 bottles 135 mL volumetric. Before corking, two different amount of potassium metabisulfite (Sigma-Aldrich St. Louis, MO,USA) were added to give the final concentration respectively of 25 and 50 mg/L of free SO_2, 1 mL of freshly made up solution of Fe(II) sulfate heptahydrate (E. Merck, Darmstadt) was added to all the bottles to have a final concentration of 5 mg/L Fe. Furthermore, different amount of a freshly made up solution of Copper sulfate pentahydrate (E. Merck, Darmstadt) was added to give a final concentration respectively of 0, 0.05 or 0.3 mg/L, according to the experimental plan reported in Table 3.2.1.

Sample	Cu (mg/L)	SO_2 (mg/L)
(1)	0	25
s	0	50
1/2c	0,05	25
1/2cs	0,05	50
c	0,3	25
cs	0,3	50

Table 3.2.1: Experimental plan of the trial with copper in model solution

Two bottles for each trial equipped with sensor (sensor spot) were also filled in order to measure the oxygen content during the storage.

The following chemical-physical analyses were performed at the beginning of the experiment and 3, 6, 17 and 32 days after bottling: free and total SO_2, acetaldehyde, absorbance at 420 nm, catechins.

All chemical analyses were performed in duplicate: for each trial, 2 different bottles were sampled.

Free and total SO_2 content were determined by distillation according to EEC methods (EEC regulation 1990), copper was determined in emission at 324 nm using an atomic absorption spectrophotometer (Perkin-Elmer 5100 PC) equipped with acetylene flame (EEC regulation, 1990). The wine color (absorbance at 420, A420) was monitored by spectrophotometry on 1 mm of optic pathway after filtration with a 0.45 µm polypropylene filter.

Catechins were determined by HPLC using a method for seeds (Ummarino *et al*., 2001) and modified for wines (data not published): samples were filtered with a 0.45 µm polypropylene filter (VWR International, Milano, Italy) and injected (20 µL). The separation occurs on an ODS Hypersil RP-C18 reversed-phase HPLC column (200 mm x 2.1 mm I.D., 5 µm packing, Thermo Scientific, Waltham, MA, USA), at 25°C. The flow rate was 0.25 mL/min. Phase A was H_3PO_4 10^{-3}M and phase B was acetonitrile (HPLC grade). The signal was monitored at 280 nm, and the peaks were identified according to the external standard method. The concentrations of (+)-catechin was determined using a six point calibration curve obtained with pure standards. Each standard was injected in triplicate to assess both the linearity and repeatability of the method.

The acetaldehyde content was determined using a colorimetric method (Di Stefano & Ciolfi 1982), which consist of distilling the sample and capture the distillate in a solution containing SO_2, then the distillate is mixed with piperidine10% and sodium nitroprusside dehydrate (Sigma-Aldrich, St Louis, MO, USA), the reaction causes a color change which maximum was monitored by spectrophotometry on 1 mm of optic pathway at 570 nm.

Oxygen dissolved in the model solution, before bottling, was measured with Orbisphere Micro O2 Logger 3650 (HACH LANGE GMBH). The measure was taken on wine flowing through a flux cell containing the sensor. The oxygen is reduced in the sensor, thus, producing an electrical signal whose intensity is proportional to the concentration of dissolved oxygen.

For the measure of oxygen dissolved in bottles, a new luminescence-based technology was used: NomaSense^{TM} O_2 Trace (PreSens GmbH, Regensburg, Germany). It is a trace oxygen meter with fiber-optic oxygen minisensors based on a 2 mm polymer optical fiber (POF). The NomaSense^{TM} O_2 Trace system detects oxygen (oxygen partial pressure) in solutions (dissolved oxygen) using sensors which are glued into bottles before bottling.

3.3 Results and discussion

The influence of copper and SO_2 on the oxidation process was studied by monitoring the dissolved oxygen and by measuring over the time some chemical physical parameters related to the oxidation process.

A fast oxygen consumption was observed, on average, for all the samples during the first 15 days (Figure 3.3.1.). The slowdown in the rate of oxygen consumption occurred in the next 40 days depended on the content both of SO_2 and copper in the solution. The higher was the content of free SO_2, the faster was the oxygen consumption. The reaction between SO_2 and quinones generated from the oxidation of phenols on one hand protects wine from oxidation phenomena and on the other hand reconverts quinones back to the original phenols and thus accelerates the oxidation reactions of phenols and the oxygen consumption (Danilewicz *et al.* 2008).

A slower rate of reaction between catechol and oxygen was therefore observed in samples with lower amount of SO_2. When free SO_2 was next to 0, oxygen consumption stopped, apart metal contents. This effect is especially clear in the sample with lower amount of SO_2 and higher amount of copper: oxygen consumption radically decreases starting from the 12^{th} day of bottling, when free SO_2 was completely consumed (Figure 3.3.1.).

As observed by Danilewicz (2011), although metals are essential to start the oxidation process, they alone do not control the rate of oxidation of polyphenols. On the other hand, the presence of substances such as sulfite and other nucleophiles, capable to react with their quinones, allows oxidation to proceed at a much faster rate.

For the same amount of SO_2, the rate of oxygen consumption was related to copper concentration: a significantly (until 17°day) and an average (until 38° day) faster consumption was observed for samples with higher amount of copper.

An influence of copper content on the consumption rate of free SO_2 was also observed: the higher the copper content, the faster the SO_2 consumption. This effect was observed and was statistically significant in the first period, when free SO_2 was present, then decreased with the decreasing of SO_2, and completely diappeared after 32 days, when all the free SO_2 was consumed.

These results confirm the role of copper to catalize the oxidative process of phenolic compounds that leads to *o*-quinones and hydrogen peroxide. Free SO_2, by reacting with these products, is quickly consumed to produce sulphate (Danilewicz *et al.*, 2008)

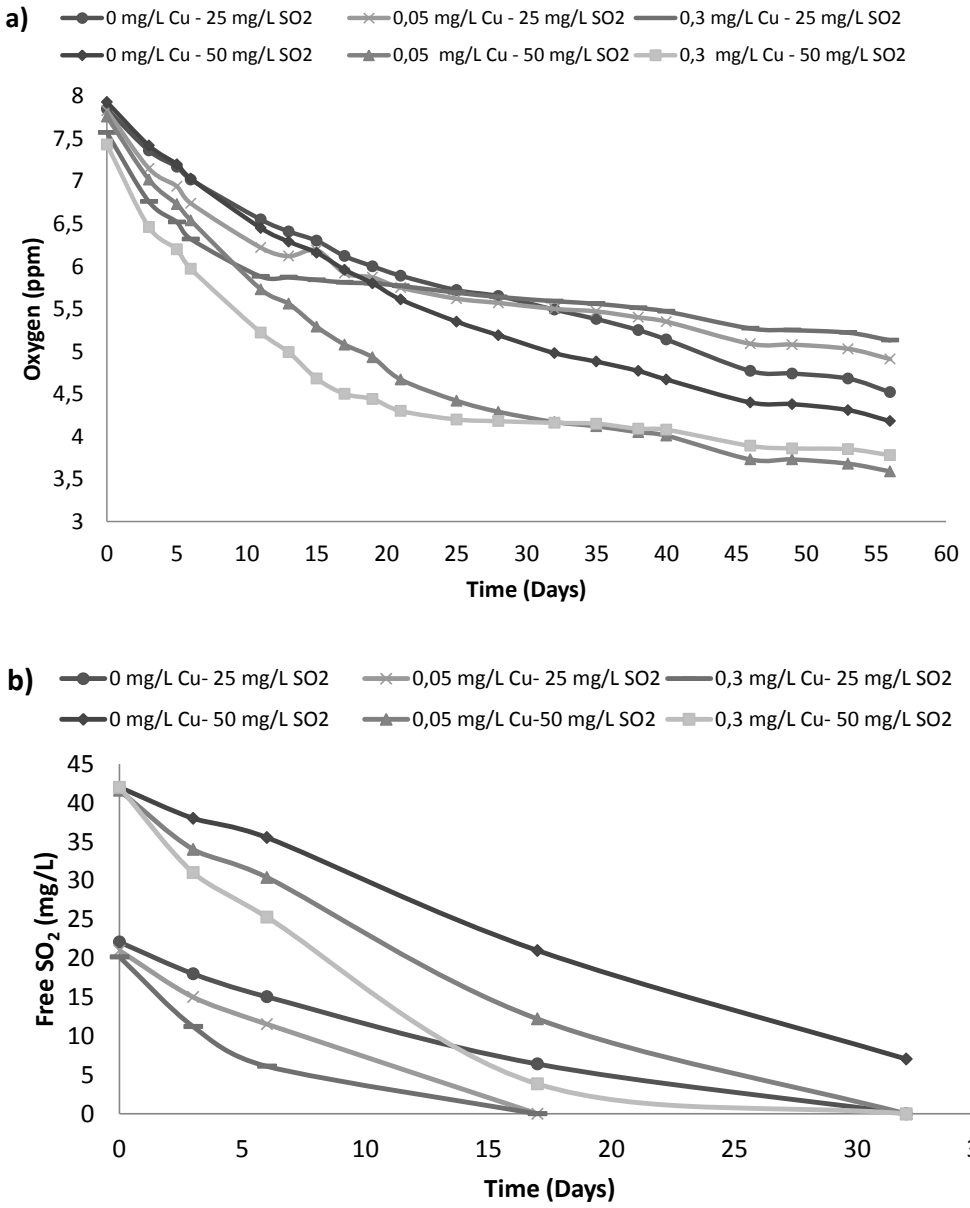


Figure 3.3.1.: kinetics of oxygen (a) and SO_2 (b) consumption in samples during storage. Effect of the different amounts of SO_2 and copper

A positive effect of SO_2 and a negative effect of copper to prevent wine browning were observed: the absorbance at 420 nm (A420) decreased in presence of higher amount of SO_2 and increased as the copper content increased.

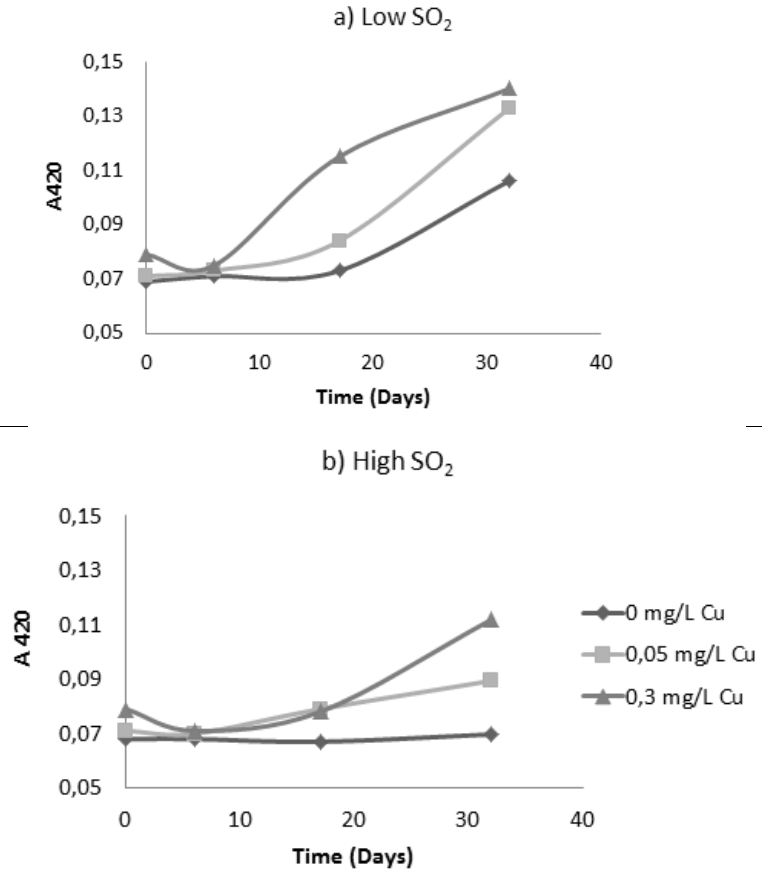


Figure 3.3.2: Evolution of the color (A_{420} parameter) during bottle aging. Effect of low (a) and high (b) SO_2 contents respectively

These results confirm the positive role played by SO_2 against wine browning and are consistent with other studies that stated a quick reaction occurs between SO_2 and *o*-quinones derived from oxidation of phenols. The consequence of this reaction is a slowdown in the polymerization reactions that lead to wine browning (Makhotkina & Kilmartin, 2009).

An influence, even if not statistically significant, was observed regarding the amount of SO_2 and copper on the content of catechins. Effectively, 32 days after the beginning of the experience, an average reduction of 5.2% in catechin content was observed. The decrease was on average higher in samples with lower amount of SO_2 and higher amount of copper, with losses varying from 6.6 to 3.7% for samples with respectively low and high content of SO_2 and changing of 2.9, 6.5, 6.1% for copper content of 0, 0.05, 0.3 mg/L.

Regardless of other works, no effect of SO_2 on the production of acetaldehyde was observed. An average increase in the production of acetaldehyde was observed with the increase of copper levels. (table 3.3.1.)

Time	**SO_2**				**Copper**				
(days)	25 mg/L	50 mg/L	F	Sig.	0 mg/L	0,05 mg/L	0,3 mg/L	F	Sig.
0	0,38	0,45	0,63	ns	0,32	0,41	0,52	1,56	ns
3	0,44	0,64	3,44	ns	0,54	0,44	0,64	1,15	ns
6	0,49	0,95	4,95	ns	0,61	0,77	0,77	0,29	ns
17	0,92	1,30	2,29	ns	0,94	0,94	1,44	1,67	ns
32	1,38	1,28	0,82	ns	1,11	1,25	1,64	8,34	ns

Table 3.3.1.: Average content of acetaldehyde in wines. Effect of different levels of copper and SO_2. ANOVA results.

3.4. Conclusions

This experiment confirmed the key role played by metals, and in particular by copper, in the oxidation of polyphenols. The O_2 cannot react directly with the reducing substances of wine, but with transition metals and free radicals. The essential catalyst is iron, but its action is markedly increased by copper. (Danilewicz, 2011).

The important role of SO_2 in protecting wine against the consequences of oxidation was confirmed. SO_2 on one hand causes an increase in the oxidation reactions (increase in the rate of oxygen consumption) which have as substrates polyphenols and on the other hand limits the browning process by converting quinones to the original phenols. Furthermore, the results of this experiment are consistent with other works carried out both in model solution and in wine where in absence of sulfite, the reaction between O_2 and catechols becomes very slow, despite the presence of metals (Danilewicz *et al* 2008, Danilewicz & Wallbridge 2010).

Danilewicz observed that, in absence of SO_2, the capacity of oxygen consumption of each polyphenol, is related to the molecular structure, that is to their redox potential. Therefore, the slowdown in the rate of oxygen consumption observed with the decrease of SO_2 could be related to the (+)-catechin and might change in presence of other phenolic molecules. On an enological point of view, it could be concluded that, since the effect of copper in accelerating the oxidation process is dose-dependent, when a wine presents high levels of copper it is recommended to increase the amount of SO_2 at bottling.

4. Effect of some antioxidant molecules on oxidative aging of a white wine

The attested antioxidant, antioxidasic and antimicrobial properties of SO_2 makes sulfure dioxide the most common additive for the preservation of wines.

However, it has been widely proven that a prolonged absorption of SO_2 can cause health problems and an allergenic effect in sensitive subjects (Ribereau-Gayon, 2004).

In the future, a decrease in the SO_2 concentration limits for wine is expected, and, in some cases, the prospective is a completely SO_2-free wine.

Other molecules are now being studied for their antioxidant and antiradical properties, such as reduced glutathione (GSH) and enological tannins (ellagitannins and gallotannins).

Recently, several works have been published (Elias *et al*. 2010; Danilewicz *et al*. 2008; Danilewicz *et al*. 2010; Danilewicz 2011) that deeply address the mechanisms that regulate the oxidation reactions, with the aim of defining the theoretical basis for the reduction in the use of SO_2 in wines. These experiments were mostly performed on the laboratory scale with model solutions and only in few cases with real wines.

This work, performed under enological conditions, was aimed at studying the possibility of adding GSH and/or ellagic or gallic tannins at bottling to limit the use of SO_2 in white wines.

To date, the studies on wine have been mainly focused on the effect of GSH on aroma evolution during bottle aging of wines with volatile thiols, in particular Sauvignon blanc. On the other hand, few information is available as regards the influence of GSH on the oxidative evolution of wine color.

Different level of oxygen, were also considered in order to study the effect of different bottling conditions on oxidative evolution of a white wine with different additives.

The work is composed by three experiments set up consecutively each other according to the results of the previous one.

4.1. Enological additives with antioxidant activity

4.1.1. Sodium disulfite

Sulfure dioxide (SO_2) is the most often employed additive for the preservation of wines, due to its antioxidant, antioxidase and antimicrobial properties.

Furthermore, it has the ability to add to carbonyl compounds to form non-volatile bisulfite adducts, so preventing their indesirable effect, such as the maderized notes caused by free acetaldehyde.

The mechanism of reaction between oxygen and sulfure dioxide has been widely studied in the last hundred years mainly for its importance in producing acid rain in the atmosphere where pH is about 4, similar to wine (Brandt & van Eldik, 1995).

In the atmosphere as in wine, SO_2 is hydrated and is present mostly as the bisulfite ion (HSO_3^-).

The proposed mechanism was that two molecules of SO_2 react with one of oxygen to produce two sulphate ions.

Some researchers were of the opinion that the same mechanism occurs in wine, that is, SO_2, by reacting directly with oxygen, protects polyphenols and other wine compounds from oxidation (Ribereau-Gayon *et al*., 2000; Clarke & Bakker, 2004).

However, studies on winelike sistems, have shown that the reaction rate of oxygen with SO_2 is much slower compared to the uptake of oxygen by wine itself.

It is then concluded by other authors (Boulton *et al*., 1996) that the main antioxidant activity of SO_2 is to scavenge hydrogen peroxide produced by the oxidation of polyphenols.

Actually, the interaction of SO_2 with oxygen is quite complex and the transformation of SO_2 into sulfate has important implications regarding the chemical changes it can induce in wine. As already reported in the previous paragraphs, the SO_2 cannot react directly with molecular oxygen, but could react with ferric ions deriving from the oxidation of ferrous ions by oxygen (auto-oxidation of SO_2).

The reaction involves a metal-catalyzed radical chain reaction in which the FeIII initiates the process by oxidizing bisulfite to the sulfite radical ($SO_3^{\cdot-}$). This radical reacts quickly with oxygen yielding the potent oxidizing peroxomonosulfate radicals ($SO_5^{\cdot-}$) which react with bisulfite through two pathway (Figure 11 reaction A and B) producing sulfate and regenerating sulfite radicals that continue the chain process (Brand *et al* 1994, Brand & van Eldik 1995, Connik *et al* 1995). On this point of view, the SO_2 should become a pro-oxidant molecule, but, in wine, this reaction is stopped by catechol, which has also an antiradical activity.

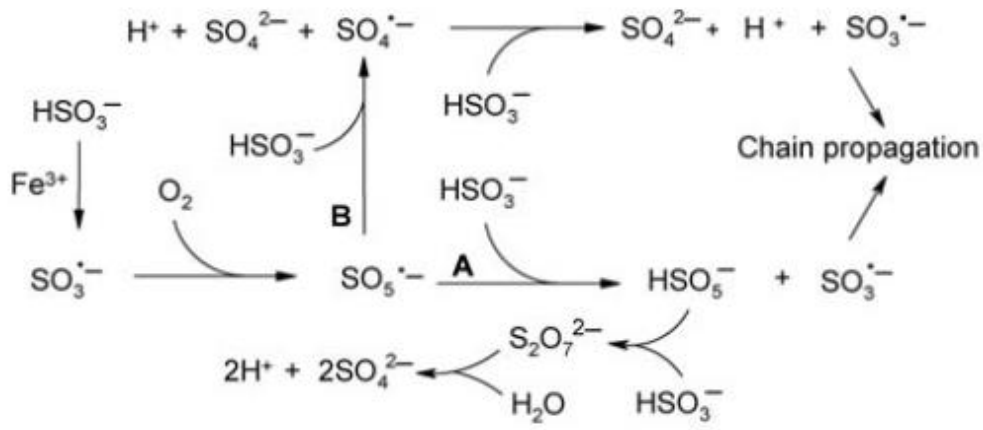


Figure 4.1.1.: Radical chain reaction involved in bisulfite oxidation (Danilewicz 2008)

In the oxidative process of wine, the reaction between catechol and oxygen catalized by iron and copper (Danilewicz, 2007) yields quinone and hydrogen peroxide. Bisulfite reacts quickly with hydrogen peroxide, and this reaction is the theory on which is founded the official method to determine SO_2 in wines. The rate of sulfite reaction is related to 4-MeC and (+)-catechin concentration in model wine and differs for different catechols (Danilewicz, 2007; Danilewicz & Wallbridge 2010). Furtheremore, as observed in the cyclic voltammetry of polyphenols in model systems and wine (Makhotkina & Kilmartin 2009), SO_2 also reacts with quinones reducing them back to their original molecules or generating some addition compounds (Michael-type 1,4 addition to give the sulfonic acid (LuValle, 1952; Youngblood, 1986; Danilewicz, 2007) (Figure 4.1.2.). These oxidation reactions can slow down the production of brown pigments in wines caused by polymerization reactions involving quinones and semiquinones. The reactivity of SO_2 both with quinones and with hydrogen peroxide is confirmed by the O_2:SO_2 molar reaction ratio1:2.

