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Investigation on cysteinyl thiol compounds from yeast affecting wine properties

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"Thought: Why does man kill? He kills for food.
And not only food: frequently there must be a beverage."
(Woody Allen)

Abstract

Thiol compounds carry out several activities in must and wine. Among them, our attention was focused on the cysteinyl thiols (glutathione and free and protein bound cysteine).
The glutathione (GSH) properties in oenological matrices are well known: it reduces the *o*-quinones arising from the hydroxycinnamic acids esters, limiting the formation of brown polymers. GSH can decrease the loss of thiol-related aromas and it prevents the sotolon (3-hydroxy-4,5-dimethyl-2(5H)furanone) formation, the main responsible of the atypical white wine ageing. The GSH constitutes the main thiol compound on grape, in must and wine and its content is affected by the winemaking practices.
The cysteinyl residues on the yeast cell wall can limit the reduced/oxidized defects in wine ageing as they occur when the ageing *sur lies* or the addition of commercial yeast cell-wall fractions (YCWF) are carried out. The addition of such adjuvants is allowed by the European law (EU Regulation 2165/2005) and they exert several activities improving the sensorial properties of wine. The evaluation of their cysteinyl thiols level was the object of this research. The analytical approaches proposed in the literature are hard-to-apply for the routine analysis since the thiols quantification of YCWF was not reliable applying the methods described for the biological system proteins.
The aims of this research were to set up and validate a sensitive and reliable analytical method for the thiols content determination in the YCWF. The cysteinyl thiols content of some commercial preparations and active dry yeast was assessed and it was correlated to the heat damage occurring during the industrial preparation. The same analytical approach was applied and validated to the quantification of GSH in grape juice, must and wine. The winemaking practices affecting the GSH level were evaluated in real processes both in vintage 2009 (8) and vintage 2010 (10). Finally, also the oxidation rate in white wine was estimated through the evaluation of the interactions between GSH, sulfur dioxide and phenols.
The analytical approach proposed was based on the reaction between the thiol and *p*-benzoquinone (pBQ); the thio-substituted hydroquinones were separated by liquid chromatography and detected by spectrophotometry. The pBQ was added in excess and the unreacted amount was bound to an excess of 3-mercaptopropanoic acid. The derivatization was fast, accurate and stoichiometric at room temperature.
The YCWF showed an heterogeneous thiols content; the lysate samples had the highest thiols concentration, as well as the GSH which could have been added by the producer to increase the antioxidant properties. The lowest amount of cysteinyl thiols was detected in the mannoprotein samples which were also able to deplete the free cysteine and GSH. Such a behavior can decrease the content of the low molecular weight thiols (e.g. flavour related thiols) naturally occurring in wine. Moreover, the heat damage was higher in the mannoprotein samples and it could be correlated to the low thiols level.

The GSH content was assessed in some grape and wine samples. The sample preparation is reliable, fast and easy-to-apply. The levels detected were in accordance to the literature. The GSH level during the winemaking was low after pressing according to the must exposure to air. The GSH increased during the fermentation dependent to the yeast strain, the fermentation course and must aeration, as well as to the readily assimilable nitrogen and the copper content. At the end of the alcoholic fermentation, the decrease of GSH concentration was faster if the must preparation was performed in oxidative condition.

The oxidation rate in white wine was evaluated through 2 experiments: in the first one the attention was focused on the interactions between GSH, sulfur dioxide and caffeic acid in depleted phenols wine and synthetic wine solution (12% ethanol, 2.5 g L^{-1} tartaric acid and pH adjusted at 3.5). In the second experiment, 13 South Africans Sauvignon blanc wines were added with sulfur dioxide and the phenols oxidation was evaluated. The quantification of GSH, caffeic acid and catechin was carried out by the validated UPLC method which was previously described. The statistical analysis showed the oxidation rate was mainly affected by the sulfur dioxide content; when the level of the latter was high the oxygen consumption decreased faster in presence of GSH, too. When GSH and sulfur dioxide were added at higher concentration, the comparison between wine and synthetic wine solution was allowed. The oxygen consumption was at least 2 times higher when the sulfur dioxide was added for 12 of the 13 Sauvignon blanc analyzed. Among the 21 parameters considered, it was mainly affected by the copper, free sulfur dioxide and ferulic acid level which constituted the 68% of the variation of the oxidation rate.

Further investigations are needed to better understand the role of copper either during the grape pressing or the wine ageing as well as the interactions between GSH, sulfur dioxide and phenols occurring in white wine.

Indagine sul contenuto dei composti tiolici cisteinici derivanti dal lievito che influenzano le proprietà del vino

I composti tiolici svolgono numerose attività in mosto e vino. Tra di essi, abbiamo focalizzato la nostra attenzione sui tioli cisteinici (glutatione e cisteina, libera e legata alle proteine).
Le proprietà enologiche del glutatione (GSH) sono ben note: riduce gli *o*-chinone derivanti dagli esteri degli acidi idrossicinnamici, limitando così la formazione di polimeri bruni. Il GSH può ridurre le perdite di aromi tiolici e previene la formazione di sotolone (4,5-dimetil-3-idrossi-2(5H)-*furanone*), il principale responsabile dell'invecchiamento atipico dei vini bianchi. Il GSH rappresenta il principale composto tiolico nell'uva, nel mosto e nel vino ed il suo contenuto è influenzato dalle operazioni di vinificazione.
I residui cisteinici della parete cellulare del lievito possono limitare i difetti di ridotto e ossidato durante l'invecchiamento del vino così come la pratica di invecchiamento *sur lies* o l'aggiunta di frazioni parietali di lievito (FPL) commerciali. L'aggiunta di questi coadiuvanti è consentita dalla legge europea (Regolamento CE 2165/2005) e sono numerose le attività che esse svolgono aventi effetto positivo sulle caratteristiche sensoriali. La valutazione del loro livello in tioli cisteinici è stata oggetto di questa ricerca. Gli approcci analitici proposti in letteratura sono difficilmente applicabili per l'analisi routinaria dal momento che la quantificazione dei tioli delle FPL non è stata realizzabile applicando i metodi descritti per le proteine di natura biologica.
Gli scopi della ricerca sono stati la validazione di un metodo analitico sensibile e realizzabile per la determinazione dei composti tiolici nelle FPL. È stato stimato il contenuto dei tioli cisteinici di alcune preparazioni commerciali e lieviti secchi attivi ed è stato correlato con il danno termico dovuto alla preparazione industriale. Lo stesso approccio analitico è stato applicato e validato per la quantificazione del GSH in succo d'uva, mosto e vino. Sono state valutate le fasi della vinificazione che influenzano il livello di GSH in condizioni di processo reali per le annate 2009 (8) e 2010 (10). Infine, è stata stimata la velocità di ossidazione del vino bianco attraverso la valutazione delle interazioni che intercorrono tra GSH, anidride solforosa e fenoli.
L'approccio analitico proposto è basato sulla reazione tra i tioli e il *p*-benzochinone (pBQ); gli idrochinoni tiosostituiti sono stati separati per cromatografia liquida e rivelati per via spettrofotometrica. Il pBQ è stato aggiunto in eccesso e la quota non reagita è stata rimossa con l'acido 3-mercaptopropanoico aggiunto in eccesso. La derivatizzazione è veloce, accurata e stechiometrica a temperatura ambiente.
Le FPL hanno mostrato un contenuto eterogeneo di tioli; i campioni di lievito lisato possedevano il più alto contenuto in tioli e di GSH; quest'ultimo è stato probabilmente aggiunto dal produttore per aumentarne il potenziale antiossidante. I livelli più bassi di tioli cisteinici sono stati rilevati nei campioni di mannoproteine per i quali è stata osservata la loro capacità di adsorbire cisteina e GSH. Tale caratteristica potrebbe comportare una diminuzione del contenuto di tioli a basso peso molecolare (ad

esempio gli aromi tiolici) naturalmente presenti nel vino. Inoltre, tali campioni hanno mostrato un danno termico elevato che potrebbe essere correlato con il loro basso contenuto in tioli.