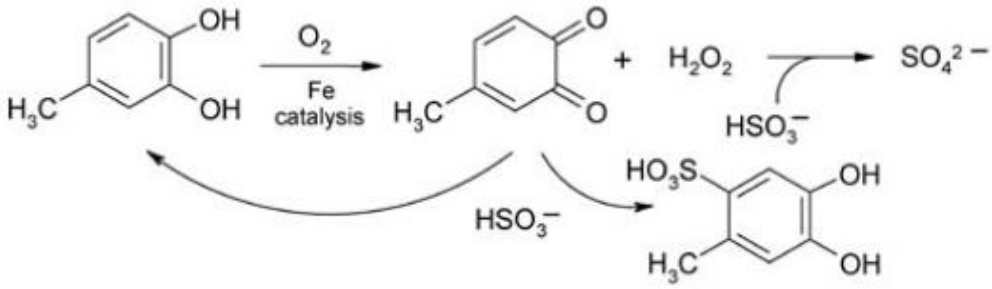


Figure 4.1.2.: Interaction of bisulfite with products of catechol oxidation: hydrogen peroxide and quinones (Danilewicz, 2008)

The regeneration of polyphenols from quinones or the presence of nucleophiles such as sulfite, cause an acceleration in the oxygen consumption rate, in spite of the presence of metals (Danilewicz *et al*., 2008; Danilewicz & Wallbridge 2010).

4.1.2. Glutathione

Glutathione is a tripeptide of L-glutamate, L-cysteine and glycine. Its biological importance is mainly related to its free sulfhydryl moiety of cysteine residue, which confers unique redox and nucleophilic properties (Penninckx, 2000).

Generally, more than 90% of glutathione is present in cells in the reduced form (GSH) (Li *et al*., 2004); the oxidation of GSH leads to the oxidized form, that is glutathione disulfide (GSSG). (Figure 4.1.3.). Glutathione reductase can reduce GSSG back to GSH at the expense of NADPH (Carmel-Harel *et al*., 2000).

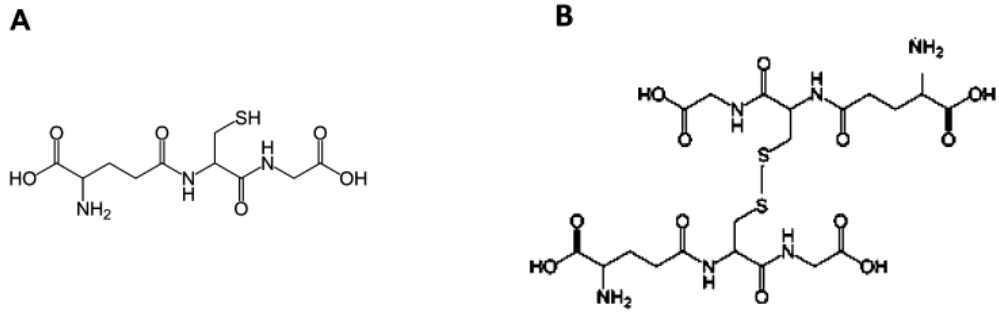


Figure 4.1.3.: Molecular structures of A) glutathione (GSH) and B) glutathione disulfide (GSSG).

Glutathione is present in many prokaryotic organisms, in plant and mammalian cells (Anderson, 1998) and it plays several physiological and biochemical roles. Mainly GSH is an antioxidant, immunity booster and detoxifier (Pastore *et al*., 2003).

Glutathione is also an important constituent of grapes, musts and wines and it was first quantified in grapes in 1989 (Cheynier *et al*., 1989).

The concentration of glutathione in grapes varies from few tens to few hundreds of µmols/Kg. and it is influenced by grape varieties, vintage, location, and technological practices (Cheynier *et al*., 1989). Furthermore, the GSH content in grapes is strictly related to the vine nitrogen status estimated as yeast assimilable nitrogen content of grape juice. The GSH content of must originating from nitrogen-deficient vines was significantly lower than of the must from vines that were fertilized after bloom (Chonè *et al*., 2006).

The range of GSH in must varies from non detectable values to about 100 mg/L (Cheynier, 1989; Park *et al*., 2000) and it is influenced by several factors: must exposure to oxygen, tyrosinse activity, grape skin maceration and the kind of pressing (Du Toit, 2007; Maggu, 2007; Patel, 2010). The reductive techniques maintained higher GSH levels both in musts and in wines. Comparing traditional and reductive pressing on four Italian grapes, Motta *et al* (2014) reported average values ranging from 3.5 to 77.7 µM for musts pressed with traditional technique (under air) and average values ranging from 37.9 to 117.3 µM for musts pressed in reductive conditions (under nitrogen).

During alcoholic fermentation a decrease in the content of GSH was observed as the consequence of the use of glutathione by yeast, but, at the end of alcoholic fermentation, yeast autolysis cause an increase in GSH content in wine (Lavigne & Duburdieu 2004).

The content of GSH in wine can also be influenced by the yeast strain used for alcoholic fermentation (Lavigne *et al*., 2007; Rauhut, 2009)

Generally, GSH levels in wines are lower compared to the corresponding grapes and range from 3 to 20 mg/L (Cassol & Adams, 1995; Du Toit *et al*., 2007), but when the grapes are poor in GSH, it can be higher in wines compared to grapes as the consequence of the release by yeasts. Other authors described values ranging from nondetectable to 70 mg/L (Fracassetti *et al*., 2011; Kritzinger, 2012).

The GSH concentration decreases during wine aging (Ugliano *et al*., 2011; Kritzinger, 2012; Lavigne *et al*., 2007; Penna *et al*., 2001). The concentration of GSH in wine during bottle aging can be influenced by the different oxygen exposure (Ugliano *et al*., 2011). For examples, a study showed that Sauvignon blanc wines exposed to lower oxygen level during bottle aging had higher amount of GSH compared to the same wine exposed to higher levels of oxygen during storage. This difference is due to the lower oxidation degree in wine with lower oxygen levels.

The concentration of GSH in wine is therefore highly influenced by winemaking techniques and the winemaker can act by reducing oxidation during vinification and aging process.

The importance and the role of glutathione in enology have been studied only recently, first of all for its effect on the control of oxidative phenomena in wines. It has, indeed, an anti-oxidant effect both on color and on several aromatic compounds in wine (Dubourdieu *et al*., 2008; Ugliano *et al*., 2011). It is considered that the important role played by GSH to prevent must oxidation consist of scaveging o-quinones formed from oxidation of phenols and thus limiting the wine browning (Singleton *et al*., 1985; Singleton *et al*., 1995; Du Toit *et al*., 2006).

Using cycling voltammetry, Makhotkina et al. (2009) found out that, in winelike sistem containing polyphenols, the behavior of GSH was similar to SO2 and supposed that GSH not only can react directly with *o*-quinones in a nucleophilic addition, but also can reduce *o*-quinones back to the original molecules by oxidizing itself to glutathione disulfite (GSSG). It is also hypothesized that GSH can also react with hydrogen peroxide and other peroxides or radicals in musts and wine producing GSSG and removing these oxidants (Okuta *et al*., 1999).

However, the effect of GSH against white wine browning is not yet clear. Lavigne *et al*. (2005) observed that GSH added at 10 mg/L at bottling slows down wine browning: during the first three year aging period, the wine with GSH showed significantly less color (A420) compared to the control wine. On the contrary, Roussis *et al* (2000) and El Hosry *et al* (2009) did not observe any effect of GSH against wine browning during accelerated browning test.

Sonni et al (2011) showed that high amounts of GSH could inhibit browning by delaying the formation of carboximethine-bridged (+)-catechin dimers because of GSH ability to form addition products with carbonyl compounds, such as glyoxylic acid. The inhibition of carbonyl-derived polymerization reactions protects wine from the formation of unwanted pigments such as yellow xanthylium cation, which may induce color changes that impaired wine quality.

The role of GSH on preserving wine aroma during aging was also studied.

In particular, it was observed a protective effect of GSH on some esters, (responsable of fruity aroma, Etievant, 1991) and terpenes (responsible of floral, rose-like, coriander, campherous characters, (Marais, 1983) during wine storage (Papadopoulou *et al*., 2008; Papodopoulou *et al*., 2001; Roussis *et al*., 2007). This protection is due to GSH free sulfhydryl (SH) moiety (Roussis et al 2009), which confers redox and nucleophilic properties.

Furthermore, some studies have shown a key role played by GSH in protecting volatile thiols during bottle aging (Dubordieu *et al*., Lavigne-Cruge *et al*., 2002). Volatile thiols, highly oxidable molecules, have an essential importance for the aroma mainly of Sauvignon blanc (Darriet *et al*., 1995; Tominaga *et al*., 1996, Tominaga *et al*., 1998), but are also present in other cultivars, such as Riesling, Merlot, Cabernet Sauvignon (Tominaga, 2000; Murat, 2001).

As reported in previous paragraphs, the enzymatic oxidation of hydroxycinnamic acids in musts yields *o*-quinones which can react with thiols via Michael addition reaction (Cheynier *et al*., 1986) or go through coupled reactions that generate peroxides which have oxidative properties against thiols (Wilderandt *et al*., 1974). It is supposed that GSH exerts a proptective effect on thiols because of, being a thiol, may compete with aromatic thiols to react with *o*-quinones and thus limiting the loss of varietal aroma (Fracassetti, 2010; Tirelli *et al*., 2010).

Moreover, studies have proved the effect of GSH on slowing down the formation of both sotolon and 2-aminoacetophenone (Dubordieu *et al*.), which are responsable of atypical aging off-odors during wine storage (Lavigne-Cruege *et al*., 2002; Escudero *et al*., 2000; Ferreira *et al*., 2003).

GSH is supposed to be a potential source of H_2S in wines for the degradation of cysteine to H_2S (Tokuyama *et al*., 1973) or for the antioxidant capacity of GSH to cause reductive conditions which promote the production of H_2S (Ugliano *et al*., 2011).

H_2S contribute to the "reductive" off-flavor in wines, with an odor described as rotten egg or putrefaction.

4.1.3. Gallic and ellagic tannins

Tannins are, by definition, substances capable of producing stable combinations with proteins and other plant polymers such as polysaccharides. On a chemical point of view, they are relatively weighty phenol molecules produced by the polymerization of elementary molecules with phenolic functions. The configuration affects their reactivity: the molecular weights of active tannins range from 600 to 3500.

Tannins in wine can be divided into condensed and hydrolysable tannins which derive from grapes and oak respectively.

Condensed tannins, also known as proanthocyanidins, are polymerised flavanol units of catechin, epicatechin, gallocatechin, epigallocatechin and epicatechin gallate (Prieur *et al*., 1994; Souquet *et al*., 1996) as described at the paragraph 1.3.

Hydrolysable tannins include ellagitannins and gallotannins, that release respectively ellagic acid and gallic acid after acid hydrolysis. They also contain a glucose molecule.

Hydrolyzable tannins are the main commercial tannins legally authorized as wine additives, in spite of not being naturally present in grapes.

Ellagitannins constitute up to 10% of the dry weight of oak heartwood. The two most common ellagitannins in oak used for cooperage are vescalagin and castalagin, there are then six other less important compounds called roburins A-E and grandinin (Herv'e du Penhoat *et al*., 1991). These molecules include a hexahydroxydiphenic and a nonhydroxydiphenic acid, esterified by a non-cyclic glucose. The partial hydrolysis of vescalagin and castalagin, with the loss of hexahydroxydiphenic acid, lead to the formation of vescalin and castalin (Figure 4.1.4.)

These molecules are water soluble and dissolve rapidly in diluted alcohol media, such as wine (Moutunet *et al*., 1989). The oxidizability (Vivas & Glories 1993, 1996) and their flavour properties (Pocock et al., 1994) play a key role on the aging of both white and red wines in wood barrels. They can favour the increase of the rate of the condensation of procyanidins (Glories, 1993; Ribereau-Gayon & Stonestreet, 1965;, Vivas, 1993). Furthermore, they can bind covalently to grape-derived nucleophilic molecules such as ethanol, flavanols, anthocyanins and thiols (Quiedau *et al*, 2005). Vivas and Glories (1996) showed an effect of oxygen in limiting ellagitannins oxidation by competing with oxygen.

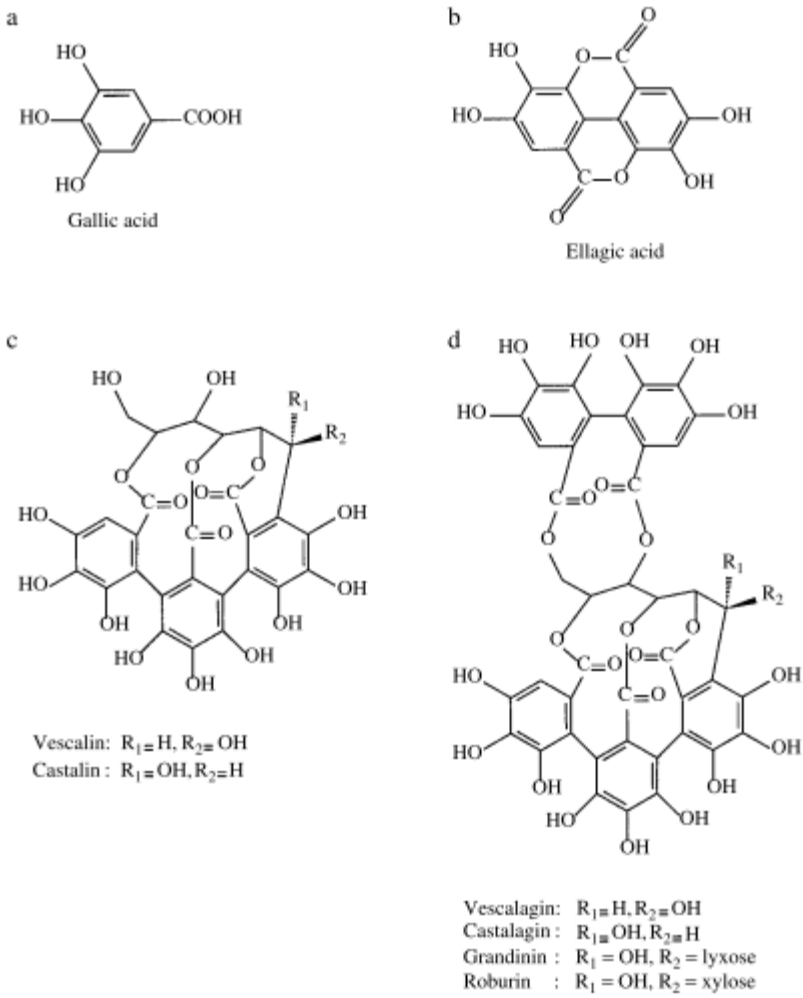


Figure 4.1.4.: Structure of phenolic acids (a and b) and ellagitannins (c and d) in extracts from the duramen of the oak and chestnut wood (Vivas & Glories, 1996)

Gallotannins are the simplest hydrolizable tannins, composed of polygallol esters of glucose (Hagerman, 2002). They are present in nutgall and can be added to wine as part of commercial tannin (Hagerman, 2002, Resolution OENO 2002).

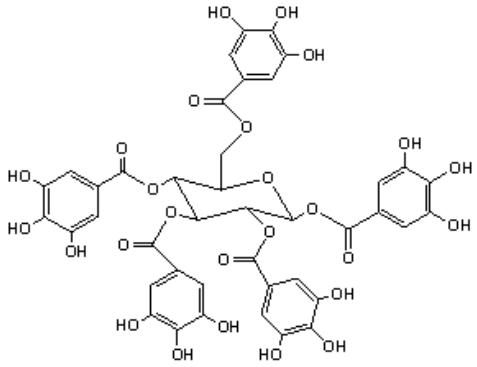


Figure 4.1.5.: Structure of gallotannin

Since hydrolysable tannins have several hydroxyl (OH) groups in the *ortho* position, they are easily oxidized and are supposed to be involved in the oxidation processes in red and white wines (Moutounet *et al*., 1989). Some studies carried out on red wines enriched with ellagitannins or catechins, showed a faster oxygen consumption rate in presence of ellagitannins than in presence of catechins (Vivas & Glories, 1996). These results can be related to the different number of hydroxyl functions of the added molecules: two for one mole of catechin opposed to 15 for one mole of ellagitannin. Furthermore, the remarkable oxidative power of ellagic tannins yields large amount of peroxides which produce an important increase in acetaldehyde, important for the condensation between condensed tannins and anthocyanins (Vivas & Glories, 1996).

The high oxidizability of hydrolysable tannins, their capacity of chelating metal ions and to react with quinones, make hydrolysable tannins acting as free radical scavengers (Puech *et al*., 1999).

Regarding the organoleptic effect of hydrolysable tannins on wine, there are controversy results which goes from no effect due to their too low concentration that is near or below their detection limit (Pocok *et al*., 1994, Puech *et al*., 1999) to an increase of the astringency (Quinn & Singleton 1985, Herv'e du Penhoat *et al*., 1991), other studies pointed out any organoleptic effect of hydrolysable tannins because of their too low concentration.

The presence of ellagitannins in oak aged wines is lower than the expected one for several reasons: the amount of tannins in wood decrease during toasting, furthermore the extracted tannins undergo chemical transformation such as oxidation, polymerization and hydrolysis (Puech *et al*., 1999).

According to Resolution OENO (2002), commercial tannins can be extracted from nutgalls, wood or grape seeds.

The use of tannins in authorized only to precipitate must and wine proteins. Tannins have several properties: they modify the redox buffer, increase the structural mouth feel, increase the phenolic substrate for micro-oxidation, limit the activity of laccase, enhance/stabilize red wine color and increase aging potential. (Zoecklein, 2005).

The schedule and the concentration depend on the purpose of the addition.

4.2.Materials and methods

4.2.1. First trial: experimental plan

A Cortese white wine produced with organic grapes at Soc. Coop. Agricola Valli Unite (Costa Vescovato, Piedmont, Italy), whose composition is reported in Table 4.2.1. was divided in two aliquotes with low and medium-low levels of SO_2 at bottling: 3.4 and 24.8 mg/L free SO_2 and 57.0 and 93.4 mg/L total SO_2, respectively.

At the same time, the effect of GSH at a dose of 20 mg/L and the effect of oak wood ellagitannins added at a dose of 20 mg/L were also studied. The chosen dose of GSH (20 mg/L) is the maximum dose considered in the Resolutions that are now being studied by OIV, and the dose of ellagitannins (20 mg/L) is the average dose normally used at bottling in commercial wineries.

The experimental plan , a complete factorial plan with 3 factors (SO_2, GSH and ellagitannins) at 2 levels, is reported in Table 4.2.2.

Free SO_2 (mg/L)	3.2
Total SO_2 (mg/L)	56
A420	0.07
Total polyphenols (mg/L)	104
p-DACA (mg/L)	8.2
GSH (mg/L)	0.3

Table 4.2.1.: Chemical-physical composition of the Cortese wine used in the first trial

Sample	Free SO_2 (mg/L)	Ellagitannins (mg/L)	GSH (mg/L)
(1)	53	0	0
s	93	0	0
t	53	20	0
g	53	0	20
st	93	20	0
sg	93	0	20
gt	53	20	20
sgt	93	20	20

Table 4.2.2: Experimental plan of the first trial

The wine was bottled at the winery following a standard bottling protocol: a rinse with micro-filtered sterile water, bottle flushing with nitrogen, filling, and a final injection of nitrogen into the bottle neck. The solutions containing the additives were added before corking, and then the bottles were manually corked. For each trial, 30 bottles (750 mL) were prepared. The bottles were then stored at 20°C neck upwards. The chemical-physical composition of the wines was determined in duplicate soon after bottling (48 hr) and after 1, 2 and 6 months of bottle aging. Since the aim was to follow the influence of the studied additives on oxidative evolution of wines, the chemical analysis were focused on some parameters significative for this purpose: free and total SO_2, acetaldehyde, GSH, HCTA, absorbance at 420 nm (A420), Cie.Lab indexes (cylindrical coordinates: L* lightness, C* chroma, h* hue), catechins, total polyphenols.

The oxygen consumption was also monitored during the storage using .

The analytical methods are described in the paragraph 4.2.4.

Regarding sensory analysis, discriminant tests (duo-trio test) (Cravero and Ubigli, 2002) were performed after 3 months of bottle aging. When significant differences were noticed, sensory analysis was deepened with paired difference tests (Ubigli, 1998) aimed at studying the effect on wine's sensory characteristics played by the addition of SO_2 (t versus s; t versus st; g versus sg; gt versus sgt), tannins (t versus s; gt versus g; st versus s; sgt versus sg) and GSH (g versus s; gt versus t; sg versus g; sgt versus g). The paired difference tests were focused on the following descriptors: color intensity, fruity, softness, bitter, and olfactory and taste preference. The tasters were asked to indicate, for each couple of trials, the sample with the highest level of the studied descriptor, and the preferred one for odor and taste. After 6 months of bottle aging, the wines were submitted to descriptive analysis, using wheels with 10 cm unstructured scales, during three sessions with a panel of 12 trained tasters. The degustations were organized in duplicate. During the first session, 7 aroma descriptors were chosen among those proposed by Guignard & Noble (1986): acacia flowers, citrus, acetaldehyde, honey, caramel, exotic fruit, cooked vegetable. The chosen olfactory descriptors were partly varietal (acacia flowers, citrus), partly related to oxidative aging: acetaldehyde and honey (Silva, Ferreira et al. 2003), caramel and cooked vegetable (Escudero *et al*. 2002). In the form, 1 visual (color intensity) and 4 taste descriptors (acidity, bitter, softness and structure) were added. The wine samples were presented randomly and tasted within 1 hr after pouring.

The tasting panel was made up of 12 trained tasters from CRA-ENO. The wines were served at the temperature of 16±1°C in ISO (3591-1977) approved glasses in an ISO (8589- 2007) tasting room.

4.2.2. Second trial: experimental plan

This experiment was performed, on a small scale compared to the previous one, at CRA-ENO's Experimental Cellar using a Cortese white wine provided by Cantina Sociale di Nizza (Nizza Monferrato, AT, Italy).

The composition of the wine is reported in Table 4.2.3.

Free SO_2 (mg/L)	11.5
Total SO_2 (mg/L)	45
A420	0.06
Total polyphenols (mg/L)	90
p-DACA (mg/L)	15.6
pH	3.30
Copper (mg/L)	0.19
GSH (mg/L)	6.4

Table 4.2.3: Chemical-physical composition of Cortese wine used in the second trial

The aim of the work was to study the oxidative kinetics of the wines in presence of two different amount of SO_2, GSH and dissolved oxygen at bottling, according to the experimental plan reported in Table 4.2.4.

The wine was bottled at CRA-ENO's Experimental Cellar using 135 mL bottles flushed with nitrogen before and after filling.

The additives were added in each bottle before corking, and then the bottles were corked with crown caps, two bottles for each trial equipped with sensor (sensor spot) were also filled in order to measure the oxygen content during the experiment.

The trials were stored at 20°C neck upwards.

The following chemical analyses were performed at the beginning of the experiment and 2, 7, 14, 20, 30 days and 1 year after bottling: free and total SO_2, GSH, absorbance at 420 nm, catechins, total polyphenols, copper.

Sample	SO_2 (mg/L)	O_2 (ppm)	GSH (mg/L)
o	20	3.3	0
so	60	3.3	0
(1)	20	1.3	0
s	60	1.3	0
og	20	3.3	20
sog	60	3.3	20
g	20	1.3	20
sg	60	1.3	20

Table 4.2.4. Experimental plan of the second trial

4.2.3. Third trial: experimental plan

This experiment was carried out at CRA-ENO's Experimental Cellar using a Cortese white wine provided by Cantina Sociale di Nizza (Nizza Monferrato, AT, Italy), the composition at the beginning of the experiment is reported in table 4.2.5. The wine was limpid and stable against tartaric and proteic precipitations.

Alcohol (%)	11.72	Acetaldehyde (mg/L)	17.6
Total extract (g/L)	16.7	Copper (mg/L)	0.07
Total acidity (g/L)	5.25	Iron (mg/L)	0.63
pH	2.96	Tartaric acid (mg/L)	1.91
Free SO2 (mg/L)	25.3	Malic acid (mg/L)	1.38
Total SO2 (mg/L)	72.3	Shikimic acid (mg/L)	0.045
Total polyphenols (mg/L)	62	Lactic acid (mg/L)	0.39
p-DACA (mg/L)	6.2	Volatile acidity (g/L)	0.24
A420	0.053	GSH (mg/L)	3.62

Table 4.2.5.: Chemical-physical composition of the Cortese wine used in the third trial

The wine was divided into two aliquotes and oxygenated respectively till 3 ppm and 5.5 ppm, then 2 different amount of SO_2, GSH and gallic tannins were added to both the aliquotes, according to the experimental plan reported in Table 4.2.6.

The different levels of oxygen were chosen to simulate two different bottling conditions, grounded on the data of the first trial: 3 ppm means a medium condition, while 5.5 means bad bottling conditions.

Sample	SO_2 (mg/L)	GSH (mg/L)	Gallic tannins (mg/L)
(1)	20	0	0
s	60	0	0
t	20	0	40
st	60	0	40
g	20	20	0
sg	60	20	0
gt	20	20	40
sgt	60	20	40

Table 4.2.6.: Experimental plan of the third trial

Samples at 3 ppm were bottled in 750 mL bottles, flushed with nitrogen before and after filling, closed with syntetic closure, and stored neck upwards at 20°C.

The dissolved oxygen and the headspace oxygen were measured at bottling and their evolution were followed over time (12 months). The measurement were performed two days a week during the first month after bottling, 1 day a week during the second and the third month of storage and once a months in the remaining months. For the measure of oxygen was used the luminescence-based technology NomaSenseTM O2 Trace (PreSens GmbH, Regensburg, Germany) described at the paragraph 3.2. In this trial, both the oxygen in solution (dissolved oxygen) and the gaseous phase (headspace) were monitored using separate sensors glued into bottles before bottling.

Chemical controls (free and total SO_2, volatile acidity, acetaldehyde, absorbance at 420 nm, CIELAB, total polyphenols) and sensory analysis were performed after 15 months of bottle aging.

Samples with 5.5 ppm of dissolved oxygen where bottled in 135 ml bottles, sealed with crown cap and stored at 20°C. Two bottles for each sample equipped with sensor (sensor spot) were also filled in order to measure the oxygen content during the experiment.

Chemical analysis performed during the storage period (1, 3, 8 and 12 months after bottling), regarded some parameters interesting to follow the oxidative evolution of the wines: free and total SO_2, GSH, HCTA, absorbance at 420 nm, CIELAB, catechins, total polyphenols.

Furthermore, the accelerating browning test (Simpson, 1982), a test to predict the tendency of wine to browning, was performed after 1 and 8 months of bottle aging.

This experiment was similar to the previous ones for the wine tipology and the amount of GSH, but was different from the others for the higher amount of dissolved oxygen, the use of gallic tannins instead of ellagic at also higher amounts (40 mg/L instead of 20 mg/L)

After 15 months of bottle aging, the wines were submitted in duplicate to descriptive analysis, during two tasting sessions, following a method previously described (Cravero *et al*., 2012; Guaita *et al*., 2013).

Two preliminary tasting sessions were also carried out to choose the descriptors of the wheel. During the first preliminary session, the tasters were asked to indicate the olfactory descriptors of the wine using as reference a predefined odor list (Guinard and Noble 1986). After the first session, 15 descriptors with a frequence of citation at least equal to 28 (8 wines*14 tasters/4, that is the 25%) were chosen.

During the second preliminary tasting session, the tasters were asked to investigate the presence of the 15 chosen descriptors in the wines helping themselves with reference standards prepared as reported in Table 4.2.7.

Eleven terms were finally selected on the basis of their frequency. Four were varietal (acacia flowers, lemon, pineapple, golden apple) and six were related to oxidative aging (cut apple/acetaldehyde, honey, licorice, nut, water of green beans, hay/ straw. To ensure unique and common meanings for all the terms, reference standards were proposed as reported in Table 4.2.7.

In the form, 1 visual (straw yellow) and 4 taste descriptors (acidity, bitter, softness and structure) were added. The descriptors were rated by using wheels with 10 cm unstructured scales. The wine samples were presented randomly and tasted within 1 hr after pouring.

Attributes	Standards
Acacia flowers	Acacia flowers essence
Lemon	1 lemon cut into pieces*
Yellow golden delicious apple	1 yellow golden delicious apple cut into pieces*
Green apple	1 granny smith apple cut into pieces*
Oxidized apple/Cut apple	1 yellow golden apple cut into pieces and exposed to air
Pineapple	pure pineapple juice*
Honey	honey (20 mL)
Liquorice	liquorice root
Almond	some almonds cut into pieces*
Walnut	some walnuts cut into pieces*
Huzelnut	some huzelnuts cut into pieces*
Cut grass	cis-3-hexen-1-ol (2 mg/L)*
Green beans	vegetation water of cooked green beans (100mL)*
Hay	a few strands of hay
Straw	some straw

***in white wine (about 300 mL) for about 24 hours**

Table 4.2.7.: Aromatic terms used in sensory analysis and reference standard compositions

The tasting panel was made up of 14 trained tasters from CRA-ENO. The wines were served at the temperature of 16±1°C in ISO (3591-1977) approved glasses in an ISO (8589- 2007) tasting room.

4.2.4. Chemical analyses

All chemical analyses were performed in duplicate: for each trial, 2 different bottles were analyzed. The ethanol concentration, total extract, pH, total acidity, volatile acidity, free and total SO_2, copper, iron, were determined according to EEC methods (EEC Regulation 1990). Organic acids were determined by HPLC (Cane, 1990) and acetaldehyde by a colorimetric method as reported at paragraph 3.2. (Di Stefano & Ciolfi, 1982). The phenolic composition (total polyphenols and catechins) was determined by spectrophotometry (Di Stefano *et al*., 1989). The wine color was monitored by spectrophotometry on 1 mm of optic pathway after filtration with a 0.45 μm polypropylene filter. The absorbance at 420 nm and CIELab indexes (cylindrical coordinates: L* lightness, C* chroma, h* hue) were determined according to Piracci (1994).

The hydroxycinnamyl tartaric acids (HCTA) and 2-S-glutathionilcaftaric acid (GRP) were determined by high-performance liquid chromatography (Di Stefano & Cravero, 1991) after filtration with a 0.45 μm polypropylene filter (VWR International, 145 Milan, Italy); the injection volume was 20 μL, and the signal was monitored and recorded at 320 nm. The peaks were identified according to the retention time and the shape of the UV spectrum, compared with references reported in the bibliography (Baranowski & Nagel 1981). HCTAs were quantified using an external standard curve:the calibration, due to the lack of availability of commercial standards for caftaric, coutaric and fertaric acids, was performed using caffeic, coumaric and ferulic acids, respectively. Each standard was injected in triplicate to assess both the linearity and the repeatability of the method.

The reduced glutathione (GSH) was quantified using the method proposed by Park et al. (2000) and modified to use twice the amount of the derivatizing agents as the authors.

Pre-column derivatization of glutathione with o-phthalaldehyde (OPA) and 2-aminoethanol was performed. The resulting isoindole derivatives were separated on a Synergy Hydro RP-C18 reversed-phase HPLC column (150 mm x 4.6 mm I.D., 4μm packing, Phenomenex, Torrance, CA, USA) and detected by a fluorescence detector with excitation and emission wavelengths of 340 and 450 nm, respectively. An Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump, a DAD UV-Vis detector and a fluorescence detector (FLD) was used. The identification of the GSH peak was made by comparison with the retention time of a pure standard that was injected under the same analytical conditions. The GSH concentration was determined with a calibration curve obtained by adding increasing quantities (6 levels) of pure reference compound to a model solution. Each point of the curve was injected in triplicate in order to assess both the linearity and reproducibility of the method. The quantification of GSH was performed using the Chemstation software (Agilent Technologies, Palo Alto, CA, USA).

The methanol for the HPLC mobile phase (HPLC grade), p-coumaric acid, GSH, N-acetyl-L-cysteine, EDTA, OPA, and 2-aminoethanol were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). The caffeic and ferulic acid standards were purchased from Extrasynthese (Genay, France). Ultrapure water from a Milli-Q gradient A10 instrument system (Millipore Corporation, Billerica, MA, USA) was used throughout this experiment.

The accelerated browning test consists in measuring the absorbance at 420 nm of the wine before and after 6 days at 50°C in a thermostat, in 50 mL flask filled for 2/3 and closed with

cotton in order to allow oxygen to diffuse in wine. The difference between the two measures give an index of the tendency to browning (Simpson, 1982).

To measure the oxygen content was used a luminescence-based technology already described at paragraph 3.2.. When 135 mL bottles were used, the measure regarded only oxygen in solution (dissolved oxygen); while in trials carried out using 750 mL bottles, both dissolved oxygen and oxygen in the gaseous space (headspace) were detected using separate sensors.

4.2.4. Statistical analyses

Chemical data were processed using a complete 3-factors ANOVA (first experience: SO_2, ellagic tannins, GSH; second experience: SO_2, GSH and oxygen; third experience: SO_2, gallic tannins and GSH) with a complete factorial model to study the main effect of the 3 factors considered in each trial and their interactions. For this purpose was used SPSS for Windows version 15.0 (SPSS Inc., Chicago, Il USA, 2004).

The results of the duo-trio tests and paired difference tests were compared to tables reporting the threshold values for statistical significance (Ubigli, 1998), whereas the sensory data obtained with the wine aroma wheels were processed with complete ANOVA: 4 factors in the first experience (SO_2, ellagitannins, GSH and tasters), 5 factors (SO_2, gallotannins, GSH, tasters, tasting session) and 3 factors (wines, tasters and tasting session) in the third experience.

4.3. Results

4.3.1. Results of the first trial

Evolution of the oxygen content in bottled wines

The trend of the dissolved oxygen is reported in Figure 4.3.1. All the samples had a high starting amount of oxygen (on average 3.2 ppm) which indicated not optimal bottling conditions.

The rate of oxygen consumption was significantly faster for the samples with a lower amount of SO_2 (-SO_2samples). The tannins had a slight effect on oxygen consumption: the presence of tannins in samples with higher amount of SO_2 (+SO_2 samples) slowed down the oxygen consumption compared to the samples without tannins.

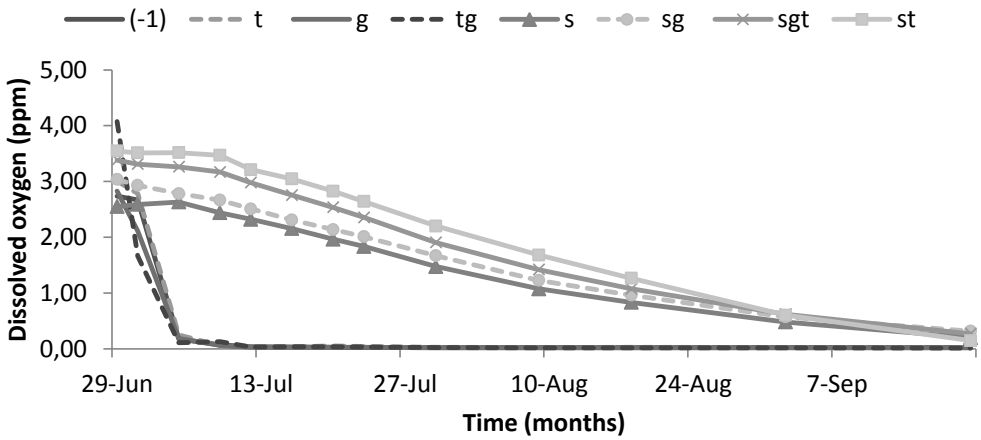


Figure 4.3.1.: Kinetic of dissolved oxygen consumption during bottle aging.

These results are in contrast with what is usually reported in literature where, SO_2 increases the rate of oxygen consumption. Regarding tannins, since they act as a catalyst of chemical oxidation, the rate of oxygen consumption should proportionally increase with the amount of added tannins.

The opposite effect is normally observed in the presence of laccase activity, that is during an enzymatic oxidation. In this case, SO_2 or tannins, slow down the rate of oxygen consumption. This could be the explanation of our results: since the wine used in the experiment derived from partially botrytized grapes and did not undergo any treatment, such as clarification with bentonite, a residual oxidasic activity could be present in this case.

Evolution of chemical-physical parameters of the wines during bottle aging

Effect of SO_2. The content of SO_2 significantly influenced the evolution of some chemical-physical parameters of the wine during bottle aging. The SO_2 consumption rate was faster in samples with higher level of SO_2 (+SO_2 sample) compared to the samples with lower SO_2 (-SO_2 sample). As regards the + SO_2 samples, a similar trend for both free and total SO_2 was observed in the first two months and then a slightly higher decrease in free SO_2 was observed probably for the release of SO_2 from its bound forms (Figure 4.3.2.).

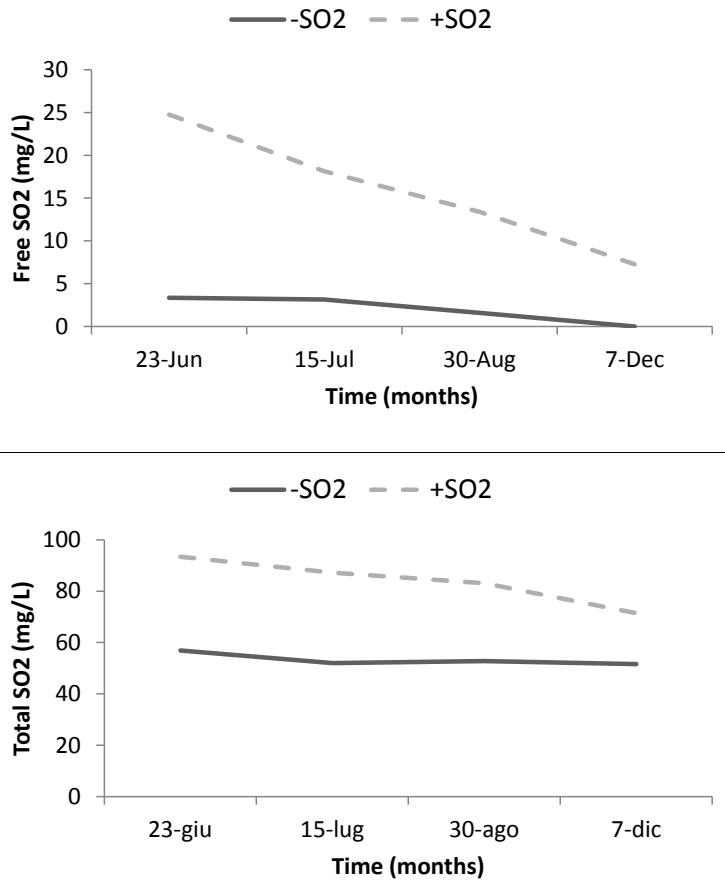


Figure 4.3.2.: Evolution of the content of free (a) and total (b) SO_2 during bottle aging in realation with the different amount of SO_2 at bottling

Wine color was significantly influenced by the level of SO_2: higher SO_2 dosages (+SO_2 trials) caused a reduction of color intensity (lower A420 and Chroma). The differences were already statistically significant immediately after the addition, and such they remained during bottle aging (Figure 4.3.3./a and b). The observed results confirmed the effect played by SO_2 on the reduction of quinones which takes part in the formation of polymers responsible for wine browning, back to the original phenols with the consequence to slow down the browning process (Danilewicz *et al*, 2008). The effect on the other color parameters, luminosity (L*) and hue (h*) were modest, even if sometimes significant, with average lower L* and h* values in –SO_2 trials (Table 4.3.1.). Furthermore, SO_2 had a significant effect on the amount of acetaldehyde, another important parameter related to wine aging. Two months after bottling

(second sampling), the -SO_2 trials started to show higher amounts of acetaldehyde compared to +SO_2 trials. (Figure 4.3.3./c)

The SO_2 has an important role in controlling the production of acetaldehyde by reacting with hydrogen peroxide and thus removing it from the Fenton reaction, that leads to the production of hydroxyl radical, able to oxidize ethanol to acetaldehyde.