Il contenuto di GSH è stato determinato in alcuni campioni di uva e di vino, la cui preparazione è risultata veloce e facilmente applicabile. La concentrazione di GSH rivelata era in accordo con i dati descritti in letteratura. Il livello di GSH durante la vinificazione era spesso basso dopo la pressatura in funzione dell'esposizione all'aria. Il GSH è aumentato durante la fermentazione in correlazione al ceppo di lievito, al decorso fermentativo e all'areazione del mosto, così come alla quantità di azoto prontamente assimilabile e di rame. Al termine della fermentazione alcolica, è stata osservata una diminuzione di GSH, generalmente più veloce se il mosto è stato ottenuto in condizioni ossidative.

La velocità di ossidazione del vino bianco è stata valutata con 2 esperimenti: nel primo, l'attenzione è stata focalizzata sulle interazioni tra GSH, anidride solforosa e acido caffeico in un vino impoverito di fenoli e in una soluzione di vino sintetico (12% etanolo, 2.5 g L^{-1} acido tartarico, pH 3.5). Nel secondo esperimento, 13 vini Sauvignon blanc sudafricani sono stati addizionati di anidride solforosa ed è stato valutato il loro contento di fenoli. La quantificazione di GSH, acido caffeico e atachina è stata possibile grazie alla validazione del metodo analitico, descritto in precedenza, in UPLC. L'analisi statistica ha mostrato che la velocità di ossidazione era principalmente influenzata dal contenuto di anidride solforosa; quando la concentrazione di anidride solforosa è elevata, il consumo di ossigeno diminuisce più velocemente se è presente anche GSH. Il confronto tra vino e vino sintetico è possibile quando GSH e anidride solforosa sono state aggiunte a elevata concentrazione. Il consumo di ossigeno era almeno 2 volte più alto quando l'anidride solforosa era aggiunta per 12 dei 13 vini analizzati. Tra i 21 parametri considerati, esso è stato principalmente influenzato dalla contenuto di rame, solforosa totale e acido ferulico che costituivano il 68% della variazione della velocità di ossidazione.

Ulteriori indagini sono necessarie per la miglior comprensione del ruolo del rame sia durante la pressatura e l'invecchiamento, così come una più approfondita ricerca sulle interazioni che possono avvenire in vino bianco tra GSH, anidride solforosa e fenoli.

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0 PREFACE

Le vin et l'homme me font l'effet de deux lutteurs amis sans cesse combattant,
sans cesse réconciliés.
Le vaincu embrasse toujours le vainqueur.
(C. Baudelaire)

Il vino e l'uomo mi appaiono come lottatori amici
che senza tregua si combattono e si riconciliano.
Il vinto bacia sempre il vincitore.

The wine is the product obtained by the *Vitis vinifera* pressing and fermented by *Saccharomyces cerevisiae*. It is the results of complex mechanisms involving several compounds which can affect the aroma, the colour and the stabilization during the shelf-life.
In particular, the wine protection against the oxidation represents an essential factor in order to preserve its initial characteristic during the winemaking and to increase the shelf-life. A natural antioxidant compound in wine is the glutathione which performs this activity in cell and plant as well. The sulphydryl group is the functional group responsible for antioxidant effect and it can be present in the yeast cell-wall proteins which are released during the cell lysis and the ageing *sur lies*. The proteins are located in the outer layer of the yeast cell-wall and the cysteinyl groups exposition is possible afterwards the β-glucanase enzyme action. The yeast proteins carry out several functions in wine and the addition of yeast mannoproteins or cell-wall fractions is allowed by law (UE Regulation 2165/2005). Little is known on the antioxidant content of these preparations and on their potential effect against the wine oxidation.
The first step of this work has been the development of an analytical method to evaluate the antioxidant property of the yeast cell wall-fractions. The yeast cell-wall fractions comprehend mannoprotein, hull, lysate and yeast extract all of them having a common objective: they improve and stabilize the wine sensorial characteristics. These preparations are commonly employed in the winemaking, but there is still a little knowledge about the antioxidant property. The thiols are the compounds with this characteristic; they are cysteine, free and linked to the protein, and glutathione and they are contained in the yeast cell-wall fractions.
Glutathione is also the main thiol compound in grape juice, must and wine. Its content is strictly correlated to the cultivar, agronomic practices, environmental conditions, pre-fermenting operations and winemaking techniques as well as the *S. cerevisiae* strain employed in fermentation. An analytical method was validated for the glutathione quantification and several winemakings, conducted in real conditions process, were followed.

Glutathione decrease during the ageing is linked to the phenols, sulfur dioxide and oxygen content. To better understand the interactions between these compounds in white wine, a Sauvignon Blanc wine was added in oxygen, left at high temperature to increase the oxidation rate for 2 months and then analyzed.
Wine cannot exist without the yeast action as well as without chemical compounds interactions. In particular, the yeast role is key in the winemaking.
I hope this research could represent a useful tool for a rapid evaluation of the antioxidant potential either of the yeast enological adjuvants or the must and wine during the winemaking and the shelf-life. I also hope this thesis could explain mechanisms already known but which have not been investigated before in real conditions process and real wine.
A good wine is the result of hard work and passion just like this work is the result of three years full of hard work and dedication to the study of glutathione.

Daniela Fracassetti

1 STATE OF THE ART

1.1 Thiol compounds: general

Thiol compounds can strongly affect the sensorial properties of must and wine and their concentration can be influenced by the winemaking and the storage conditions.
The thiol compounds in must and wine are mainly represented by reduced glutathione, which can be correlated to the grape, the pre-fermenting operations and the yeast metabolism. It can carry out several activities in must and wine, such as the protection against the oxidation, the off-flavour formation and on the aroma content.
Low concentrations of cysteine and methionine, amino acid containing sulfur which can participate in sulfur metabolism, were also detected (Park et al., 2000a).
Some volatile thiol compounds are related to either reduced defects or olfactive perceptions like grapefruit, guava, passion fruit or *cassis*. The concentrations of these compounds are correlated to the cultivar, agronomic practices and pedoclimatic factors; they are released during the alcoholic fermentation.
The addition of yeast cell wall fractions, (e.g. mannoproteins, lysates and hulls) as well as the ageing *sur lies* can affect the thiol content in wine and increase shelf-life of wine.

1.2 Glutathione

The reduced glutathione (GSH) is a tripeptide constituted by L-cysteine, γ-glutammic acid and glycine (Figure 1.1). It exerts antioxidant and detoxifying activities in the cell (Friedman, 1994; Noctor et.al., 1998).

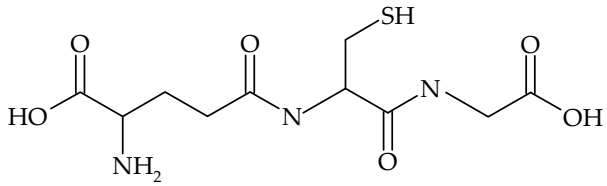

Figure 1.1: Molecular structure of the glutathione.