Furthermore, 2 months after bottling SO_2 started to show an influence on volatile acidity, with significantly lower amount for the +SO_2 trials (Figure 4.3.3./d). Again, this effect has to be related to the the role of SO_2 in the Fenton reaction to compete with hydrogen peroxide and thus to reduce the production of acetaldehyde which can then be oxidized to acetic acid.

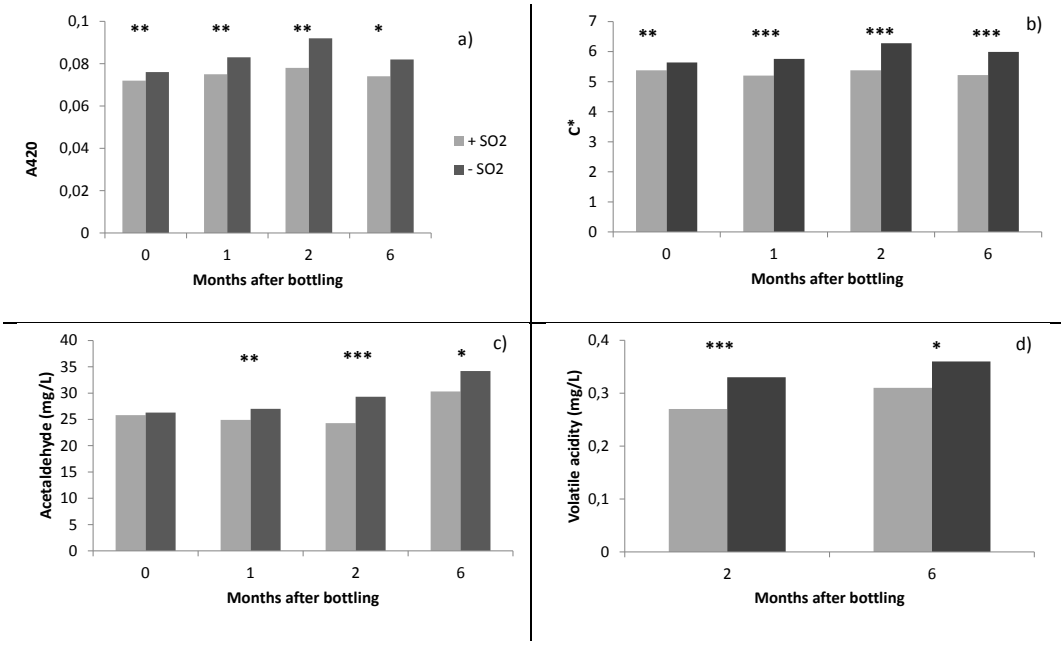


Figure 4.3.3.: Influence of different levels of SO_2 on respectively (a) absorbance at 420 (A420) , (b) Chroma (C*), (c) acetaldehyde and (d) volatile acidity during bottle aging. *;**;*** indicate significant differences at P≥ 95%; 99% and 99.9%.

The interactions between the studied factors (SO_2, GSH and tannins) were also investigated. Interesting and significant interaction, from an enological point of view, was observed 6 months after bottling between SO_2 and tannins as regards volatile acidity: in presence of ellagitannins, high level of SO_2 caused the reduction of the production of volatile acidity. It can be said that the addition of ellagitannins caused a slight increase in volatile acidity that can be controlled by adding SO_2 (Figure 4.3.4.).

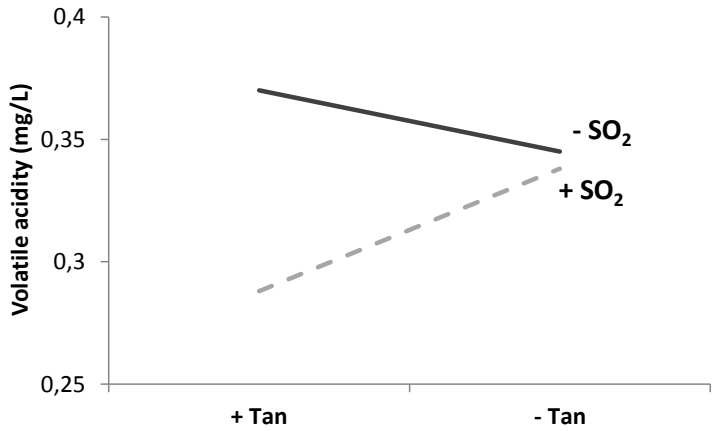


Figure 4.3.4.: Representation of the interaction between the factors "tannins" and "SO_2" as regards volatile acidity.

No significant differences were observed as regards the other studied parameters for the different levels of SO_2 (Table 4.3.1.)

	After bottling			After 1 month			After 2 months			After 6 months		
	+SO_2	-SO_2	F-value	+SO_2	-SO_2	F-value	+SO_2	-SO_2	F-value	+SO_2	-SO_2	F-value
Free SO_2 (mg/L)	24,76	3,35	208***	18,13	3,15	1919***	13,38	1,06	555***	7,28	0,02	285***
Total SO_2 (mg/L)	93,4	56,95	1792***	87,2	52	737,7***	83,08	52,75	1335***	71,4	51,6	1960***
A420, 1 mm	0,072	0,076	14,5**	0,075	0,083	26,6**	0,078	0,092	26,1**	0,074	0,082	8,4*
L*	99,54	99,39	6.7 *	99.1	98.9	2.3 n.s.	99.02	98.66	7.2 *	99.1	99.15	0.18 n.s.
h*	-1.29	-1.33	5 n.s.	-1.32	-1.36	96.4 ***	1.34	-1.37	18.6 **	-1.32	-1.32	0.09 n.s.
C*	5,38	5,64	26.5 **	5.2	5.76	144.1 ***	5.38	6.28	69.1 ***	5.22	5.99	36.1 ***
Acetaldehyde (mg/L)	25.8	26.3	1.5 n.s.	24.9	27	15.3 **	24.3	29.3	161***	30.3	34.2	5.7 *
Total poliphenols (mg/L)	90	91	0.1 n.s.	84	84	0.0 n.s.	90	93	4.4 n.s.	97	101	7.2 *
Catechins (mg/L)	7.8	8.1	14.4 **	7.8	8.3	69 ***	7.4	8.2	249 ***	7.6	8.4	165 ***
GSH (mg/L)	7.7	8.5	1.1 n.s.	4.9	4.1	0.4 n.s.	3.3	2.3	112***	1.2	1.2	0.04 n.s.
Volatile acidity (g/L)	n.d.	n.d.		n.d.	n.d.		0.27	0.33	52.9 ***	0.31	0.36	8.1 *
t-caftaric acid (mg/L)	43.5	42.8	0.2 n.s.	45.6	46.5	2.2 n.s.	47.7	47.6	0.8 n.s.	35.9	36.6	0.4 n.s.
c-coutaric acid (mg/L)	1.8	1.78	0.3 n.s	1.8	1.8	1.7 n.s.	1.84	1.89	23.4 **	1.6	1.7	2.0 n.s.
t-coutaric acid (mg/L)	2.4	2.4	1 n.s.	2.3	2.3	0.4 n.s.	2.5	2.5	1.8 n.s.	2.3	2.3	0.0 n.s.
GRP (mg/L)	17.9	17.5	0.3 n.s	18.0	18.6	5.9 *	17.6	17.9	15.6 **	5.7	5.9	0.4 n.s.
c+t fertaric acid (mg/L)	2.2	2.1	0.7 n.s.	2.1	2.1	0.7 n.s	2.2	2.1	5.6 *	1.8	1.8	0.2 n.s.

Table 4.3.1.:Average values of the main chemical-physical parameters of wines during bottle aging in trials with different SO_2 content ("+SO_2" and "–SO_2" trials). ANOVA results. *;**;*** and n.s. indicate significance at P ≥ 95%, 99%, 99.9% and not significant, respectively.

Effect of ellagitannins:

The addition of ellagitannins significantly increased the rate of free and total SO_2 consumption starting one month after bottling (second sampling). The differences between the trials with different ellagitannins contents remained significant until the end of the experiment, except for free SO_2 two months after bottling (third sampling) (Figure 4.3.5.).

The faster consumption of SO_2 in samples enriched with ellagitannins was probably due to the faster production of hydrogen peroxide as a consequence of the oxidation of ellagitannins.

Danilewicz reported that the consumption rate of SO_2 has to be related both to the amount and the kind of catechol present in the solution (Danilewicz, 2007).

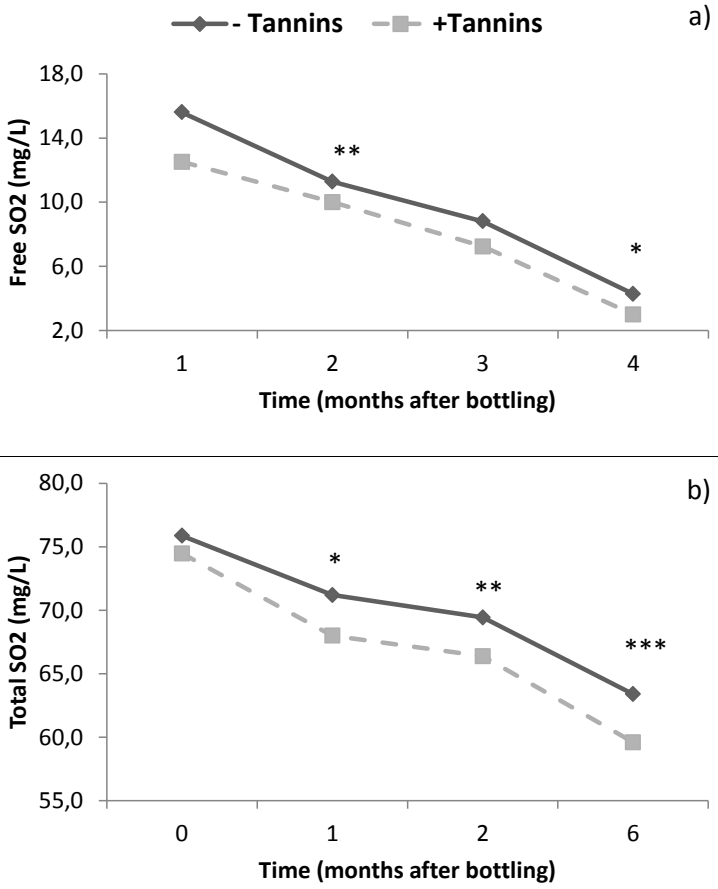


Figure 4.3.5.: Evolution of the average content of free (a) and total (b) SO_2 in the "+Tannins" and "–Tannins" samples during bottle aging. *, **, and *** means differences significant respectively at P ≥ 95%, 99%, 99.9%.

The addition of ellagitannins increased wine color, and the differences between the trials with or without tannins remained constant during aging: the effect of tannins was probably due only to their natural color.

A slight increase in the production of acetaldehyde in the trials with ellagitannins, significant only two months after bottling (third sampling) was observed. This result was most likely the consequence of the increase in the production of hydrogen peroxide caused by the added tannins. For the same reason higher losses in SO_2 were observed for samples with ellagitannins.

It is known that tannins can also increase the production of acetaldehyde. They do this by regenerating ferrous iron from the ferric state in presence of high amount of oxygen. In this condition hydroxyethyl radical cannot do it because it is quickly oxidized by molecular oxygen (Elias *et al*., 2010). However, this second pathway of the production of acetaldehyde occurs mainly in wine stored in tanks and above all in wood barrels where there are higher amounts of dissolved oxygen. Therefore, the effect of tannins on the production of acetaldehyde could be lower when the addition occurs at bottling rather than in previous stages of the winemaking process.

Instead no effect of ellagitannins on HCTA, total polyphenols, catechins, GSH and volatile acidity were observed (Table 4.3.2.).

	After bottling			After 1 month			After 2 months			After 6 months		
	+Tannins	-Tannins	F-value	+Tannins	-Tannins	F-value	+Tannins	-Tannins	F-value	+Tannins	-Tannins	F-value
Free SO_2 (mg/L)	12,5	15,6	4.3 n.s.	10,0	11,3	13.9 **	6.18	8.8	4.1 n.s.	3.0	4.28	8.8 *
Total SO_2 (mg/L)	74,5	75,9	2.6 n.s.	68,0	71,2	6.1 *	66,4	69,4	13.5 **	59,6	63,4	72.2 ***
A_{420}	0,078	0,070	52.5 ***	0,083	0,075	26.3 **	0,088	0,082	4.2 n.s.	0,082	0,075	6.3 *
L*	99,40	99,52	4.3 n.s.	98,90	99,10	2.1 n.s.	98,82	98,86	0.1 n.s.	98,97	99,27	3.4 n.s.
h*	-1,31	-1,30	0.5 n.s.	-1,35	-1,34	2.8 n.s.	-1,36	-1,35	0.0 n.s.	-1,33	-1,31	4.5 n.s.
C*	5.73	5,23	81.4 ***	5.72	5.24	109.1 ***	6.03	5.62	14.1 **	5.71	5.49	2.9 n.s.
Acetaldehyde (mg/L)	25.7	26.4	3.2 n.s.	26.2	25.7	0.6 n.s.	27.3	26.4	5.8 *	32.7	31.7	0.4 n.s.
Total poliphenols (mg/L)	90,00	91,00	0.4 n.s.	87,00	81,00	1.6 n.s.	92,00	91,00	1.0 n.s.	100,00	99,00	0.2 n.s.
Catechins (mg/L)	8,00	8,00	0.1 n.s.	8,00	8,00	0,5 n.s.	8,00	8,00	1.5 n.s.	8,00	8,00	1 n.s.
GSH (mg/L)	0,30	0,34	0.2 n.s.	4.5	4.5	0.0 n.s.	2.8	2.8	1.3 n.s.	1.2	1.2	0.1 n.s.
Volatile acidity (g/L)	n.d.	n.d.	-	n.d.	n.d.	-	0.31	0.30	2.5 n.s.	0,02	0,02	0.6 n.s.
t-caftaric acid (mg/L)	44.6	41.7	3.3 n.s.	45.4	46.7	4.6 n.s.	47.1	48.2	31.8 ***	35.9	36.6	0.4 n.s.
c-coutaric acid (mg/L)	1.8	1.8	3.0 n.s.	1.8	1.8	0.4 n.s.	1.9	1.9	0.2 n.s.	1.6	1.6	0.1 n.s.
t-coutaric acid (mg/L)	2.4	2.4	1.8 n.s.	2.3	2.3	3.1 n.s.	2.5	2.5	0.0 n.s.	2.3	2.4	0.9 n.s.
GRP (mg/L)	18.3	17.1	4.1 n.s.	18.1	18.5	3.4 n.s.	17.7	17.9	4.7 n.s.	5.8	5.9	0.1 n.s.
c+t fertaric acid (mg/L)	2.2	2.1	3.0 n.s.	0,09	0,09	0,5 n.s.	2.2	2.2	0.7 n.s.	1.8	1.8	0.4 n.s.

Table 4.3.2.: Average values of the main chemical-physical parameters of wine during bottle aging in trials with different tannins content ("+Tannins" and "–Tannins" trials). ANOVA results. *;**;*** and n.s. indicate significance at $P \geq 95\%$, 99%, 99.9% and not significant, respectively.

Effect of GSH: no significant differences were observed in any chemical-physical parameter for the addition of GSH, except for total SO_2 6 months after bottling (fourth sampling) which was significantly higher in the "-GSH" trial. However, even if significant, the difference was so low that it cannot have any practical interest (Table 4.3.3.). Therefore, the main difference between GSH and other antioxidant molecules, such as ellagitannins or ascorbic acid is the fact that GSH did not consume SO_2 during bottle aging. This is probably due to the fact that GSH, differently from other additives, does not increase the production of hydrogen peroxide, which consumes SO_2. The GSH is supposed to participate to nucleophilic addition reactions with *o*-quinones, as proposed by Makkhonika et al., or reconvert them back to the original phenols oxidizing itself to oxidate glutathione (GSSG).

However, the addition of GSH did not influence wine color: no statistically significant differences were observed regarding A420 and Chroma (Table 4.3.3.), according to other works (Roussis *et al*., 2000, El Hosky *et al*, 2009).

Another mechanism suggested for GSH as antioxidant molecule is the reaction with hydrogen peroxide, like the well known role of SO_2. Some results obtained by studying the interactions between these two molecules seemed to confirm this mechanism. Significant interaction was observed two months after bottling between SO_2 and GSH as regards the parameter acetaldehyde: the $–SO_2$ samples produced less acetaldehyde in the presence of GSH than in the absence of GSH. This difference can be explained by a reactivity of GSH with hydrogen peroxide, but further investigation is needed. (Figure 4.3.6.).

	After bottling			After 1 month			After 2 months			After 6 months		
	+GSH	-GSH	F-value	+GSH	-GSH	F-value	+GSH	-GSH	F-value	+GSH	-GSH	F-value
SO_2 libera (mg/L)	14.28	13.84	0.09 n.s.	10.60	10.68	0.1 n.s.	8.44	6.55	2.1 n.s.	3.80	3.48	0.6 n.s.
SO_2 totale (mg/L)	75.08	75.28	0.05 n.s	69.40	69.80	0.1 n.s.	68.68	67.15	3.4 n.s.	60.80	62.20	9.8 *
E_{420}	0.075	0.073	2.6 n.s	0.080	0.078	0.8 n.s.	0.84	0.085	0.1 n.s.	0.079	0.078	0.01 n.s.
L*	99.42	99.50	1.9 n.s.	98.94	99.04	0.9 n.s.	98.85	98.83	0.02 n.s.	99.16	99.07	0.3 n.s.
h*	-1.32	-1.30	1.7 n.s.	-1.35	-1.34	8.3 *	-1.35	-1.36	0.04 n.s.	-1.32	-1.32	0.2 n.s.
C*	5.51	5.51	0.0 n.s.	5.49	5.47	0.2 n.s.	5.82	5.83	0.02 n.s.	5.66	5.55	0.7 n.s.
Acetaldehyde (mg/L)	25.68	26.42	2.9 n.s.	25.93	26.05	0.2 n.s.	26.84	26.84	0.0 n.s.	32.09	32.33	0.02 n.s.
Total poliphenols (mg/L)	89	92	2.3 n.s.	83.90	82.75	0.3 n.s.	91.99	91.28	0.2 n.s.	99.30	99.50	0.02 n.s.
Catechins (mg/L)	8	8	0.0 n.s.	8	8	0.8 n.s.	8	8	0.06 n.s.	8	8	0.2 n.s.
GSH (mg/L)	15.8	0.4	366 ***	8.80	0.21	58.1 ***	5.15	0.43	2244 ***	2.05	0.38	229 ***
Volatile acidity (g/L)	n.d.	n.d.		n.d.	n.d.		0.31	0.30	2.5 n.s.	0.34	0.33	0.2 n.s.
t-caftaric acid (mg/L)	43.4	42.9	0.1 n.s.	45.9	46.2	0.2 n.s.	47.6	47.7	0.0 n.s.	37.3	35.1	3.7 n.s.
c-coutaric acid (mg/L)	1.8	1.8	0.1 n.s.	1.8	1.8	1.6 n.s.	1.9	1.9	0.5 n.s.	1.7	1.6	0.2 n.s.
t-coutaric acid (mg/L)	2.4	2.4	1.2 n.s.	2.3	2.3	1.5 n.s.	2.5	2.5	0.1 n.s.	2.4	2.3	2.1 n.s.
GRP (mg/L)	17.8	17.6	0.1 n.s.	18.2	18.3	0.2 n.s.	17.8	17.8	0.0 n.s.	6.0	5.6	2.0 n.s.
c+t fertaric acid (mg/L)	2.16	2.15	0.0 n.s	2.1	2.1	0.8 n.s.	2.2	2.2	0.1 n.s.	1.9	1.8	3.2 n.s.

Table 4.3.3.: Average values of the main chemical-physical parameters of wine during bottle aging in trials with different GSH content ("+GSH" and "–GSH" trials). ANOVA results. *;**;*** and n.s. indicate significance at $P \geq 95\%$, 99%, 99.9% and not significant, respectively.

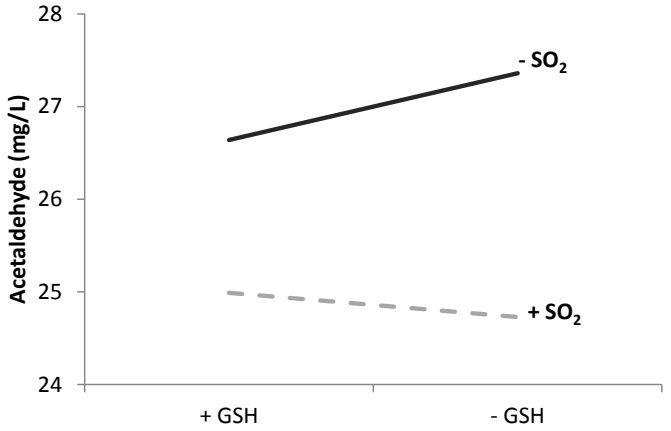


Figure 4.3.6: Representation of the interaction between the factors "SO_2" and "GSH" as regards acetaldehyde.