GSH can perform several activities in must and wine: antioxidant activity against wine browning during winemaking and ageing, improvement of wine aroma limiting both the thiol-related aromas oxidation and the off-flavour formation. The *Saccharomyces cerevisiae* strain can affect the GSH concentration in must and wine due to its metabolism; GSH represent the 0.5-1% of the dried weight of *S. cerevisiae* (Pennicks, 2002).
GSH can avoid the enzymatic and non-enzymatic browning in must and wine and, for this reason, it is added to fruit juices and different type of food in order to increase the quality of the final product and the shelf-life (Molnar-Perl & Friedman, 1990; Friedman, 1994; Son et al., 2001).

1.2.1 Glutathione in grape

Grape represents the first potential source of GSH in winemaking; GSH concentration in grapes can exceed 100 mg kg^{-1} according to grape cultivar, environmental conditions and viticultural practices (Cheynier et al., 1989).
The GSH accumulation in grape is affected by several agronomic and climatic conditions; in particular, treatments increasing the nitrogen amount in the soil during the ripening seem to affect the GSH concentration in grape (Lavigne & Dubourdieu, 2004). Nevertheless deeper researches should be carried out.
The GSH concentration is correlated with the readily assimilable nitrogen: an increased concentration of nitrogen in the vineyard may originate juice with a high content of assimilable readily nitrogen (120-200 mg L^{-1}), caused by high GSH concentrations in berries. Juice from grape showing a poorer nitrogen concentration may lead to the opposite situation (Lavigne & Dobourdieu, 2004).
The nitrogen starvation causes the grapevine leaves to become yellow and leaves the plant with poor vigor. A nitrogen addition performed with sufficient concentration and advance on the harvest, allows increasing the GSH content in the berries. The GSH transport and accumulation occur through the vascular system carrying water and sugars during the ripening. The GSH concentration increases after the veraison, independently from the variety, and it is proportional to the sugars content, approximately until 16° Brix. For higher sugars content, their concentration increases faster than the GSH (Adams & Liyanage, 1993). The GSH amount can vary from different bunches and even from different berries in the same bunch.

1.2.2 Glutathione in must and wine

The GSH content in must ranges from 10 to 100 mg L^{-1} (Cheynier et al., 1989) and it can be affected by exposure to oxygen, tyrosinase activity and pre-fermentative grape skin maceration (du Toit et al., 2007; Maggu et al., 2007).
At the beginning of the alcoholic fermentation GSH almost disappears and then its concentration increases as an effect of the yeast cell synthesis and lysis. The GSH level becomes stable one month after the alcoholic fermentation is started (Lavigne & Dubourdieu, 2004).
After fermentation, the GSH content in wine can be affected by the yeast strain (Lavigne et al., 2007).
The concentration of GSH in wine is lower than in juice and grapes and it ranges from 3 to 20 mg L^{-1} (Cassol & Adams, 1995; du Toit et al., 2007) and it can be increased through the choice of an adequate yeast strain (Rauhut, 2009). No GSH is released from yeast under nitrogen starvation during the alcoholic fermentation (Lavigne & Dubourdieu, 2004).
Moreover, the yeast cell can release compounds other than GSH after lysis, such as fatty acids, amino acids, peptides, polysaccharides and glycoprotein, all of them performing different functions.

1.2.3 Glutathione properties

1.2.3.1 Antioxidant activity

The antioxidant property of GSH is well known: it can reduce the *o*-quinone deriving from the enzymatic oxidation carried out by the polyphenoloxidase enzymes (PPO) on the tartaric esters of hydroxycinnamic acids. During ageing, the *o*-quinones are produced as a result of the non-enzymatic oxidation (also known as chemical oxidation) of *o*-diphenols (Li et al., 2008). The level of *o*-diphenols in wine is correlated to the browning of white wines (Margalit, 2004; Riberau-Gayon et al., 2006; Li et al., 2008). Quinones are electrophilic molecules capable to react with nucleophilic thiol compounds like cysteinyl residues of the yeast mannoprotein (Cheynier et al., 1990).
The reaction between GSH and the respective *o*-phenols (Figure 1.2) prevents the phenols polymerization and limits the formation of brown compounds (Salgues et al., 1986).

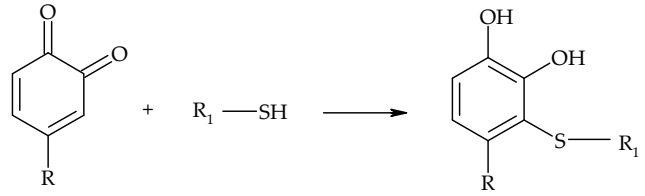

Figure 1.2: Reaction scheme of *o*-quinones formation.

Caffeoyl-tartaric acid (caftaric acid) and coumaric-tartaric acid (coutaric acid) are some of the most abundant hydroxycinnamic acid in must (Riberau-Gayon, 1996) and, so these phenols are the mainly oxidized substrate by the enzymic action.
The GSH can reduce the oxidized caftaric acid, generating 2-*S*-glutathionyl caftaric acid, also known as Grape Reaction Product (GRP) (Singleton et al., 1984). The position 2 of the caftaric acid benzyl ring is the most electrophile position and the nucleophile attack occurs preferably in that position (Figure 1.3) (Cilliers & Singleton, 1990). The GRP is not a substrate of the PPO and it can trap the *o*-quinone, limiting the formation of brown polymers which are responsible in colour changes of white must and wine. GRP can be oxided enzymically by the *Botrytis cinerea* laccase and chemically by the caffeoyl-tartaric acid quinone. In this way, the GRP quinone is originated; it can be a substrate of condensation reaction with the phenols. This molecule is the responsible of the brown compounds formation. The high GSH concentration allows a second nucleophile attack, in position 5 of the benzyl ring. The 2,5-diglutathionyl caftaric acid (GRP2) is formed and it is a substrate of the laccase action (Salgues et al., 1986). With low GSH concentration, the GRP can be oxidized by the excess of caftaric acid quinones which can cause an intense browning (Figure 1.3) (Cheynier & Van Hulst, 1988; Cheynier & Ricardo da Silva, 1991).

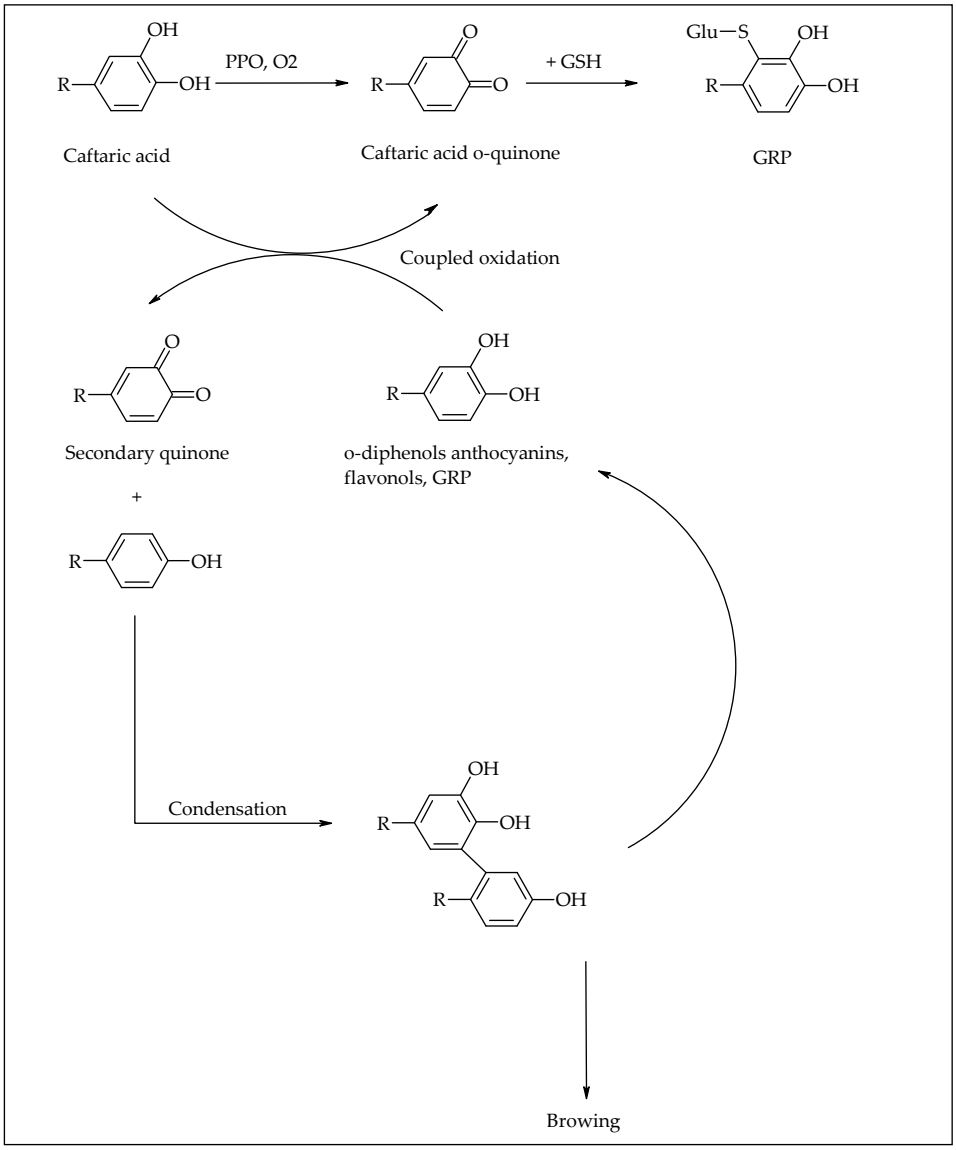

Figure 1.3: Scheme of GRP and browning compounds formation.

1.2.3.2 Interactions with aroma

GSH exerts a protective effect towards the flavoring volatile thiols of wine acting as a competitor for the reduction of the quinones (Lavigne & Dubordieu, 2004). Since GSH concentration is about a thousand times higher, it can also preserve thiol-related aroma compounds from oxidation. GSH concentration ranging from 6 mg L^{-1} to 10 mg L^{-1} can have a positive effect slowing down the decrease of the thiol-related aroma (Lavigne & Dubourdieu, 2004). Additionally, other aromatic compound such as isoamyl acetate (3-

methyl-1-butyl acetate), ethyl hexanoate and linalool (3,7-dimethylocta-1,6-dien-3-ol), are better protected during bottle storage (Papadopoulou & Roussis, 2008), especially if caffeic acid is present in wine at certain levels (15-30 mg L^{-1}) (Roussis et al., 2007; Ioannis et al., 2008; Roussis & Sergianitis, 2008).
GSH can reduce the formation of sotolon (3-hydroxy-4,5-dimethyl-2(5H)furanone), a compound responsible for the atypical ageing character of white wine (Lavigne & Dubordieu, 2004). It confers aromas descriptor as dried fig and rancid and its perception threshold is 7 μg L^{-1} (Cutzach et al, 1999). The sotolon synthesis is probably due to the chemical mechanisms occuring with the Maillard reaction (König et al., 1999). These interactions happen especially when the reduced sugars concentration is high.
Besides sotolon, 2-aminoacetophenone (1-(2-aminophenyl)ethanone) is also responsible for the atypical ageing; this aroma has a lower perception threshold than sotolon, corresponding to 1 μg L^{-1}.
The exposition of wine to oxygen during the bottling could have an increasing effect on the oxidative defects formation (Lavigne et al, 2008). GSH can have a protective action on the wine aroma during the oxidative ageing. GSH reduces the formation of both these off-flavours during storage (Table 1.1).

Atypical ageing compound	*Wine tested*	*Wine added to GSH (10 mg L^{-1})*
Sotolon	9 μg L^{-1}	3 μg L^{-1}
2-aminoacetophenone	215 ng L^{-1}	125 ng L^{-1}

Table 1.1: Effect of GSH on the formation of atypical aging off-flavours during wine storage (Lavigne & Dubourdieu, 2004).

Moreover, GSH can have a positive effect on the white wine colour which appears to be more stable during the ageing (Lavigne & Dubourdieu, 2004; Hosry et al., 2009).

1.2.4 Glutathione and yeast

GSH was discovered in yeast by Hopkins and Kendall in 1921 (Kocková-Kratochvílová, 1990) and it represents 0.5-1% of the dry weight of *Saccharomyces cerevisiae*, 95% of the intracellular pool of sulfur compounds having low molecular weight and occurs in high concentrations up to 3 g L^{-1} (10 mM) in yeast cells (Elskens et al, 1991; Mehdi and Pennincks, 1997; Pennicks, 2002).
GSH is involved in the oxidative stress response through glutathione peroxidase and detoxification processes (Rauhut, 2009). It prevents the cellular destruction by maintaining certain thiols in their reduced stage by its SH-group of the cysteine (Cys). GSH can react with some heavy metals and other toxic compounds (Duncan and

Derek, 1996; Pennicks, 2002; du Toit et al, 2007). The stress resistance is ensured by the vacuolar transport of metal derivates by the tripeptide (Pennicks, 2002).
GSH may be involved in the maintaining of the mitochondrial and membrane integrity either in *Saccharomyces* or in non-*Saccharomyces* yeast and it can be metabolized during the sulfur and nitrogen starvation as well as the reproduction (Rauhut, 2009).

1.2.4.1 Glutathione synthesis

GSH is synthesized by the consecutive action of γ-glutamylcysteine synthetase and L-γ-glutamylcysteine-glycine γ-ligase (Figure 1.4) (Pennicks, 2002).
The γ-glutamylcysteine synthetase is a highly regulated enzyme (Lee et al., 1999) and its activities are feedback-inhibited by GSH, preventing an over-accumulation of the tripeptide (Meister & Anderson, 1983; Penninckx & Elskens, 1993). The glutathione synthetase is a constitute unregulated enzyme (Inoue et al., 1998).

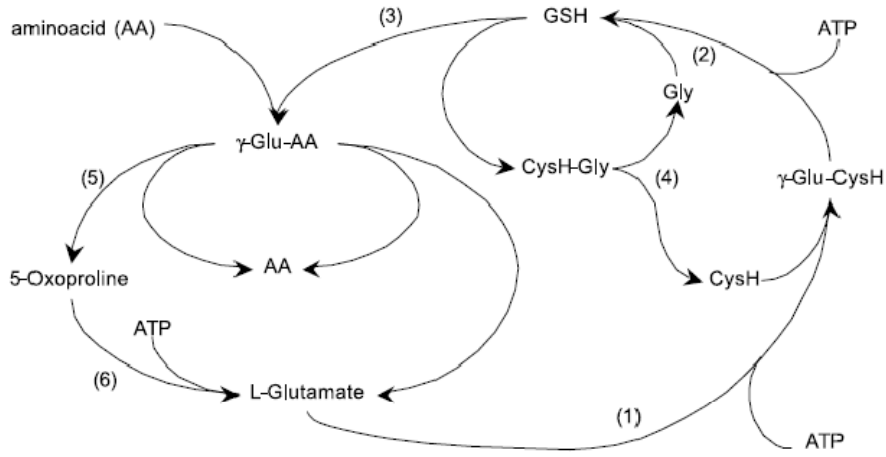

Figure 1.4: The γ-glutamyl cycle (Meister and Anderson, 1983): (1) γ-glutamylcysteine synthetase; (2) glutathione synthetase; (3) γ-glutamyltranspeptidase; (4) γ-cysteinyl glycine dipeptidase; (5) γ-glutamyl cyclotransferase; (6) 5-oxoprolinase.