The GSH was hightly unstable (Figure 4.3.7.): its concentration had fallen by up to half after one month of bottle aging and was reduced to one fifth after 2 months. After 6 months, the GSH content was one tenth of the added amount, regardless of the presence of high or low levels of SO_2. The instability of GSH molecules has been already described in other works (Sonni *et al* 2011).

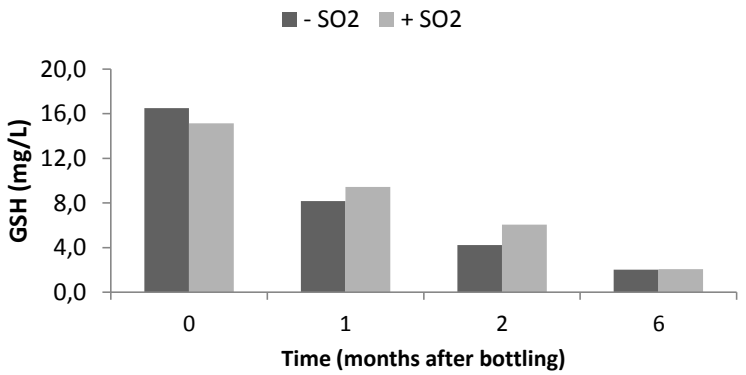


Figure 4.3.7.: Effect of different SO_2 levels at bottling on the evolution of the GSH content ("+GSH" samples) during bottle aging.

Sensory analysis

Only the trials with different levels of SO_2 were statistically distinguishable in the duo-trio test performed three months after bottling. The results of the paired difference tests confirmed the role of SO_2 in protecting color and odor, already observed by other authors (Godden *et al*.,

2001). Effectively, $+SO_2$ trials had a significantly lower color and were judged, on average, more fruity.

Regarding the taste, a contrary effect was observed for the addition of ellagitannins in the presence or the absence of SO_2: $+SO_2$ trials with ellagitannins were judged softer and less bitter; on the other hand, $-SO_2$ trials with ellagitannins scored less compared to $+SO_2$ trials as regards the taste.

The sensory profile of wines evaluated six months after bottling with a wine aroma wheel confirmed the positive effect of SO_2 in protecting wine during bottle aging Figure 4.3.8.).

The + SO_2 trials had lower color and resulted statistically different from the - SO_2 trials for the more intense notes of citrus and acacia flower (related to freshness of young wines, Silva Ferreira *et al*, 2003), and for the less intense notes of cooked vegetables and acetaldehyde (descriptors of oxidation, Escudero *et al*., 2002).

Regarding the taste, the + SO_2 trials resulted significantly softer and more structured than the – SO_2 trials.

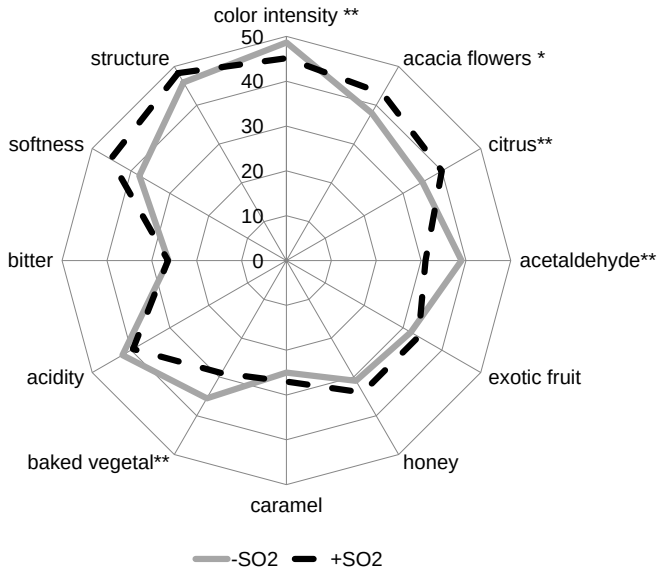


Figure 4.3.8.: Sensory profile of wines after 6 months of bottle aging. Effect of SO_2* and ** indicate significant differences at P≥ 95% and 99% respectively.

No noteworthy results were observed either for ellagitannins or for GSH regarding their protective effect on oxidative evolution of wines. In samples enriched with GSH, it was even observed a decrease in the intensity of citrus notes, related to freshness.

4.3.2. Results of the second trial

Evolution of the oxygen content in bottled wines

The rate of oxygen consumption was fast during the first days after bottling and positively related to the initial oxygen content of each sample: the higher the oxygen content at bottling, the faster its consumption. However, after 15 daays, the complete consumption of oxygen was observed only in samples characterized by a low oxygen content: they consumed all the oxygen during the first 8 days. Regarding samples with higher oxygen content, the complete oxygen consumption was observed only in the sample with added GSH and with 60 mg/L of SO_2 (sgo), (Figure 4.3.9.).

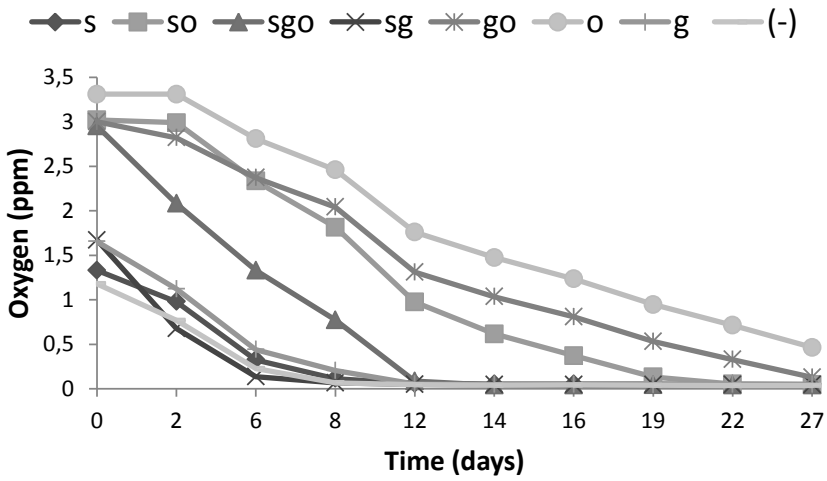


Figure 4.3.9.: Kinetic of oxygen consumption during the first month of bottle aging.

The trend of the curves were, effectively, influenced by the content of free SO_2. SO_2 caused a statistically significant increase in the rate of the oxygen consumption and the effect of SO_2 was more evident in the trials with the highest level of dissolved oxygen. A significant acceleration of oxygen consumption was also observed in samples enriched with GSH. In trials with higher SO_2 content, the initial oxygen (3 mg/L at bottling), was completely consumed within 12 and 21 days in the presence or the absence of added GSH, respectively. The effect on the consumption rate was similar for both the additives, but higher for SO_2 (Table 4.3.4.).

Statistically significant interactions were observed between SO_2 and GSH as regards dissolved oxygen: the rate of oxygen consumption increased when GSH was added in the presence of high amounts of SO_2. Furthermore, statistically significant interactions were observed between

SO_2 and oxygen and between GSH and oxygen (Table 4.3.4.). The higher the concentration of dissolved oxygen, the higher the influence of both SO_2 and GSH.

The increase in the rate of oxygen consumption in presence of SO_2 has been already described in some model solutions (Danilewicz *et al*, 2010) and in our first experiment (paragraph 3.1). This effect can be related to the capacity of SO_2 to reduce the concentration of quinones by reconverting them back to the original phenols that can undergo further oxidations.

A reduction in the concentration of quinones occurs also with other nucleophilic compounds, such as benzensulfinic acid sodium salt (BSA) and GSH (Cheynier *et al*., 1986; Makhotkita *et al*. 2009; Sonni *et al.*, 2011), which form additional compounds with quinones. The losses of quinones accelerate the oxidation process and the oxygen consumption rate (Danilewicz, 2011).

Sampling	-SO2	+SO2	F	Sig.	-O2	+O2	F	Sig.	-GSH	+GSH	F	Sig.	F_{SO2*O2}	Sig.	$F_{SO2*GSH}$	Sig.	F_{GSH*O2}	Sig.
T 0	2.29	2.24	0.2	ns	3.07	1.46	297	***	2.21	2.32	1.4	ns	1.8	ns	0.08	ns	10	*
T 2	2.00	1.68	6.8	*	1.67	2.01	236	***	2.01	1.67	7.3	*	2.7	ns	4.7	ns	9	*
T 6	1.46	1.03	42	***	0.28	2.21	848	***	1.42	1.07	29	**	24	**	13	**	31	**
T 8	1.19	0.69	141	***	0.12	1.77	1539	***	1.11	0.77	66	***	116	***	23	**	84	***
T 12	0.79	0.29	389	***	0.05	1.03	1498	***	0.71	0.37	175	***	397	***	20	**	174	***
T 14	0.65	0.19	493	***	0.05	0.79	1287	***	0.55	0.29	150	***	503	***	3.6	ns	150	***
T 16	0.53	0.13	273	***	0.05	0.61	533	***	0.43	0.23	61	***	278	***	0.7	ns	59	***
T 19	0.39	0.07	200	***	0.05	0.41	258	***	0.29	0.16	31	**	207	***	11	*	31	*
T 22	0.28	0.05	135	***	0.05	0.28	139	***	0.21	0.11	24	**	140	***	19	**	25	**
T30	0.17	0.04	44	***	0.04	0.17	44	***	0.15	0.06	21	***	48	***	17	**	22	**

Table 4.3.4.: Average content of dissolved oxygen in wines in relation to the different amount of the studied parameters. ANOVA results and 1st order interactions between the factors "SO_2", "oxygen" and "GSH". *;**;*** and n.s. indicate significance at P ≥ 95%, 99%, 99.9% and not significant, respectively.

Evolution of chemical-physical parameters of bottled wines

Effect of O_2

The consumption of both free and total SO_2 was, on average, faster in the samples with higher levels of oxygen at bottling, regardless of the amount of SO_2. The differences started to appear 7 days after bottling (Figure 4.3.10.).

The consumption of free SO_2 significantly rose with the increasing of dissolved oxygen, while only a slight influence was observed for the presence of GSH (Figure 4.3.10., a and b). Also the consumption of total SO_2 increased for high levels of oxygen, but the differences were not significant (Figure 4.3.10. c and d).

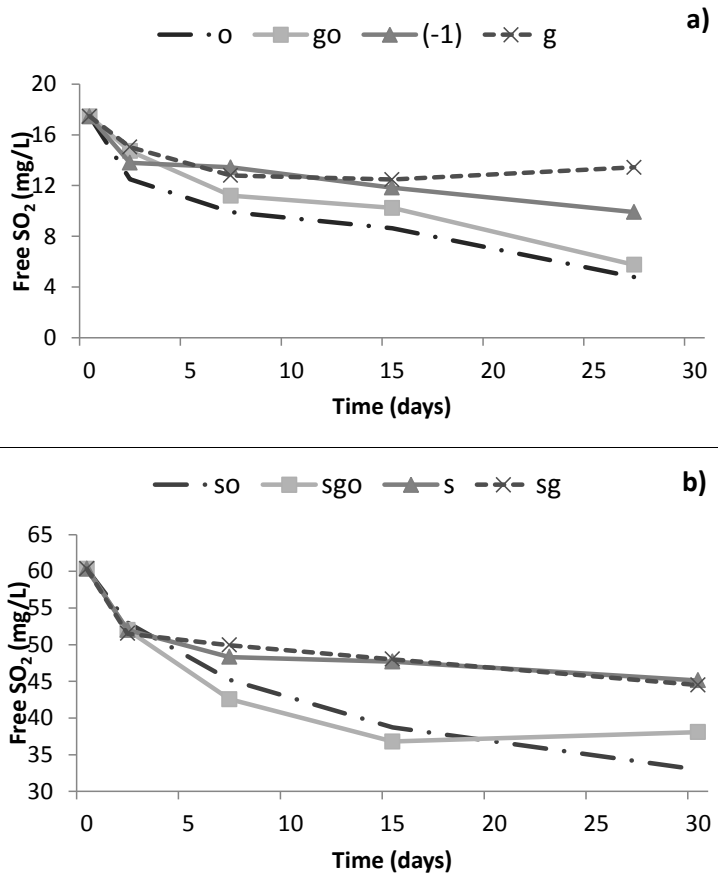


Figure 4.3.10: Kinetic of free SO_2 consumption in samples with low level of SO_2 (a), and high level of SO_2 (b). Kinetic of total SO_2 consumption in samples with high level of SO_2 (c) and low level of SO_2 (d)

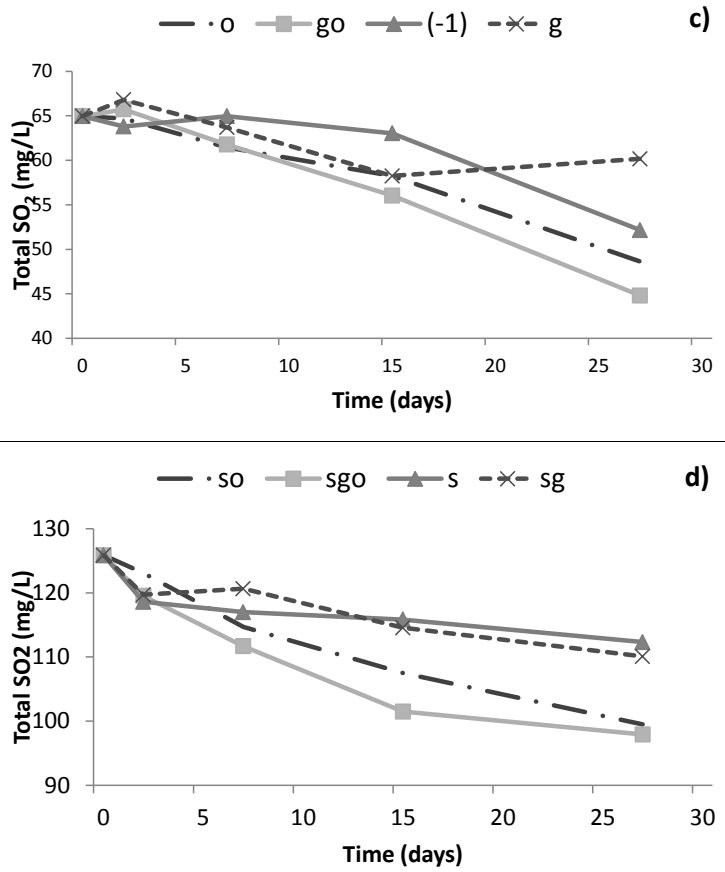


Figure 4.3.10.: Kinetic of total SO_2 consumption in samples with low level of SO_2 (c) and high level of SO_2 (d).

A significant effect of dissolved oxygen on A420 (higher value of A420 in presence of higher level of oxygen), was observed only 15 days after bottling (4th sampling).

Sampling	-SO2	+SO2	F	Sig.	-O2	+O2	F	Sig.	-GSH	+GSH	F	Sig.	F_{SO2*O2}	Sig.	$F_{SO2*GSH}$	Sig.	F_{GSH*O2}	Sig.
T 0	0,05	0,05	0,23	ns	0,05	0,05	0	ns	0,05	0,05	0.14	ns	0.05	ns	0.29	ns	0.34	ns
T 2	0,05	0,05	1,76	ns	0,05	0,05	0.04	ns	0,05	0,05	0.55	ns	0.36	ns	0.55	ns	0.21	ns
T 7	0,05	0,04	6,76	ns	0,05	0,05	1.53	ns	0,05	0,05	0.66	ns	0.78	ns	0.06	ns	0.31	ns
T 15	0,05	0,04	6778,77	*	0.04	0,05	3802.78	*	0,05	0.04	3325.44	*	336.11	*	205.44	*	529.00	*

Table 4.3.5.:Average value of A420 parameters. ANOVA results. Influence of the different amount the stodied parameters on the color development

Effect of SO_2. A positive effect of SO_2 in protecting wine color was observed both in the short and in the long term: the +SO_2 trials had lower values of A420. These differences became significant 15 days after bottling (4th sampling) and remained hightly significant 1 year after bottling (Figure 4.3.11.). These results confirmed the studies of Danilewicz et al (2008) as

regards the protective effect of SO_2 in controlling the formation of brown polymers by polyphenols condensation.

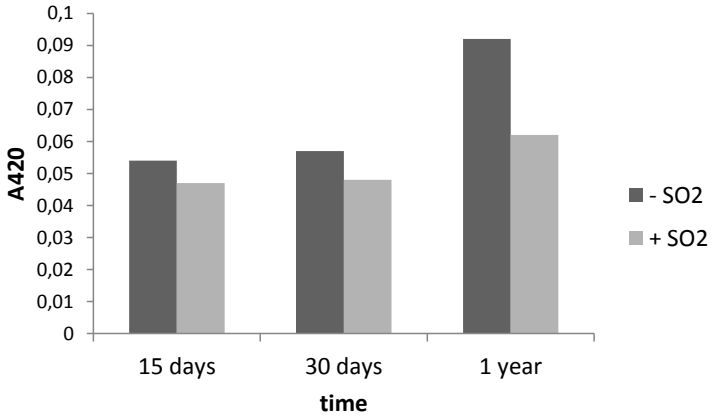


Figura 4.3.11.: Evolution of wine color during aging. Influence of different levels of SO_2. * and ** indicate significant differences at P≥ 95% and 99% respectively.

Effect of GSH: The GSH did not influence the consumption rate either of free or of total SO_2. No significant influence was observed as regards A420 parameter, except 15 days after bottling (4^{th} sampling), where, a significantly lower A420 value was observed in wines enriched with GSH.

The GSH added to wines was quickly consumed in all the samples, the amout had fallen by up to about half within 15 days after bottling. The amount of oxygen or SO_2 showed just a slight influence on the stability of GSH. Only after 1 month of bottle aging a significant protective effect was observed for these two factors, but with scarce practical interest (Figure 4.3.12.)

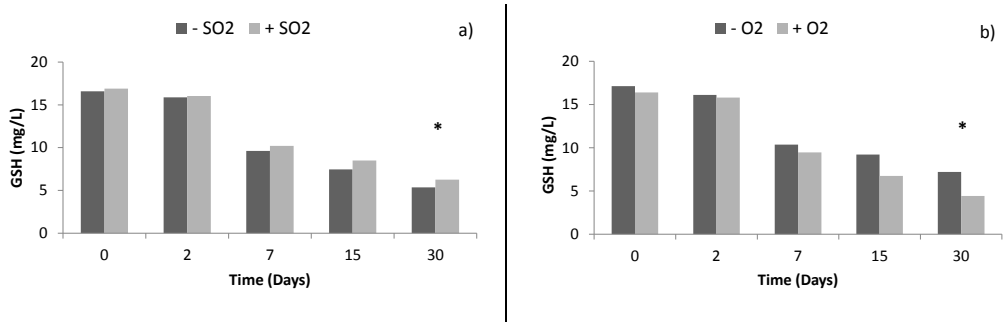


Figure 4.3.12.:GSH consumption during bottle aging in relation to the different level of SO_2 (a) and oxygen (b) *indicate differences between thesis sifnificant at P≥ 95%

No effect of the studied additives was observed either for total polyphenols or for catechins.

Chemical-physical analysis 1 year after bottling (Table 4.3.6.): the chemical-physical analysis performed 1 year after bottling confirmed the important role of SO_2 in protecting wines from browning. No protecting effect against oxidation was observed for GSH. Furthermore, wines with higher level of oxygen at bottling ($+O_2$ samples) showed significantly higher A420 values compared to $–O_2$ samples. These differences, however, were not visually perceivable.

The different levels of oxygen at bottling have significantly influenced the concentration of free and total SO_2 during bottle aging, with higher losses in both free and total SO_2 for wines with higher level of oxygen. After 1 year of bottle aging, the wines with 3 mg/L of dissolved oxygen at bottling ($+O_2$ samples) had 10 mg/L of free SO_2, while the wines with 1.5 ml of dissolved oxygen at bottling ($-O_2$ samples) had 16 mg/L of free SO_2. These differences in the content of free SO_2 mean that, if the $-O_2$ samples can be considered protected against the development of oxidative characters, the +O2 samples had a higher possibility to develop that unwanted characteristic. These differences are thus very important, because they can influence wine evolution and shelf life.

	$+SO_2$	$-SO_2$	F-value	$+O_2$	$-O_2$	F-value	+GSH	-GSH	F-value
Free SO_2 (mg/L)	23.28	2.6	185***	9.6	16.28	19**	13.56	12.32	0.7 ns
Total SO_2 (mg/L)	87.2	40.38	345***	56.8	70.78	31**	61.86	65.72	2.3 ns
$A_{420,\,1\,mm}$	0.062	0.092	212***	0.079	0.074	5.6*	0.077	0.077	0.03 ns
Total polyphenols (mg/L)	80	83	2.9 ns	81	81	0.00 ns	82	80	0.9 ns
Catechins (mg/L)	10	11	11*	10	10	1 ns	10	10	0.6 ns

Table 4.3.6.: Chemical-phisical composition of the wines after 1 year of bottle aging, and ANOVA results

4.3.3. Results of the third trial

Evolution of the oxygen content in bottled wines

The trend of oxygen consumption was consistent with the results of the previous experiments: samples with higher levels of SO_2 ($+SO_2$ samples) consumed oxygen faster than $–SO_2$ samples. The same trend was observed both in 135 mL and in 750 mL bottles (Figure 4.3.14.)

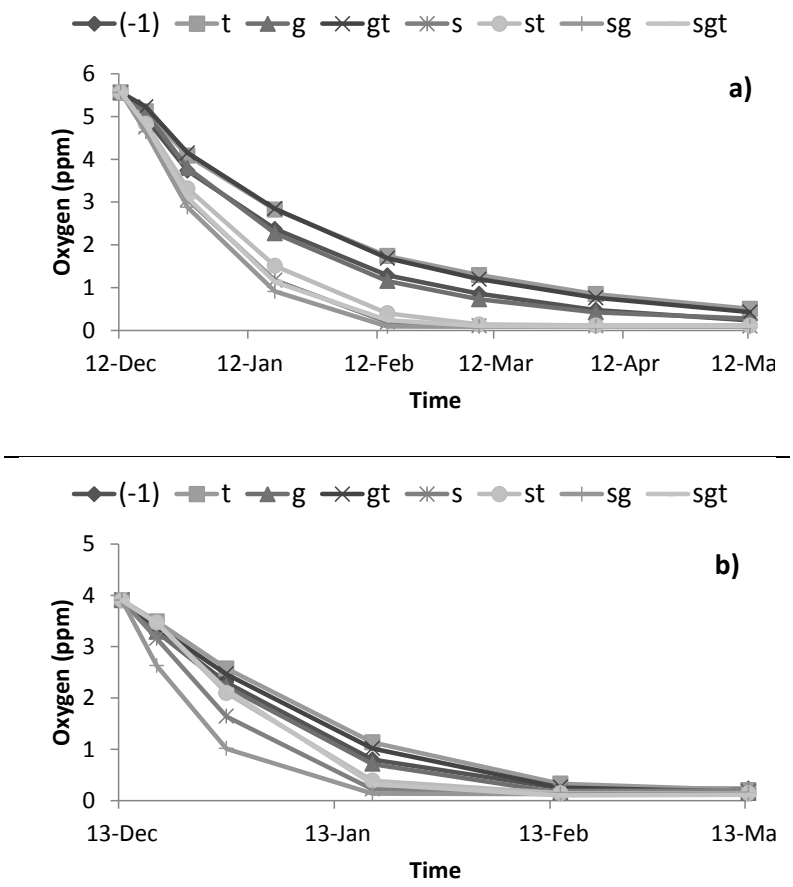


Figure 4.3.14.:Evolution of the content of dissolved oxygen during bottle aging in 135 ml (a) and 750 mL (b) bottles respectively

The figure 4.3.15. reports the different tendency to consume oxygen of the samples related to each additive in 135 mL bottles. Significant results during all the storage period were observed only as regards the different levels of SO_2, with a faster consumption of oxygen for higher content of SO_2. The GSH did not influence the content of dissolved oxygen, even if it was on average slightly faster in "+GSH" samples.

As regards tannins, differently from what is reported in literature, an increase in the rate of oxygen consumption was observed in "–Tannins" samples. This effect was modest and only sometimes significant.

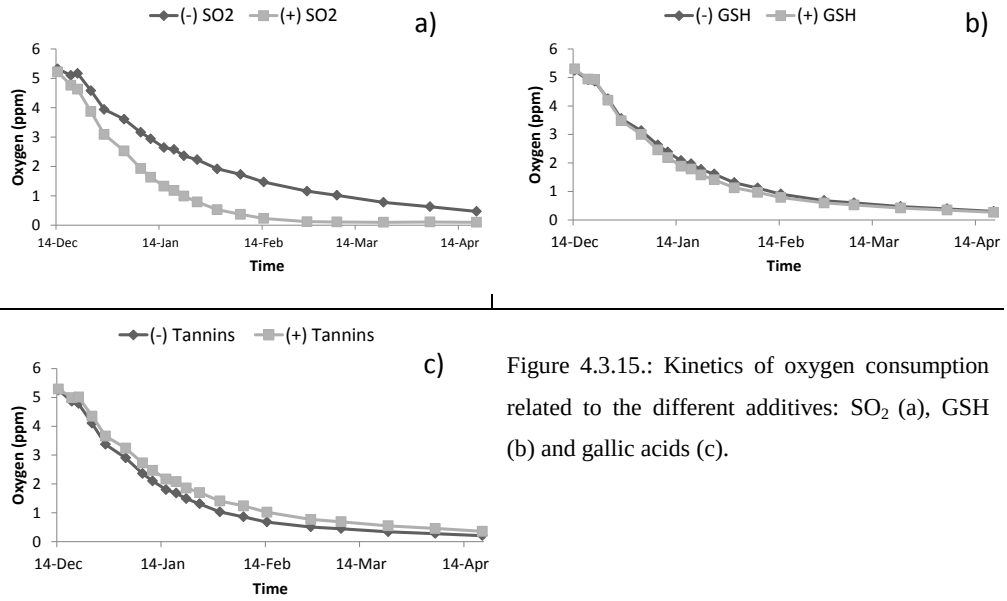


Figure 4.3.15.: Kinetics of oxygen consumption related to the different additives: SO_2 (a), GSH (b) and gallic acids (c).

Evolution of chemical-physical parameters of bottled wines

Effect of SO_2: the positive effect of SO_2 in protecting wine from the browning process was confirmed. Significant lower values of A420 were observed from the first month after bottling (first sampling) and the differences between "–SO_2" and "+SO_2" trials increased during the storage period (Table 4.3.7.).

The other color parameters were also influenced by the content of SO_2, the higher the SO_2, the higher the L* and the lower the h* and c*. The differences were almost always significant and increased during the aging period.

	bottle aging											
	1 month			3 months			8 months			12 months		
	-SO_2	+SO_2	F value	-SO_2	+SO_2	F value	-SO_2	+SO_2	F value	-SO_2	+SO_2	F value
Free SO2 (mg/L)	11,1	24,63	***	4,1	20	***	3,68	18,84	***	1,72	15,28	***
Total SO2 (mg/L)	42,49	71,11	***	30,1	62,2	***	30,24	56,02	***	27,7	63,72	***
p-DACA (mg/L)	5,7	5,69	n.s.	5,79	5,85	n.s.	5,13	5,27	n.s.	5,27	5,89	**
Total polifenols (mg/L)	66,86	67,1	n.s.	73,5	69,1	*	57,4	57,06	n.s.	59,49	60,23	n.s.
A420	0,041	0,036	***	0,047	0,041	***	0,058	0,046	***	0,066	0,046	***
L	99,51	99,67	*	99,47	99,64	n.s.	99,12	99,42	**	99,06	99,58	**
h	-1,35	-1,3	n.s.	-1,36	-1,31	*	-1,37	-1,33	*	-1,37	-1,32	***
c	2,93	2,65	*	3,19	2,78	*	4,18	3,38	***	4,78	3,44	***
GSH	8,8	10,8	n.s.	8,8	10,8	n.s.	0,5	0,71	n.s.	nd	nd	-

Table 4.3.7.: evolution of the chemical-physical parameters during bottle aging. Effect of different levels of SO_2 at bottling. ANOVA results. *;**;*** and ns indicate differences at P≥ 95%; 99%; 99.9% and not significant respectively

The content of HCTA was studied from the first month of bottling. The Cortese used in this experiment had an amount of HCTA very low also compared to the characteristic of the cultivar. For example, the content of t-Caftaric acid was less than a quarter compared to the Cortese of the first trial (Table 4.3.1. and 4.3.8.) During the aging period in bottle the amount of HCTA remained nearly costant in all the samples (Table 4.3.8.). However, statistically significant differences between samples were observed in some case. These differences were more due to an excellent repeatability of the analysis which determined very low values of the variance of error (high F values) than to the real differences between the trials and do not have any practical interest.

Time	HCTAmg/L	-SO2	+SO2	F-value	-GSH	+GSH	F-value	-Tannins	+Tannins	F-value
1 month	t-Caftaric acid	10,57	10,54	0,29 ns	10,63	10,48	4,81 ns	10,58	10,53	0,74 ns
	c-Coutaric acid	0,64	0,62	1,5 ns	0,65	0,61	3,42 ns	0,64	0,62	1,08 ns
	t-Coutaric acid	0,34	0,36	0,11 ns	0,34	0,36	0,44 ns	0,35	0,35	0,29 ns
	GRP	6,22	6,11	8,24 *	6,19	6,14	1,8 ns	6,19	6,14	2,15 ns
	c+t Fertaric acid	1,56	1,58	0,1 ns	1,62	1,52	2,55 ns	1,62	1,52	2,26 ns
3 months	t-Caftaric acid	11,56	10,99	0,56 ns	10,85	11,69	1,2 ns	11,18	11,37	0,06 ns
	c-Coutaric acid	0,62	0,56	10,19 *	0,6	0,58	1,96 ns	0,61	0,57	4,55 ns
	t-Coutaric acid	0,38	0,38	0,003ns	0,4	0,36	1,43 ns	0,42	0,34	7,7 *
	GRP	6,05	5,86	13,28**	5,99	5,9	1,93 ns	6,05	5,86	12,24**
	c+t Fertaric acid	1,68	1,58	1,66 ns	1,7	1,56	3,34 ns	1,63	1,63	0,006 ns
8 months	t-Caftaric acid	10,74	10,58	29,44**	10,67	10,65	0,46 ns	10,69	10,63	4,18 ns
	c-Coutaric acid	0,56	0,65	108***	0,6	0,61	1,14 ns	0,6	0,61	0,55 ns
	t-Coutaric acid	0,64	0,45	173,4***	0,57	0,53	7,49 *	0,55	0,54	0,77 ns
	GRP	5,71	5,77	4,18 ns	5,8	5,7	8,93 *	5,7	5,77	3,14 ns
	c+t Fertaric acid	1,54	1,54	5,17 ns	1,6	1,59	0,17 ns	1,59	1,61	0,23 ns

Table 4.3.8.: Content of HCTA in wines during bottle aging. Effect of SO_2, GSH and gallotannins. ANOVA results. *;**;*** and ns indicate differences at P≥ 95%; 99%; 99.9% and not significant respectively.

According to the previous results, no significant effect of SO_2 in reducing GSH losses was observed. (Table 4.3.9.)

The accelerated browning test performed 1 and 8 months after bottling to verify the resistance to browning, showed a protective effect of SO_2 8 months after bottling: "+SO_2" samples presented lower levels of A420.

	SO2			GSH			Gallic tannins		
	-SO2	+SO2	Sign	-GSH	+GSH	Sign	-Tannins	+Tannins	Sign
1 month	0,03	0,03	ns	0,026	0,033	**	0,028	0,037	ns
8 month	0,05	0,036	*	0,036	0,05	*	0,04	0,04	ns

Table 4.3.9.: results of the accelerated browning test in wines after 1 and 8 months of bottle aging. ANOVA results. *;** and ns indicate differences at P≥ 95%; 99%; and not significant respectively

Effect of GSH: Figure 4.3.16. shows the trend of free SO_2 consumption related to the presence or the absence of GSH. In the previous experiments no effect of GSH was observed, instead in this last trial a significant increase in the consumption of free SO_2 during the first month for +GSH samples and a reduction of SO_2 consumption was observed in the last period of bottling. The positive effect of GSH in reducing free SO_2 losses remained significant until 8 months after bottling. The kinetic of the consumption of total SO_2 followed the trend of free SO_2, but significant differences can be observed only after 8 months of bottle aging.

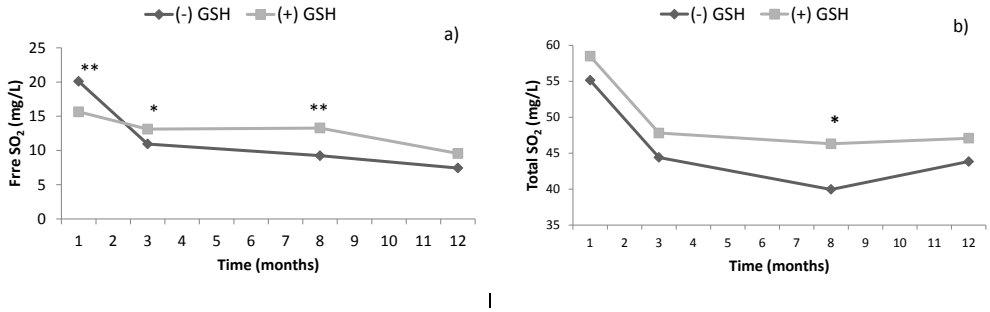


Figure 4.3.16.: Kinetic of the consumption of free (a) and total SO_2 (b) during bottle aging in relation with the GSH additions. * and ** indicate differences at P≥ 95% and 99%; respectively.

As observed in the previous experiments, the GSH was not able to reduce the wine browning during bottle aging, differently from SO_2. The trend of the A420 parameter during aging was the same in the"+GSH" and "–GSH" samples, except for 8 months after bottling, when the wines enriched with GSH presented lower values of A420 parameter than the "-GSH" samples (Figure 4.3.17.). However, these difference do not have any practical interest.

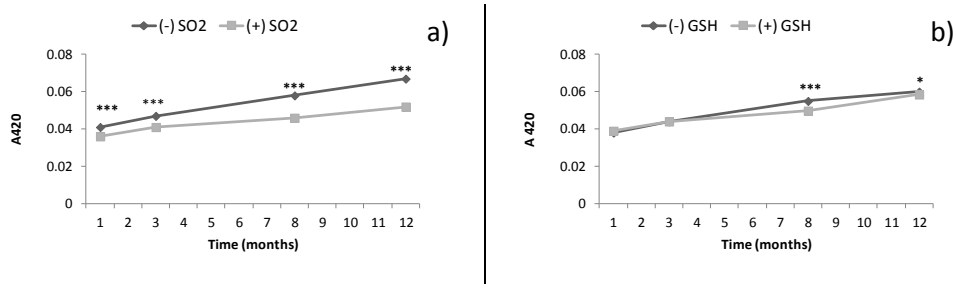


Figure 4.3.17.: Evolution of wine color during bottle aging in 135 mL bottles. Effect of SO_2 (a) and GSH (b). * and*** indicate differences at P≥ 95 and 99.9% respectively

The results of the accelerated browning test showed that the GSH increased the risk of a wine to browning during aging. Effectively, +GSH samples showed higher A420 values compared to –GSH samples after a storage of 6 days at 50°C (Table 4.3.9.)

During bottle aging, the GSH added to wines decreased in all the trials; in this experiment the losses were slower compared to the previous ones. After 1 month of bottling, the average content of GSH in +GSH samples was still 16.9 mg/L. As already observed in the previous works, SO_2 did not have any effect in protecting GSH losses.

The slower rate of GSH losses can be related to the matrix: this Cortese was effectively poor in phenols content.

Effect of gallotannins: no noteworthy effect was observed as regards gallotannins on the studied parameters except for an increase in polyphenols content in +tannins samples due to the additions.

An increase in the rate of the SO_2 consumption was observed only after 1 month of bottling in "+Tannins" sample, then the differences disappeared (Table 4.3.10.).

	bottle aging											
	1 month			3 months			8 months			12 months		
	-Tannins	+Tannins	Sign	-Tannins	+Tannins	Sign	-Tannins	+Tannins	Sign	-Tannins	+Tannins	Sign
Free SO2 (mg/L)	19,71	16,02	**	11,6	12,48	n.s.	10,88	11,64	n.s.	8,4	8,6	n.s.
Total SO2 (mg/L)	54,76	58,84	n.s.	47,36	44,88	n.s.	42,94	43,32	n.s.	43	47,92	*
p-DACA (mg/L)	5,7	5,7	n.s.	5,73	5,92	*	5,18	5,22	n.s.	5,54	5,63	n.s.
Total polifenols (mg/L)	59,46	74,46	***	64,9	77,62	***	55,96	58,5	n.s.	54,84	64,88	***
A420	0,038	0,039	n.s.	0,04	0,04	n.s.	0,053	0,051	*	0,057	0,055	n.s.
L	99,62	99,56	n.s.	99,56	99,56	n.s.	99,2	99,34	n.s.	99,3	99,33	n.s.
h	-1,32	-1,33	n.s.	-1,33	-1,33	n.s.	-1,36	-1,34	n.s.	-1,35	-1,34	n.s.
c	2,78	2,8	n.s.	2,97	3	n.s.	3,86	3,7	n.s.	4,25	3,98	**
GSH	8,9	9,99	n.s.	3,12	2,9	n.s.	0,46	0,75	n.s.	nd	nd	-

Table4.3.10.: Average values of the main chemical-physical parameters in the trials with different tannin conent ("+tannins" and "-tannins" trials. ANOVA results. *;**;*** and ns indicate differences at P≥ 95%; 99%; 99.9% and not significant respectively

Chemical-physical composition 15 months after bottling: table 4.3.11. shows the chemical-physical composition of wines stored in 750 mL bottles for 15 months at 20°C. The amount of SO_2 at bottling has influenced the evolution of wine color: "+SO_2" samples were distinguished from the –SO_2 samples for the significant lower values of A420, h*, c* and for the higher value of L*. No influences were observed as regards the other studied parameters.

The addition of GSH at bottling caused a significant increase in L*, h* and c* parameters.

As regards gallotannins, the only significant difference, except the higher value of total polyphenols, consisted of an increase of L* in presence of tannins.

	-SO2	+SO2	Sign	-GSH	+GSH	Sign	-Tannins	+Tannins	Sign
Free SO2 (mg/L)	1,72	8,8	***	4,72	5,8	n.s.	4,68	5,84	n.s.
Total SO2 (mg/L)	22,36	55,16	***	36,24	41,28	n.s.	35,24	42,28	n.s.
Total polifenols (mg/L)	60,94	59,74	n.s.	60,99	59,68	n.s.	56,83	63,85	***
A420	0,072	0,052	***	0,06	0,06	n.s.	0,06	0,06	n.s.
L	99,08	99,55	***	99,25	99,38	**	99,27	99,36	*
h	-1,38	-1,32	***	-1,32	-1,36	**	-1,35	-1,35	n.s.
c	5,39	3,97	***	3,97	4,61	n.s.	4,67	4,69	n.s.
Volatile acidity (mg/L)	0,15	0,15	n.s.	0,15	0,16	n.s.	0,16	0,14	n.s.
Acetaldehyde (mg/L)	17,5	18	n.s.	17,9	17,5	n.s.	18,2	17,3	n.s.

Table 4.3.11.: Chemical-physical composition of wine after 15 months of bottle aging. Influence of SO_2, GSH, and Gallotannins. ANOVA results. *;**;*** and ns indicate differences at P≥ 95%; 99%; 99.9% and not significant respectively

Sensory analysis: the sensory analysis confirmed the positive role of SO_2 in protecting wine during aging, according to the results of chemical-physical analysis. The average sensory profile for the $+SO_2$ and $–SO_2$ samples is reported in Figure 4.3.18.

In table 4.3.12. are reported the ANOVA results and the average sensory scores of the descriptors related to the different amounts of the studied antioxidants ("SO_2", "GSH", "gallic tannins").

The samples with a higher level of SO_2 at bottling were described as significantly less colored and with more intense notes of lemon and acacia flowers (freshness descriptors) compared to the $–SO_2$ samples. Furthermore, the $+SO_2$ samples resulted statistically different from the $–SO_2$ samples for lower intense notes related to oxidative evolution: acetaldehyde, licorice, walnut, green beans. No differences were observed between the two samples for taste descriptors.

The different amounts of GSH at bottling influenced only the descriptor pineapple evaluated significantly more intense in -GSH samples, and licorice judged significantly more intense in +GSH samples (table 4.3.12). No effect was observed as regards the content of tannins.