1.2.4.2 Nutrients starvation

Saccharomyces can degrade GSH in response to the nitrogen and sulfur starvation as source of these elements (Elsken et al, 1991). GSH can be taken up by the yeast cell through two transport systems, GSH-P1 (high affinity, regulated system) and GSH-P2 (low affinity, unregulated system). About 50% of the yeast GSH is stored in the cytoplasm and the remaining is stored in the central vacuole during growth when nitrogen is available.
The main role in the sulfur flows regulation is played by the GSH cycle, which is closely correlated to the yeast sulfur metabolism (Figure 1.5) (Pennickx, 2000). The

cytoplasmic GSH is used as endogenous sulfur source (Elskenk et al., 1991) in case of total sulfur deficiency, until it reaches a residual concentration of about 10% of its normal value (Pennickx, 2002).
If the yeast is in nitrogen starvation, more than 90% of GSH is transported to the central vacuole (Mehdi & Pennickx, 1997) where it is splitted in the respective amino acids by the γ-glutamyl transpeptidase, the only GSH degrading enzyme currently characterized in yeast (Penninckx er al., 1980; Mehdi et al., 2001). In this condition, a smaller amount of GSH could be released in the growth medium.

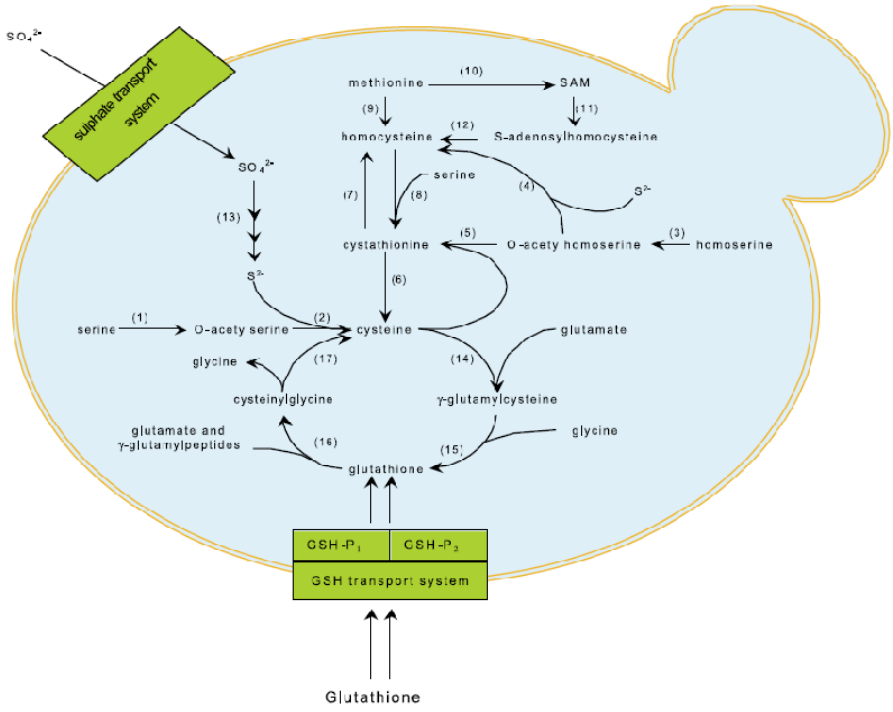

Figure 1.5: Transport and metabolism of sulfur in *S. cerevisiae* (Intyre & Curthoys, 1982; Miyake et al., 1998). (1) Serine acetyltransferase; (2) cysteine synthase; (3) homoserine acetyltransferase; (4) homocysteine synthase; (5) γ-cystathionine synthase; (6) γ-cystathionase; (7) L-cystathionase; (8) L-cystathionine synthase; (9) homocysteine methyltransferase; (10) S-adenosylmethionine synthase; (11) S-adenosylmethionine demethylase; (12) adenosylhomocysteinase; (13) sulfate-reducing pathway; (14) γ-glutamylcysteine synthetase; (15) glutathione synthetase; (16) γ-glutamyltranspeptidase; (17) L-cysteinyl glycine dipeptidase.

1.2.4.3 Oxidative stress

Yeast produces toxic oxidative compounds during its metabolism such as H_2O_2, alkylhydroperoxide (ROOH), superoxide anion ($O_2^{\bullet -}$) and hydroperoxide lipid

(LOOH). These compounds can degrade in very reactive radicals which can cause metabolic imbalances and the cell death.
The glutathione peroxidase is the key enzyme in the defense mechanisms against the hydroperoxides. This enzyme is induced by oxidative conditions in *S. cerevisiae* (Galiazzo et al., 1987). It detoxifies the cell from peroxide compounds catalyzing the reduction of hydrogen peroxide to water and the organic peroxide to the corresponding stable alcohols. The glutathione peroxide performs the reduction using the GSH as equivalents source, according to the reaction as follows:

$$2\ GSH + ROOH \longrightarrow GSSG + H_2O + ROH$$

The glutathione peroxidase is localized in cytoplasm and mitochondria, where high amounts of peroxides are produced. Exposure of yeast to high thermal condition or oxidative shock increases the respiration rate and the formation of peroxides. In order to overcome this critical condition, *Saccharomyces* metabolizes higher GSH concentration to preserve the cell structure integrity and the metabolism (Spector et al, 2001).

1.2.4.4 Detoxification of heavy metals and xenobiotics

Unfavorable yeast growth conditions and environmental stress cause an accumulation of toxic heavy metals and xenobiotics (Pennickx, 2000).
GSH can chelate the heavy metals present in the cytoplasm (i.e. copper, zinc, silver, lead and cadmium) and the complexes formed are transported in the central vacuole and then eliminated (Ortiz et al., 1992; Duncan & Derek, 1996; Penninckx, 2002). The vacuolar enzymic pool is able to degrade the toxic compounds through mechanisms strain-dependent which employ the GSH (Ramsay & Gadd, 1997). The reaction between GSH and the heavy metals and xenobiotics is catalyzed by the glutathione S-transferase enzyme, according to the following scheme:

$$GSH + RX \longrightarrow GSX + RH$$

In this way, the yeast cell is protected through the toxic action of the heavy metals and both the degradation and the elimination of xenobiotics are guaranteed.

1.2.5 The ageing on the lees

The ageing *sur lies* has a protective effect against the oxidation and improves the sensorial properties of wine. It also increases the concentration of amino acids, peptides and proteins due to the passive release or the yeast lysis. Yeast lees are composed by the cell wall debris, mainly constituted by ramified glucans and mannoproteins (Fornairon-Bonnefond et al., 2001).

Yeast lees can absorb the oxygen (Salmon et al., 2000), limiting the formation of the oxidative components.
The yeast lees can also absorb several organic substances, as polyphenols (Mazauric & Salmon, 2005), toxins, pesticides, antifoaming and volatile compounds (Perez-Serradille & de Castro, 2008). Moreover, the yeast lees can absorb several volatile compounds related with wine aroma, as esters, aldehydes and norisoprenoids, which have positive aroma impact of wine (Gallardo-Cachón et al., 2009). The yeast lees can stabilize the thiol-related aromas, either during the ageing or the storage (Baumes, 2009). Wine aged on the lees showed a lower formation of off-flavours such as sotolon and 2-aminoacetophenone (Lavigne & Dubourdieu, 2004). The positive effect of the yeast lees on the white and sparkling wine is well known: when the lees are removed, non-enzymatic browning, oxidation and decrease of thiol-related aromas occur faster (Feuillat, 2003; Lavigne & Dubourdieu, 2004).
The antioxidant properties of reducing protein Cys on the yeast lees has not been extensively investigated in wine, but the effect of cysteinyl residues of mannoproteins in the reduction of oxidative molecules is already described (Jaehrig et al., 2007; Gallardo-Chacón et al., 2010).
The periodical stirring of wine allows the release of mannoproteins and amino acids, improves the roundness and decreases the astringency (Feiullat, 2003; Caridi, 2006).