	-SO_2	+SO_2	Sign.	-GSH	+GSH	Sign.	-Tannins	+Tannins	Sign.
Straw yellow	49,79	39,61	***	45,05	44,39	ns	45	44,45	ns
Acacia flower	28,13	34,32	***	31,12	31,29	ns	31,17	31,24	ns
Lemon	21,92	31,6	***	25,62	27,85	ns	25,8	27,67	ns
Pinepple	24,71	27,75	ns	27,89	24,57	*	26,09	26,36	ns
Golden apple	35,67	33,8	ns	35,14	34,34	ns	33,61	35,86	ns
Acetaldehyde	45,85	32,48	***	39	39,28	ns	40,96	37,44	ns
Honey	30,6	28,19	ns	30,69	28,12	ns	30,21	28,6	ns
Licorice	24,12	19,61	***	19,84	22,9	*	20,95	21,79	ns
Walnut	29,21	23,26	***	25,56	26,93	ns	26,85	25,65	ns
Green beans	21,52	16,2	***	18,56	19,18	ns	18,86	18,88	ns
Hay/Straw	29,35	28,32	ns	29,38	28,3	ns	29,14	28,54	ns
Acidity	43,88	43,51	ns	43,89	43,51	ns	43,37	44,03	ns
Bitter	26,88	25,73	ns	26,74	25,88	ns	25,82	26,79	ns
Softness	41,79	42,6	ns	41,27	43,11	ns	42,62	41,77	ns
Structure	42,61	43,35	ns	43,13	42,83	ns	43,23	42,72	ns

Figure 4.3.12.: Average scores of the sensory descriptors in relation to the different amounts of the studied antioxidants: SO_2, GSH and gallotannins. ANOVA results. *;*** and ns indicate differences at P≥ 95%; 99.9% and not significant respectively

A complete ANOVA for the factors "wine", "tasting session" and "taster" was done to study the behavior of the panel, that means the repeatability in the judgments for each descriptors and the relative agreement between the tasters. For this purpose, the interactions of I level between the three factors were studied (table 4.3.13). The presence of significative interactions was an index of low consistency of the descriptor. As reported in the table, the most solid descriptors, that is, the ones for which the panel was more consistent with their judgments (no significant interactions), were: acacia flowers, lemon, golden apple, walnut as regards the odor and acidity, bitter and softness as regards the taste. For each descriptors, statistically significant differences between the tasters were also observed. These differences are due to a different use of the scale by the tasters but they do not influence the ANOVA results.

	Taster	Wine	Session	Taster*Session	Taster*Wine	Wine*Session
Straw yellow	***	***	*	***	*	ns
Acacia flowers	***	ns	*	ns	ns	ns
Lemon	***	***	*	ns	ns	ns
Pinepple	***	ns	***	*	*	ns
Golden apple	***	ns	ns	ns	ns	ns
Acetaldehyde	***	***	ns	*	*	ns
Honey	***	ns	ns	*	ns	ns
Licorice	***	**	ns	*	ns	*
Walnut	***	**	ns	ns	ns	ns
Green beans	***	**	**	*	ns	ns
Hay/Straw	***	ns	*	**	ns	**
Acidity	***	ns	ns	ns	ns	ns
Bitter	***	ns	*	ns	ns	ns
Softness	***	ns	ns	ns	ns	ns
Structure	***	ns	ns	*	ns	ns

Table 4.3.12.: Interactions between tasting session and taster, taster and wine, wine and tasting session. ANOVA results. *;**;*** and ns indicate differences at p≥ 95%; 99%; 99.9% and not significant respectively

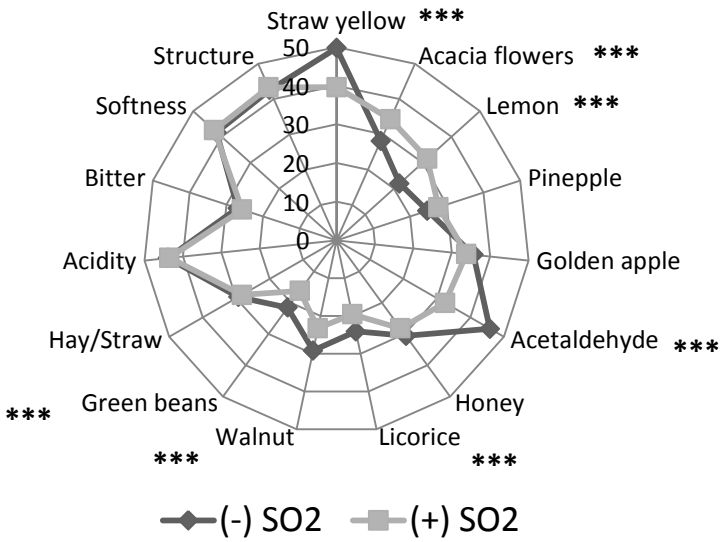


Fig 4.3.18.: Sensorial profile of Cortse wine 15 month after bottling. Influence of different amounts of SO_2 at bottling.

4.4. Discussion

The work regarded the study of the evolution of physical-chemical composition and sensory characteristics of three different white wines from the Cortese cultivar, during bottle aging.

The effect of antioxidant molecules, GSH or ellagitannins, both at a dose of 20 g/hL as partial substituents of SO_2 (1st experience, paragraph 4.3.1.) was investigated in wines from organic grapes with low (3.35 mg/L) and medium-low (24.8 mg/L) levels of SO_2 at bottling. It was then studied the role of GSH (2nd and 3rd experience, paragraphs 4.3.2. and 4.3.3. respectively) and gallotannins (3rd experience) on the protection against oxidation of wines with doses of SO_2 equal to those of commercial use (medium-high and high). Moreover, during the 2nd experiment, it was studied the effect of two different level of oxygen at bottling: medium and high (different bottling conditions).

The results of both chemical and sensory analysis highlighted the positive and irreplaceable role of SO_2 in protecting white wines from oxidative aging during storage in bottles.

No effect on the natural oxidative evolution, and in particular on the color browning and on the appearance of olfactory notes of oxidized, was observed either for GSH or for tannins in any trial. The chemical data were in agreement with the results of sensory evaluation.

When the content of SO_2 at bottling were low and medium-low (1st experience), the oxidative notes were significantly perceived from 3 months after bottling, regardless of the presence of GSH or ellagitannins.

The SO_2 plays several interesting roles on the oxidation process: it causes the acceleration of the rate of oxygen consumption during chemical oxidations (paragraphs 3.3.; 4.3.2.; 4.3.3.) and a slowdown in the rate of oxygen consumption during enzymatic oxidations (presence of residual laccase activity in wines) (paragraph 4.3.1.), moreover SO_2 reduces the color intensity of white wines and controls the process of browning during bottle aging.

The increase in the rate of oxygen consumption during the reaction is the consequence of the acceleration of phenols oxidation, which is due to the reduction of quinones back to phenols, or to the formation of additional compounds with quinones or to the consumption of hydrogen peroxide. The effect is clear only in the presence of free SO_2, when the content of free SO_2 decreases, the rate slows down regardless of the composition of the mean (paragraph 3.3).

On the other hand, the GSH increased the rate of oxygen consumption only in one case (2nd trial, paragraph 4.3.2.). The GSH is a nucleophilic molecule, able to form additional compounds with quinones produced from phenols. This mechanism has been studied for a long time in

musts and only lately in wines.

The mechanism of action supposed for GSH is the same as for free SO_2: GSH reacts with hydrogen peroxide and with quinones reconverting them back to the phenols. Even so, the data collected suggests that its reactivity is still negligible compared to that of SO_2, since there was no significant effect of GSH on the color intensity and on the oxidative browning of wines during bottle aging .

Finally, no effect on the rate of oxygen consumption was observed after the addition of tannins. These results are in contrast with those reported in other studies (Danilewicz *et al*., 2008) where it was observed an increase in the rate of oxygen consumption proportional to the amount of added tannins. The main cause of this different behavior may be linked to the amount of oxygen present in the mean at the moment of the tannins addition. In all the experiments the addition occurred at bottling and the wine during bottle storage generally undergoes lower oxygen uptakes (nano-oxygenation) compared to the preceding phases of winemaking.

As already reported in the previous paragrphs, the role of tannins in the Fenton reaction varies depending on the amount of dissolved oxygen in the wine. In the presence of high levels of oxygen, tannins allow to regenerate the FeII from FeIII and accelerate the reaction of formation of the hydroxyl radical ($HO^{\bullet}$). The $HO^{\bullet}$ is known to be responsible for the oxidation of some alcohols into aldehydes or ketones, first of all of ethanol to acetaldehyde. The increase in the production of acetaldehyde after the addition of tannins, was observed only during the 1st experiment, when the amount of oxygen was higher than the third trial where this increase was not detected. These different results could not be considered dependent either on the type of tannins used (ellagitannins in the first case and gallotannins in the second one) or on the dose (even double for gallotannins than the ellagitannins), but rather on the content in dissolved oxygen in the wine. Effectively, during the first experiment the non optimal bottling conditions and the kind of stopper (cork closures in 1st trial and synthetic stopper in third trial) caused both a higher uptake of oxygen at bottling and during storage.

However, the ability to cause an increase in the rate of consumption of oxygen could not be used as an indicator of the effectiveness of an antioxidant molecule.

An increase in the rate of the consumption of both free and total SO_2 was observed after the addition of tannins. This means that, when tannins are added to wine it is recommended to increase the amount of free SO_2, as is done for ascorbic acid, at bottling in order to protect wine.

On the contrary, the addition of GSH did not increase the SO_2 consumption and this could be

due to the fact that GSH, unlike tanninns and ascorbic acid, does not cause an increase in the production of hydrogen peroxide, which oxidized SO_2 to sulphates.

This should be a necessary characteristic for an additive to be used in place of SO_2.

The different levels of SO_2 used in the dfferent trials did not have any protective effect against GSH losses. The GSH decreased rapidly and in relation to the composition of the mean, probably to the polyphenols content (faster in the 1st and 2nd experiements compared to the 3rd experiment).

The study of the evolution of the polyphenolic component of wines, determined by spectrophotometry with the reaction of Folin Ciocalteu (total polyphenols) and with the p-DACA (catechins and flavans low MW) and HPLC, during the storage, showed that this was stable over time, only slightly influenced by the factors studied.

As regards the content of HCTA, it decreased during bottle aging in the first trial, while it remained stable in the 3rd trial. Even during a previous experiment of conservation in the bottle carried out on wine Montepulciano (Guaita *et al*., 2013) it was observed a reduction of the content in HCTA over time. In that experiment the losses were independent of the oxygen content dissolved in wine (comparison between closures with different oxygen permeability), but probably were due to hydrolysis of HCTA, in particular the hydrolysis of caftaric acid to the respective caffeic acid. In the present work, the evolution of HCTA, was observed only in the Cortese with the higher amounts of it and was not related to the oxidation process (different content of SO_2). These results seem to confirm that the decrease of HCTA during storage can depend on the chemical reactions of hydrolysis, the importance of which depends on the concentration of reagents. This should prove the scarce role of HCTA in the chemical oxidations of the wine during bottle aging, differently from what happens during the enzymatic oxidation of musts.

The uptake of oxygen at bottling (bottling conditions) and thus the amount of dissolved oxygen in the bottle represents a factor that affects in an important way the rate of consumption of free and total SO_2. In the 2nd experiment we observed how an increase of only 1.5 mg/L of dissolved oxygen could significantly affect the amount of free SO_2 present in wine after 1 year of bottle aging and thus influence the further preservation of wine over time.

5. Conclusions

The results of this thesis on one hand confirmed the key role of SO_2 in prolonging the shelf life of bottled wines and in preserving their organoleptic characteristics, but on the other hand showed the low effectiveness of the other studied additives (GSH and tannins) in preserving wines.

The amount of SO_2 to be added to the wine at bottling, must be sufficient to react with oxygen which enters into the bottle during the bottling phases and during the storage (type of closure used). Neither tannins nor GSH can be used as a complete or part substitute to SO_2, at least at the tested amounts (20 g/L for ellagitannins and GSH and 40 g/L for gallotannins).

On a practical point of view, to date the only way to extend the shelf life of wines without adding too high an amount of SO_2 at bottling, is to control the oxygen uptake in bottled wine, for example by improving the bottling systems to limit the amount of oxygen that enters in the bottle and is dissolved in the wine. Furthermore, it is important to lead the winemakers to the correct choice of closures, since different kinds of closures perform differently regarding their oxygen permeability (OTR = Oxygen transfer rate).

Finally, since the free SO_2 takes part directly to the oxidative process, the reduction of the doses of total SO_2 depends on the possibility to limit, during winemaking, the production of molecules that can combine free SO_2.

5. Study of the Fenton reaction in a model solution

5.1. Introduction

It is known that the poor reactivity of molecular oxygen with organic molecules is overcome by the generation of reactive oxygen species (ROS) that constitute a reductive ladder of oxidation (Figure 5.1.1.)

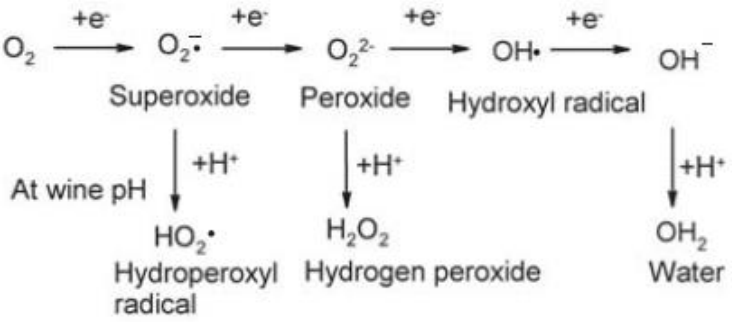


Figure 5.1.1.: Ladder of oxygen reduction (Waterhouse & Laurie, 2006)

A key role in the oxidative chain is played by metals. The first step is the transfer of an electron from transition metal ions, such as Fe^{2+} or Cu^{+}, to molecular oxygen. This reduction leads to formation of superoxide ion $^{\cdot}O^{-}_{2}$, which at wine pH exists as the hydroperoxide radical ($^{\cdot}OOH$).
The transfer of a second electron would then produce a peroxide ion (O^{2-}_{2}), which at wine pH exists as hydrogen peroxide (H_2O_2).
The reduction of oxygen to hydroperoxide radical needs an high activation energy, which is supplied by transition metals (Danilewicz, 2003).
Considering the reduction potential and the capacity to react directly with phenols, the hydroperoxide is an oxidant much stronger than oxygen (Danilewicz, 2003).
The further reduction, called Fenton reaction, occurring between hydrogen peroxide and ferrous ions, produces an oxidative agent even more reactive than the previous one, namely the hydroxyl radical ($^{\cdot}OH$) and ferric ions (Figure 5.1.2.) (Green & Hill 1984; Boulton, 2003; Danilewicz, 2003; Waterhouse & Laurie 2006).
The final product of oxygen reduction is water.
The iron reactions described require the ferrous form of iron and yield ferric iron. The ferric iron is reduced back to ferrous by phenols.
The other product of this reaction is quinone, which provides electrophiles for the reaction

described.

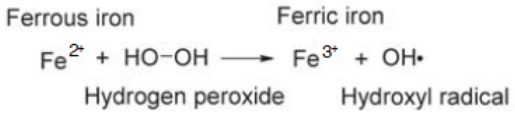


Figure 5.1.2.: Fenton reaction

The hydroxyl radical is an highly unstable radical which reacts almost immediately. Therefore, it does not react selectively with antioxidants, such as phenolics, but reacts with all the hydroxylic saturated compounds present in solution in proportion to their concentration, first of all ethanol to give acetaldehyde and organic acid to yield keto acids (Figure 5.1.3.)

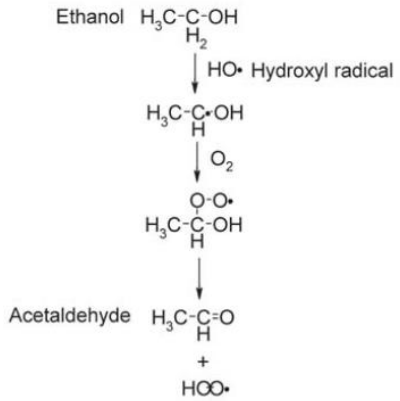


Figure 5.1.3.: Oxidation of ethanol by hydroxyl radical (Waterhouse & Ellias, 2006)

However, the fate of H_2O_2 is dependent on several factors. In wine (acidic conditions), and in presence of excess SO_2, hydroperoxyl radical appears to react quickly and irreversibly by a two electron reaction (nonradical) with bisulfite ions (HSO_3^-) to produce sulfate (HSO_4^-/H_2SO_4) and water. The H_2O_2 is then subtracted from the Fenton reaction and the organic fraction of wines remains protected (Figure 5.1.4.)

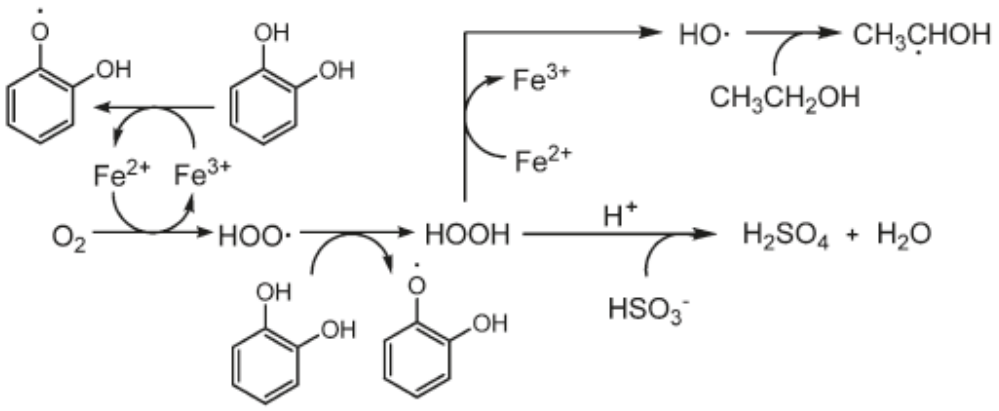


Figure 5.1.4.: Proposed metal-catalyzed wine oxidation scheme and role of SO_2 (Elias & Waterhouse 2010)

Therefore, in wine, in presence of H_2O_2, SO_2 and transition metals, the fate of H_2O_2 depends on the competition between SO_2/H_2O_2 and Fe^{2+}/H_2O_2 reactions. The prevalence of the second one leads to the oxidation of the main compounds of wine.

The rate of the Fenton reaction is influenced by several parameters: the amount of compounds capable of complexing metal ions (organic acids, polyphenols) and thus reducing its reactivity with H_2O_2 (Voelker et al., 1996), the pH of the wine which can influence the rate of SO_2/ H_2O_2 reaction (McArdle et al., 1983) and thus the amount of H_2O_2 available to the Fenton reaction, the dissolved oxygen (Voelker et al., 1996).

Furthermore, the relative concentrations of H_2O_2 and SO_2 in wine are dynamic and related to several factors.

The formation of H_2O_2 in wine at any given time is a function of the rate at which hydroperoxyl radicals react with polyphenols; the kinetics of this reaction depends on several factors: the composition of the polyphenolic fraction, pH, temperature, amount of dissolved oxygen. Furthermore, the concentration of available HSO_3 ions is dynamic in wine and is known to decrease steadily as it is consumed by H_2O_2 and/or trapped by quinones (Boulton et al., 1996). Reduced glutathione, like SO_2 is a reducing agent stronger than polyphenols. Previous works employing cyclic voltametry (Makhotika et al 2009) verified that when GSH is added to model solutions containing polyphenols, its behavior is similar to SO_2. The same authors hypothesized that GSH, besides reacting with o-quinones (nucleophilic addition), can be oxidized to glutathione disulfite (GSSG) through a reaction coupled with the reduction of o-quinones to catechol, as for SO_2.

Furthermore, Okuta et al. (1999), hypothesized that GSH can remove hydrogen peroxide and other peroxides and/or radicals from musts and wines by producing GSSG, though the effect of GSH against white wines browning seems to be less important than the effect of SO_2.

5.2. Aim of the work

To date no efficiency of GSH added at bottling to white wines was observed to prevent wine oxidation during aging period in any trials carried out in our laboratory.

An hypothesis could be that, since the molecule of GSH is much heavier than the molecule of metabisulphite, the addition of the same amount in mg/L of the two molecules means a lower molar concentration of GSH compared to metabisulfite.

The effect of the SO_2 and the GSH, used at the same molarity, on the Fenton reaction was compared in this study by measuring the amount of acetaldehyde produced at the end of the Fenton reaction.