1.3 Aromatic compound containing sulfur

Beside the GSH, a source of thiol compounds in wine is also represented by the thiol-related aromas. These molecules can be responsible of the formation of some off-flavours, such as the mercaptans, and supply the typical aromatic profile of wine, like the long-chain volatile sulphur compounds.

1.3.1 Thiol-related aromas

Volatile thiols can have different sources in wine. They come from the grape (directly or indirectly, such as nonvolatile precursors), from microbial fermentation, from chemical reactions during storage and from environment (wood) (Landaud et al., 2008).
The thiol aromas originate either from enzymatic or non-enzymatic mechanisms. The first one involves the degradation of sulfur-containing amino acids, the fermentation and metabolism products from some sulfur-containing pesticides. Non enzymatic processes involve photochemical, thermal and other chemical reaction of sulfur compounds during winemaking and storage (Mestres et al., 2000).
The sulfur compounds can affect the sensorial properties of wine and wine yeast and bacteria can metabolize these thiols, which are generally considered responsible of off-flavours (Bartowsky & Pretorious, 2009). Nevertheless, the long-chain polyfunctional sulfur compounds are one of the most important groups of aroma compounds in wine, which confer pleasant aromatic notes at trace levels, but at high concentrations these

compounds can be objectionable (Mestres et al., 2000; Swiegers et al., 2005; Dubourdieu et al., 2006; Swiegers & Pretorius, 2007). They are characterized by some fruity aromas, like *cassis* (Rigaud et al., 1986), grapefruit (Demole et al., 1982), passion fruit (Engel & Tressl, 1991), guava (Idstein & Schreier, 1985). The impact of the sulphur compounds on wine aroma is updating in the recent literature and the varietal character is affected by several of these molecules if their concentration is close to the threshold perception (Fedrizzi et al., 2007; Rauhut et al., 1998; Ugliano & Henschke, 2009).

Long-chain volatile sulphur compounds as 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) are particularly important for the wine aroma (Figure 1.6A-C). These compounds characterize the typical varietal aroma of Sauvignon blanc wine (Dubourdieu et al., 2006). Moreover, in some wine, 4-mercapto-4-methylpentan-2-ol (4MMPOH) is also detected but its concentration is lower than the thiol-related aromas above mentioned (Figure 1.6D). The 4MMP has aroma descriptor of box tree, blackcurrant, or cat urine at higher concentrations; 3MH and 3MHA impart passion fruit and grapefruit aromas and 4MMPOH is reminiscent of citrus zest and grapefruit (Tominaga et al., 1998a). The perception threshold of these compounds in wine is 0.8 ng L^{-1}, 60 ng L^{-1} and 4 ng L^{-1} for 4MMP, 3MH and 3MHA, respectively (Landaud at al., 2008). The perception threshold of 4MMPOH is 55 ng L^{-1} in aqueous alcoholic solution (Tominaga et al., 2000). 3MH is the most abundant in wine, in concentration generally higher than its perception threshold, while 4MMPOH in wine is generally lower than its perception threshold. The level of 4MMP is dependent on the grape cultivar and it change between different samples in the same cultivar (Baumes, 2009).

4MMP, 3MH and 3MHA are presents in grape as non-volatile cysteinyl-conjugated precursors (Darriet et al., 1993, Tominaga et al., 1995). These compounds are splitted by the *S. cerevisiae* and, through the enzymatic activity of carbon-sulfur lyase (Tominaga et al., 1995), the thiol aromas are liberated.

Besides the Cys, some aroma can be conjugated to the GSH. These compounds are degraded by the γ-glutamyl transpeptidase and carbon peptidase, enzymes which perform the lysis of the link between the Cys and glutammic acid and glycine. In this way the aroma is cysteinyl-conjugated and the yeast carbon-sulfur lyase action can release it in wine.

3MHA is formed by yeast from 3MH no cysteinyl-conjugated esterified with acetic acid (Swiegers et al, 2005). This founding correlated for the first time the ester and volatile thiol metabolism in yeast (Bartowsky & Pretorius, 2009). The *S. cerevisiae* capability to liberate long-chain polyfunctional thiols from their precursors is genetically determined; the yeast selection can represent an useful tool to check the 3MH and 4MMP release (Dobourdieu et al., 2006; Ugliano & Henschke, 2009). The 3MHA formation from 3MH is also dependent from the strain genetic characteristics (Swiegers et al, 2008). The characterization researches showed the enhanced capacity of different *S. cerevisiae* strains to hydrolyze the S-cysteinyl link rather than the ester

synthetic activity. The combined use of different yeast strains, one of them having hydrolyzing ability and other one with stronger esterificating ability, can represent an useful tool to affect the thiol-related aromas composition (Ugliano & Henschke, 2009).
The content of these compounds decreases during wine ageing, according to the oxidative conditions. GSH, sulfur dioxide and anthocyanins content exerts a protective effect. In contrast, increased contact with oxygen, particularly in presence of catechin derivates, promotes their degradation (Murat et al., 2003; Blanchart et al., 2004; Baumes, 2009). Likewise, the concentration of long-chain polyfunctional sulfur compounds decreases during the storage time, strictly dependently on the oxidative reaction, presence of GSH, sulfur dioxide and anthocyanins (Murat et al., 2003; Blanchart et al., 2004; Baumes, 2009).

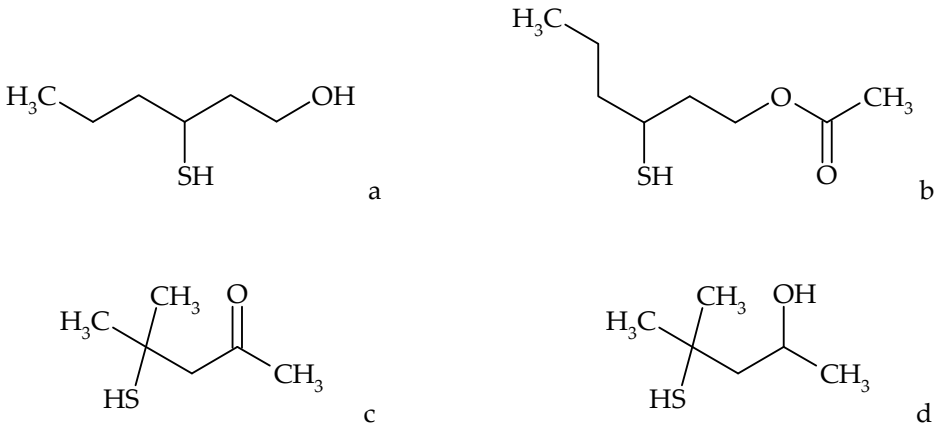

Figure 1.6: Chemical structure of 3MH (a), 3MHA (b), 4MMP (c), 4MMPOH (d).