5.3. Materials and methods

A model wine consisting of 12% v/v ethanol, 53 mM tartaric acid and adjusted to pH 3.6 with NaOH 6N was prepared and used for all the experiments. The stock solutions of Fe(II), hydrogen peroxide, SO_2 and GSH at different concentrations according to the experimental plan reported below, were prepared immediately before the use in deoxygenated water. The Fe(II) and the SO2 were introduced to the reaction flask by syringe 1 min before the start of the reaction. It is important to add the Fe(II) in advance to prevent the production of complex acid-iron or catechol-iron that can affect the reaction rates and thus the results. In all cases, the reactions started upon the addition of H_2O_2.

The experiments were carried out both in the presence and in the absence of oxygen, in particular under aerial, air-saturated and anoxic conditions. The air saturated conditions were obtained by decanting the solution from tank to tank, while the anoxic conditions were obtained by purging the model solution with ultrahigh-purity nitrogen gas for 10 min.

The temperature of the solution was keep constant at 20°C for the length of the experiment.

The antioxidant power of SO_2 and GSH was monitored by measuring the acetaldehyde produced at the end of the experiment. The method used to measure acetaldehyde is reported at the paragraph 3.2.

The duration of the experiment was 10 minutes: at the end of this time all the aceltaldehyde was produced (Ellias and Waterhouse 2010)

5.4.Experimental plans and results

First experiment. The first experiment regarded the study of the effect of SO_2 added at three different doses 10, 20, 30 mg/L on the Fenton reaction (amount of aceyaldehyde produced) compared to a sample without SO_2 (control). The experience was carried out under air using a model solution prepared as described at paragraph 5.3. and with 180 µM/L of Fe^{2+} and 300 µM/L of hydrogen peroxide. Since this trial was carried out without using catechol, an higher amount of Fe^{2+} was used compared to the amount used by Waterhouse: 180 µM against 50 µM in order to avoid the risk that a low quantity of ferrous ions could become a limiting factor in the acetaldehyde yield . In fact, normally, the Fe^{2+} is regenerated by catechol.

		Additions		Acetaldehyde	
		µM	mg/L		
oxygen	Fe^{2+}	180	10	**µM**	mg/L
	H_2O_2	300	10,2		
	SO_2	**0**	0	**250**	11,04
		156	10	**136**	5,98
		312	20	**115**	5,07
		469	30	**11**	0,5

Table 2.4.1.: Experimental plan and relative results of the first trial

Results: the results of this first trial showed a significant reduction in the amount of acetaldehyde produced during the Fenton reaction in samples with SO_2. The effect was already observed for the addition of 10 mg/L (156 µM) of SO_2 and it increased with the rising of SO_2 levels. However, the effect of SO_2 in cutting down the acetaldehyde production was not linear to the the amount of added SO_2: 10 and 20 mg/L showed a similar reduction, while 30 mg/L had a greater effect (Figure 5.4.1.).

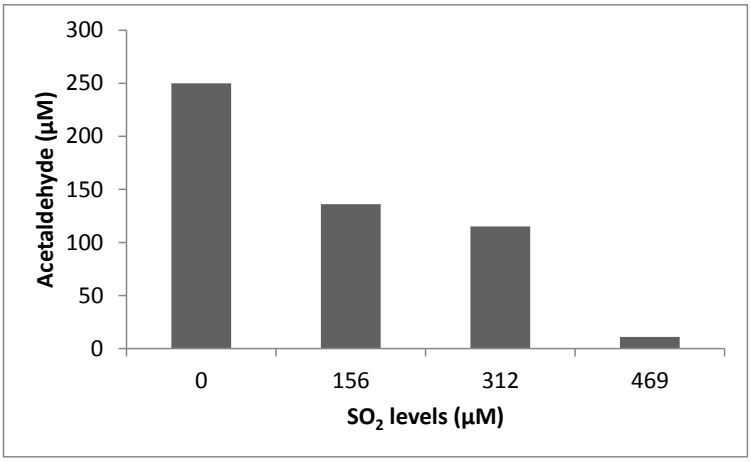


Figure 5.4.1.: Acetaldehyde produced during the Fenton reaction. Effect of the different amounts of SO_2

Second experiment: the second experiment was carried out in anoxic conditions (paragraph 5.3.). The amount of ferric ions used in this trial was higher than in the previous one and the same amount of hydrogen peroxide was used (Table 5.4.2.) The comparison was between the trials with 0, 15, 30 µM of SO_2, studying the effect on the acetaldehyde production.

	Additions			**Acetaldehyde**	
		µM	mg/L		
anoxic	Fe^{2+}	300	16,8	**µM**	mg/L
	H_2O_2	300	10,2		
	SO_2	**0**	0	**246**	10,84
		234	15	**249**	10,99
		469	30	**77**	3,45

Table 3.4.2.: Experimental plan and relative results of the second trial

Results: The amount of acetaldehyde produced in the absence of SO_2 was similar to the one observed in the first trial (246 µM and 250 µM). This result was in contrast with what is reported in literature (Ellias & Waterhouse 2010) where, in presence of oxygen, the production of acetaldehyde resulted to be limited by the initial content of ferrous ions, since they were not regenerated in that conditions. On the contrary, a significantly higher yield of acetaldehyde was observed in deoxygenated systems. In anoxic conditions it seems that the Fe^{3+} produced by the Fenton reaction was quickly reduced to Fe^{2+}, which would explain why Fe^{2+} is apparently not

limiting in this system. It is likely that in anoxic condition, in the absence of catechol, the 1-hydroxethyl radical is capable to rapidly reducing Fe^{3+} to Fe^{2+}.

The different result of the present work, could be due to the fact that, regardless of Waterhouse, the first trial was carried out under aerial conditions and not in a system saturated with oxygen, moreover the anoxic conditions were not so strict as in Warerhouse work. So, the differences between the 1st and the 2nd trial as regards the oxygen content are lower compared to the Waterhouse experiments. However, in the first trial, the production of acetaldehyde was not limited by the amount of ferrous ions. The ferrous was added at 180 µM and the acetaldehyde produced was 250. Probably a partial regeneration of ferrous ions occurs also even in the absence of catechol.

No effect of SO_2 added at 15 mg/L was observed in reducing the production of acetaldehyde, regardless of the previous trial. This result probably was due to the different composition of the mean between the two trials, in particular to the different amount of Fe^{2+}: in this second trial the dose of Fe^{2+} was higher compared to the 1st trial: 300 µM against 180 µM. The higher amount of ferrous ions can cause an increase in the competition between ferrous ions and SO_2 for the hydrogen peroxide. This competition can also explain the lower effect in reducing the concentration of acetaldehyde observed for the SO_2 at 30 mg/L compared to the previous trial (70 µM was the amount of acetaldehyde produced in the second trial and 11 µM in the first). The production of acetaldehyde is thus also influenced by the competition between the reactions SO_2/H_2O_2 and $Fe(II)/H_2O_2$.

Third experiment: in this experiment the effect of SO_2 on controlling the Fenton reaction was compared to GSH. The experiment was carried out under aerial conditions and two different amount of Fe^{2+} were used: 50 µM and 300 µM (Table 5.4.3.)

oxygen

Additions			**Acetaldehyde**	
	µM	mg/L		
Fe^{2+}	300	10	**µM**	mg/L
H_2O_2	300	10,2		
SO_2	**0**	0	**207**	9,13
	234	15	**195**	8,59
	469	30	**23,5**	1,04
GSH	**469**	144	**225**	9,89

	Additions		Acetaldehyde	
	µM	mg/L		
Fe^{2+}	50	2,8	**µM**	mg/L
H_2O_2	300	10,2		
SO_2	**0**	0	**225**	11,23
	469	30	**63**	2,77
GSH	**469**	144	**179**	7,91

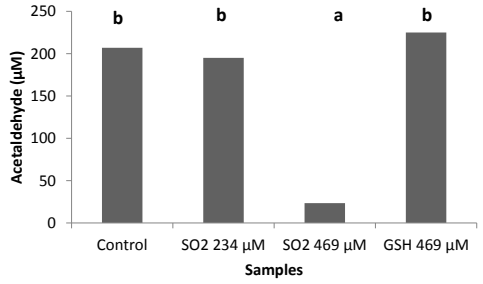
oxygen

Table 5.4.3.: Experimntal plan and results of the third trial

Results: the production of acetaldehyde in the two trials without SO_2 was similar and always lower than the molar concentration of hydrogen peroxide (respectively 207 and 225 µM compared to 300 µM of hydrogen peroxide). The Fe^{2+} did not result a limiting factor for the formation of acetaldehyde, also in the absence of catechol. A significant reduction in the production of acetaldehyde was noticed for trial with SO_2 at 30 mg/L as alredy observed in the previus experiments. On the other hand, no effect was observed when SO_2 was added at 15 mg/L. The efficiency of SO_2 in reducing the production of acetaldehyde seemed to be influenced also in this trials by the metal content: when ferrous ions were 6 fold concentrated (300 µM in the 1st trial aginst 50 µM in the 2nd trial), the cut down in acetaldehyde production was lower (63 µM against 23.5 µM).

As regards the effect of GSH in controlling the Fenton reaction and thus reducing the production of acetaldehyde, it was observed that it was also influenced by the matrix. Effectively, no effect of GSH was observed in the 1st trial when the Fe2+ levels were 300 µM, while a significant effect was observed in the 2nd trial when the amount of ferric ions was 50 µM.

In this case the sample with GSH was statistically different either from the control or from the sample with SO_2 at 30 mg/L for the lower and the higher amount of acetaldehyde respectively (Figure 5.4.2.)



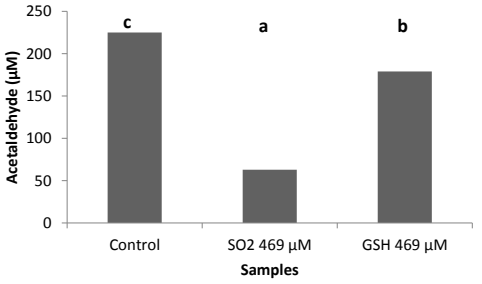


Figure 5.4.2.: Acetaldehyde produced at the end of the Fenton reaction. Effect of different levels of SO_2 and of GSH and influence of the amount of Fe2+: 300 µM (I) and 50 µM (II)

.Fourth experiment: in this experiment it was studied the influence of the presence or the absence of oxygen on the Fenton reaction. The Fe^{2+} was added at 50 µM (Table 5.4.4.)

	Additions			Acetaldehyde	
		µM	mg/L		
	Fe^{2+}	50	2,8	**µM**	mg/L
	H_2O_2	300	10,2		
oxygen	**SO_2**	**0**	0	**255**	11,23
		469	30	**63**	2,77
	GSH	**469**	144	**179**	7,91
anoxic	**SO_2**	**0**	0	**262**	11,52
		469	30	**46**	2,03
	GSH	**469**	144	**161**	7,11

Table 5.4.4.: Experimental plan and results of the fourth trial

Results: no statistically differences were noticed in the production of acetaldehyde for the presence or the absence of oxygen, as already observed in the previous trials. It is important to take into consideration that the differences in oxygen content were higher in the work carried out by Ellias and Waterhouse, compared to this study. The comparison between the effectiveness of GSH and of SO_2 confirmed the lower efficiency of GSH in controlling the Fenton reaction as already seen before.

Fifth experiment: the experiment was done using higher amount of oxygen (saturation conditions, dissolved oxygen of 7.5 ppm) and operating thus in the same conditions as Elias and Waterhouse in order to see whether the saturation of oxygen could have any influence in the studied process. The ferric ions added were 50 µM (Table 5.4.5.)

	Additions			Acetaldehyde	
oxygen 7,5 ppm		**µM**	mg/L	**µM**	mg/L
	Fe^{2+}	50	2,8		
	H_2O_2	300	10,2		
	SO_2	**0**	0	**100**	4,38
		469	30	**3**	0,14
	GSH	**469**	144	**177**	7,77

Table 5.4.5.: Experimental plan and results of the fifth trial

Results: a decrease in the production of acetaldehyde was observed in presence of high levels of oxygen, in according to what observed by Ellias and Waterhouse. The acetaldehyde produced in this conditions was 100 µM, while in presence of air was on average 240 µM.

The SO_2 at 30 mg/L had completely cut down the production of acetaldehyde, while no effect was observed for GSH.

5.5. Discussion and conclusions

The results of this experiment confirmed the key role played by SO_2 in controlling the Fenton reaction. The efficiency of SO_2 is related to both its concentration and to the composition of the matrix, in particular to the content of ferric ions which can compete with SO_2 for the consume of hydrogen peroxide at the beginning of the Fenton reaction.

The amount of oxygen present in the mean was also an influent parameter: high concentration of oxygen, next to saturation had a different influence on the Fenton reaction compared to a medium-low content of dissolved oxygen. Effectively the acetaldehyde detected at the end of the reaction was lower in the satured solution. This result was consistent with what is reported by Ellias and Waterhouse.

The GSH showed the capacity to reduce the production of acetaldehyde, but the effect was much lower than SO_2 and very variable with the composition of the mean, in particular with the content of Fe^{2+} and with the concentration of dissolved oxygen.

Since the molecular weight of GSH is nearly 5 fold higher than the molecular weight of SO_2 (307.33 GSH and 64.04 SO_2), in the best conditions, it is necessary to add to wine an amount of GSH of about 150 mg/L to obtain a result similar to the result obtained with value of free SO_2 of about 20 mg/L.

These doses are absolutely unacceptable on an enological point of view.

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Appendix 1 List of pubblications

ISI Journals, first author

Effect of reductive pressing on the content of reduced glutathione and phenols in the musts of four Italian cultivars.
S. Motta, M. Guaita, M. Petrozziello, L. Panero, A. Bosso
Lavoro accettato da American Journal of Enology and Viticulture (2014): in press

ISI journals, co-author

Analytical and sensory characterization of the aroma of "Langhe D.O.C. Nebbiolo" wines: influence of the pre-fermentative cold maceration with dry ice
M. Petrozziello, M. Guaita, **S. Motta**, L. Panero, A. Bosso.
Journal of Food Science, 2011, Vol. 76, Nr. 4, 525-534. ISSN 0022-1147.

Influence of the submerged-cap vinification on the polyphenolic composition and the volatile compounds of Barbera wines.
A. Bosso, L. Panero, M. Petrozziello, R. Follis, **S. Motta**, M. Guaita.
American Journal of Enology and Viticulture, 62:4 (2011), 503-511. ISSN 0002-9254.

Effect of the closure type on the evolution of the physical-chemical and sensory characteristics of a Montepulciano d'Abruzzo rosé wine
M. Guaita, M. Petrozziello, **S. Motta**, F. Bonello, M.C. Cravero, C. Marulli, A. Bosso
Journal of Food Science, 78:2 (2013), 160-169. ISSN 0022-1147.

Influence of matrix composition on the volatility of 4-ethylphenols and on the perception of the "Brett character" in red wines.
Petrozziello M., Asproudi A., Guaita M., Borsa D., **Motta S**., Panero L., Bosso A.
Food Chemistry 149 (2014) 197-202
On line: http://dx.doi.org/10.1016/j.foodchem.2013.10.098

Effect of SO_2, Reduced Glutathione and Ellagitannins on the Shelf Life of Bottled White Wines.
L. Panero, **S. Motta**, M. Petrozziello, M. Guaita, A. Bosso
European Food and Research Technology.

Polyaspartate − a new additive for the tartaric stabilization of wines
A. Bosso, L. Panero, M. Petrozziello, M. Sollazzo, A. Asproudi, **S. Motta**, M. Guaita
Australian Journal of Grape and Wine Research

Congresses, poster and not ISI journals

The influence of different pressing techniques on the levels of glutathione and hydroxycinnamic acids in grape juices of some white Italian cultivars
S. Motta, M. Petrozziello, M. Guaita, L. Panero, A. Bosso
Poster presentato a "Macrowine 2010 – Third International Symposium on Macromolecules and Secondary Metabolites of Grapevine and Wines", Torino, Italia, 16-18 giugno 2010.

Influenza della barrique sulla composizione aromatica dei vini.
A. Bosso, M. Petrozziello, **S. Motta**, M. Guaita
Vitenda 2011, 288-289. ISBN 978-88-86055-23-9.

L'impiego della criomacerazione per il miglioramento qualitativo dei vini rossi.
M. Guaita, **S. Motta**, L. Panero, M. Petrozziello, A. Bosso
Rivista di Viticoltura ed Enologia, n° 1-2-3, dicembre 2011. ISSN 0370-7865.

Validazione di una metodica enzimatica per la valutazione del contenuto in mannosio di vini affinati su fecce.
M. Guaita, M. Petrozziello, **S. Motta**, L. Panero, V. Buoso, A. Bosso.
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Rivista Internet di Viticoltura ed Enologia, 2011, 12/1 (www.infowine.com). ISSN 1826-1590.

The effect of carboxymethylcellulose on tartaric and color stability of red wines.
S. Motta, M. Bertè, M. Guaita, L. Panero, M. Petrozziello, A. Bosso
Poster presentato al XXXIV Congresso Mondiale della Vigna e del Vino (OIV), Porto, 2011.
Atti del XXXIV Congresso OIV, Pubblicato su: Le Bulletin de l'OIV, 2011. Codice ISSN 0029-7127.

Reduction of the use of SO_2 in the vinification process of organic grapes.
M. Guaita, **S. Motta**, L. Panero, M. Petrozziello, R. Follis, A. Bosso
Relazione orale e poster presentati al "9ème Symposium Oeno 2011", Bordeaux, 15-17 giugno 2011.
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Caratterizzazione del quadro polifenolico ed aromatico di mosti di 5 diverse cultivar a bacca bianca, sottoposti a pressatura all'aria e sotto azoto.
A. Bosso, R. Follis, M. Guaita, **S. Motta**, L. Panero, M. Petrozziello
Presentazione orale al convegno "Territori diVini", Treviso, 24 giugno 2011.
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Copper content in organic and conventional grape musts. Effect on the fermentation trend and on wines composition.
M. Guaita, **S. Motta**, L. Panero, M. Petrozziello, A. Bosso
Poster presentato al XXXV Congresso Mondiale della Vigna e del Vino (OIV), Izmir, Turchia, 18-22/6/2012.
Atti del XXXV Congresso OIV, in corso di pubblicazione su: Le Bulletin de l'OIV, 2012.

Dealcolazione: processi, tecniche e prodotti
Antonella Bosso e **Silvia Motta**
Corriere Vinicolo n. 12 del 26 Marzo 2012, pp 46-50**.**

Study of the influence of native molecules and of some additives on white wines evolution during storage. Individuation of the conditions that can slow down the oxidative aging in bottled wines.
17th Workshop on the developments in the Italian PhD Research on Food Technology and Biotechnology.
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Vini rossi e bianchi parzialmente dealcolati: preferenza e accettabilità da parte di consumatori ed esperti.
Abstract di **Silvia Motta** pubblicato su **L'Enologo** n.12, Dicembre 2012, pag. 62-63.

Rimozione parziale dell'etanolo durante la fermentazione per ottenere vini a ridotto contenuto alcolico.
Abstract di **Silvia Motta** pubblicato su **L'Enologo** n.12, Dicembre 2012, pag. 63-64**.**

Tartaric stabilization of wines. Comparison of the effectiveness of different kinds of polyaminoacids.
M. Petrozziello, L. Panero, M. Guaita, **S. Motta**, A. Bosso*
Presentato al XXXVI Congresso Mondiale della Vigna e del Vino (OIV), Bucarest, Romania, 2-7/6/2013.
Atti del XXXVI Congresso OIV, in corso di pubblicazione su: Le Bulletin de l'OIV, 2013.

Study of the influence of different anti-oxidant molecules and of oxygen content on the oxidative aging of wines
Atti del **18th Workshop on the developments in the Italian PhD Research on Food Technology and Biotechnology**. ISBN 978-88-97385-68-4
Conegliano 25-27 Settembre 2013

Il ruolo antiossidante della SO2. Tecniche per ridurne l'impiego e prodotti alternativi.
Antonella Bosso e **Silvia Motta**
Corriere Vinicolo n. 42, 28 ottobre 2013, pp 14-15

Speaker at Congresso internazionale "**Macrowine 2012: macrovision of viticolture winemaking and markets** with the presentation: Comparing two different dechniques to dealcoholize three wine typology.
18-21 Giugno 2012, Bordeaux.

17th Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology. Cesena, September 19-21, 2012. Presentazione di un poster dal titolo: Study of the influence of the native molecules and of some additives on white wines evolution during storage

Speaker **OIV scientific meeting**, *OIV* -gruppo di esperti "tecnologia"- 46ª edizione, Parigi 4-5 Marzo 2013

Speaker al Congresso internazionale **Intervitis Interfructa**, 61st German Winegrowers' Congress. 23-27 April 2013, Messe Stuttgart