1.3.1.1 Precursors of thiol-related aroma compounds

S-cysteinyl conjugated compounds are the precursors of the thiol-related aromas. Chemically, these compounds are the *S*-substituted derivates of the Cys and only three of these precursors were identified in grape: *S*-(1-hydroxyhex-3-yl)-L-cysteine (P3MH), *S*-(4-methyl-2-oxopent-4-yl)-L-cysteine (P4MMP), and *S*-(4-methyl-2-hydroxypent-4-yl)-L-cysteine (4MMPOH) (Tominaga et al., 1995; Tominaga et al., 1998b). However, the *S*-(4-hydroxyhex-3-yl)-glutathione, the precursor of the 3MH, was identified in Sauvignon blanc must and, probably, in Gros Manseng must (Peyrot des Gachons et al., 2002a). This compound could be a precursor of P3MH, obtained as a result of the enzymatic activities of γ-glutamyltransferase, which removes glutamic acid, and of carboxypeptidase, which eliminates glycine (Peyrot des Gachons et al., 2002a). No other GSH conjugated was identified, neither corresponding to P4MMP nor P4MMPOH (Baumes, 2009). *S*-glutathione conjugates may be involved in detoxification processes in wine, as well as in other plant or organisms (Dubourdieu & Tominaga, 2009).

The level of these compounds in must does not exceed 100 μg L^{-1} and it is dependent on environmental conditions, climate and soil parameters and vineyard treatments (Peyrot des Gachons et al., 2000; Peyrot des Gachons et al., 2005). In particular, the skin contact has a more increasing effect on the P3MH concentration than P4MMP and P4MMPOH (Peyrot des Gachons et al., 2002b).
During the alcoholic fermentation, a yeast lyase releases the thiol compounds by cleavage of the carbon-sulfur bound (Tominaga et al., 1998b), activity correlated to the *S. cerevisiae* strain (Murat et al., 2001).

1.3.2 Thiol off-flavours

The sulfur-containing off-flavours impart the negative notes as cabbage, onion, rotten egg, garlic, sulfurous and rubber (Vermeulen et al., 2005). These compounds are typically found at low concentration and they have very low perception threshold (Bartowsky & Pretorious, 2009).
Hydrogen sulfide is probably the best-known sulfur compound in wine which imparts rotten egg aroma. It is highly volatile and it has a low perception threshold (10-80 μg L^{-1}). It can be removed by aeration or copper treatments (Monk, 1986). Hydrogen sulfide is a very reactive species and it can participate in reactions generating compounds as mercaptans, dimethyl sulfide and polysulfide, which have negative impact on the wine aroma. Dimethyl sulfide and polysulfide cannot be removed by copper addition (Bartowsky & Pretorious, 2009).
Mercaptans are responsible of reduced aroma in wine. Their removal is possible by racking wine in aerating conditions or by the ageing on the lees. Mercaptans are converted into the respective thiol-substituted hydroquinones as the result of the reaction with *o*-quinone or they can link to the cysteinyl residues of the yeast cell-wall to give disulfides (Lavigne & Dubourdieu, 1996).
Methionine can be metabolized by yeast to produce fusel alcohol, methionol and 3-methylthio-1-propanol imparting cabbage and cauliflower aromas. Cys is a precursor of *S*-containing heterocycle compounds; these compounds can be metabolize by *Oenococcus oeni* and aroma descriptors as sulfury, floral, fruity, toasted and roasted can be imparted to the wine (Pripis-Nicolau et al., 2004; Bartowsky & Pretorious, 2009).

1.4 Yeast-cell wall fractions

The yeast cell-wall fractions (YCWF) comprehend mannoproteins, hulls, lysates and extracts which are industrially produced and used as oenological adjuvants. These preparations carry out several activities affecting positively the wine sensorial properties. Mannoproteins fractions are glycoproteins and they can be released in wine from yeast during the fermentation and ageing on the lees due to the yeast lysis. Mannoprotein and yeast extracts are usually added to wine during the winemaking; these preparations are soluble and they have a higher protein content than hulls and lysates. Those fractions showed a high carbohydrate content and their use occurs after

the alcoholic fermentation during the barrel or tank ageing and their addition allows judging a wine mature in less time.
The antioxidant properties of the reducing protein Cys from yeast lees have not been extensively investigated in wine, but some results show the Cys residues of yeast mannoproteins to be effective antioxidants if they are accessible to oxidizing molecules (Jaehrig et al., 2007). Moreover, white wines and sparkling wines are well known to develop faster non-enzymatic browning, oxidation, and loss of odor-related thiols after yeast lees are removed (Feuillat, 2003; Lavigne & Dubourdieu, 2004). The release of mannoproteins from yeast lees as well as the addition of yeast glycoproteins to wine could increase the antioxidant Cys content. Nevertheless, little is known about the Cys content in YCWF as well as about the antioxidant behavior of yeast lysates, hulls, extracts and mannoproteins in wine. It is possible that the cysteinyl thiols bound to the YCWF exert an analogue property as GSH during the ageing and the shelf life (Gallardo-Chacón et al., 2010).

1.4.1 *Saccharomyces cerevisiae* cell wall

Saccharomyces cerevisiae cell wall (Figure 1.7) represents the 30% of the cell dried weight and it is composed by polysaccharides (about 85%) and proteins (about 15%) (Lesage & Bussey, 2006). It is a layered structure where the inner layer is made of β-1,3 glucan, β-1,6 glucan and low amounts of chitin and mannoproteins. The latter are the only constituent on the cell-wall external layer.
The glucan layer is aimed to maintain the cell shape and has mechanical strength. The outer layer determines surface properties, as electrical charge, hydrophobicity, flocculence and limiting the porosity of the cell wall (Moukadiri et al., 1997).

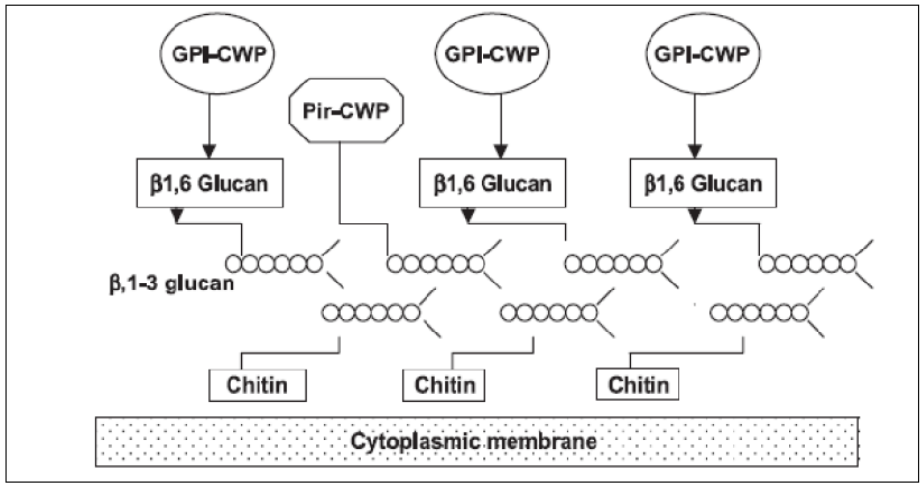

Figure 1.7: Structure of *Saccharomyces cerevisiae* cell wall (Smits et al., 1999). In the scheme, the non covalent links and the disulphide bridge are not represented. The β-1,3 glucans are in the

inner layer forming a tridimensional network. The cell wall structure is stabilized by the disulfide bridges among the glucans. In the outer layer, the Pir protein (family of covalently linked cell wall proteins of *S. cerevisiae*), are directly linked to the β-1,3 glucans, while the GPI (glycosylphosphatidilinositol) is indirectly bound with the β-1,3 glucans, through the β-1,6 glucans.

1.4.1.1 Chitin

The chitin is a linear polymer of β-1,4-N-acetyl-glucosamine (Lesage & Bussey, 2006); it can form microfibrils stabilized by hydrogen bonds (Lipke & Ovalle, 1998). It is the minor compound of the *S. cerevisiae* cell wall (1-2% of the dried weight). The chitin has an important role in the septum formation in the cell reproduction (Cabib et al., 2001). About 40-50% of the chitin is bound to the no-reducing terminal ends of the β-1,3 glucan by β-1,4 bonds with the no-reducing ends of the chitin. Its crystalline structure increases the yeast stretching resistance (Lesage & Bussey, 2006).
When the yeast cell is the substrate of exogenous β-1,3 glucanase, the cell wall can decrease its rigidity due to the β-1,3 glucan degradation. In order to resist, the cell has a rescue system which consists in the increase of the bonds between protein and the β-1,6 glucan – chitin complexes (Kapteyn et al., 1997).

1.4.1.2 Mannoproteins

Mannoproteins are constituted of about the 10% of proteins and they accounts for 35-50% of the cell-wall dried weight (Gonzales-Ramos & Gonzales, 2006). The polysaccharide fraction of the mannoproteins is mainly constituted by mannose (98%) and glucose (2%). Their molecular weight ranges from 20 kDa to 450 kDa and the glycosylation grade is strain-dependent (Nguyen et al., 1998). The mannoproteins are placed in the outer layer of the cell wall which is less permeable to macromolecules than the inner layer. This property is due to the highly branched chains of carbohydrate bound to asparagine and the disulfide bridges (Klis et al., 2002). These macromolecules can carry out cell interactions, as flocculation, killer factor and sexual reproduction, while some of them have enzymatic activity, as invertase, glucosidase and esterase.
The mannoproteins are localized only in the cell wall associated to the glucans network (Feuillat, 2003). These molecules are classified in 3 groups according to the structural bonds involved: not covalently linked, covalently linked with the glucans and forming disulfide bridges with proteins covalently bound to the glucans (Jaafar et al., 2003). Two further groups of mannoproteins were described: those bound to non-reducing end of β-1,3 glucan by β-1,6 bonds and those directly bound to β-1,3 glucan. Mannoproteins can establish also ionic interactions and disulfide bridges with the cell-wall polysaccharides (Lomolino & Curioni, 2007).
The time needed for the release of mannoproteins is strictly correlated to the interactions among the macromolecules (Charpentier et al., 2004). The chemical and

physical properties and the amount of the mannoproteins are strictly correlated to the yeast strain (Escot et al.,2001; Feuillat, 2003). The mannoproteins released following to the alcoholic fermentation as effect of the β-1,3 glucanase are partially soluble in water. This enzyme performs its degrading activity either on the lees or on the growing cells and its action is dependent to contact time, temperature and biomass shaking (Caridi, 2006). For instance, the mannoproteins released during the alcoholic fermentation are able to reduce the astringency more efficiently than those released after the cell lysis (Escot et al.,2001; Feuillat, 2003).

1.4.1.3 Cell wall proteins – polysaccharides complex

In rich growing medium, the abundant complex of yeast cell wall is formed by glycosylphosphatidilinositol (GPI) – cell wall proteins (CWPs) – β–1,6 glucan – β–1,3 glucan. In some complexes, the GPI-CWPs is directly linked with the β–1,3 glucans by a not characterized alkali sensitive bond. Moreover, the GPI-CWPs can be bound with β–1,6 glucan conjugated to the chitin, commonly produced when the yeast cell is growing in stressed conditions (Caro et al., 1997; Klis et al., 2002).
The CWP-polysaccharides complexes are summarized in figure 1.8.

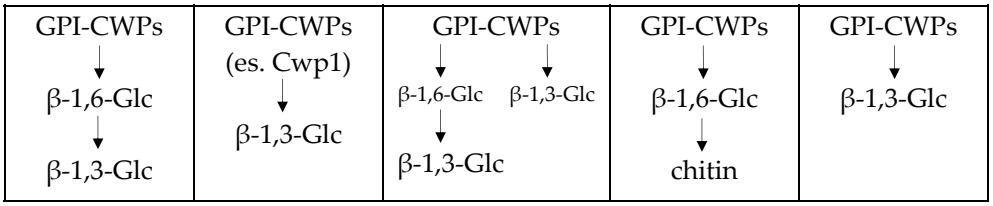

Figure 1.8: Structure of protein–polysaccharides complexes in the cell wall (Klis et al., 2002).

1.4.2 Enological properties of yeast cell wall fractions

The YCWF are often used in winemaking in form of mannoproteins, hulls, lysates, cell extracts or inactivated yeasts in order to positively affect the sensorial properties and the chemical-physical stability of wine.
The EU Regulation n° 2165/2005 permits the addition of mannoproteins for the tartaric and protein stabilization.

1.4.2.1 Inhibition of tartrate salt crystallization

During the winemaking, the tartrate salt crystallization reduces the wine acidity. Wine is a supersaturated solution of potassium hydrogen tartrate and this salt can crystallize and precipitate in bottled wine producing turbidity, sediments and colour loss in red wine (particularly in cold storage) if effective stabilizing practices are not applied. Mannoproteins can avoid such precipitation hindering the growth of the tartrate crystal nuclei. The effect corresponds to a decrease of the crystallization temperature

equivalent to 2.20-2.35°C (Feuillat et al, 1998). This property is due to some highly glycosylated mannoproteins with molecular weight ranging from 30 kDa to 50 kDa (Caridi, 2006).
The mannoproteins fractions useful against tartrate precipitation are linked to the cell wall by covalent bond and are extracted by enzymatic treatment. Their addition to the wine should be carried out just before the bottling and the effective amount should evaluate through preliminary assays. Effective mannoproteins concentration could be 5 mg L^{-1}; the optimal concentration would be 12.5 mg L^{-1}, even though higher concentration (30 mg L^{-1}) are possible, related to the protein fraction (Moine-Ledoux & Parodi, 2006).

1.4.2.2 Prevention of protein haze

The protein haze is a common problem in white wine caused by the slow denaturation and flocculation of heat unstable proteins contained in grape (Caridi, 2006). Protein haze occurs after wine bottling and it has a detrimental effect on the wine aspect. The phenolic compounds as well as the metallic ions contribute to this phenomenon increasing the aggregate dimension. Some yeast mannoproteins fractions can prevent the protein haze in wine up to 40% (Gonzales-Ramos et al., 2008). The effective mannoproteins preventing the protein haze have high molecular weight (420 kDa) but also a 32 kDa invertase fragment showed the same stabilizing property. The former protein group is present at low concentration in wine. The prevention mechanism is not completely clear, but it was suggested the mannoproteins form an hydrated neutral layer on the protein aggregates preventing their growth (not over 5 μm) and the precipitation. The proteins are adsorbed on the surface flocculus and the haze is not visible at naked eye (Dupin et al, 2000a). The proteins causing the haze can not be denatured or adsorbed on the lees, but the active invertase fragment released during the aging increases their heat stability (Dupin et al., 2000b). This enzyme can be extracted from the yeast cell wall through enzymatic digestion (Moine-Ledoux & Parodi, 2006). Some glycoproteins obtained from yeast extracts could prevent the protein haze. Their addition allows a protein haze reduction up to 20% in comparison to the untreated wine, nevertheless high protein amounts are necessary to be effective (Lomolino & Curioni, 2007). The addition of these glycoproteins to wine could decrease the amount of bentonite needed to remove the proteins causing the haze stabilization of the product.

1.4.2.3 Interactions with phenolic compounds

Some mannoproteins can protect the colour of red wine hindering the tannin-anthocyanin polymerization. The same occurs for the proanthocyanins polymerization and aggregation which are accountable for tannin precipitation in wine ageing (Riou et al., 2002; Caridi, 2007). On the contrary, recent studies showed that in wine treated

with commercial mannoproteins, the colour intensity is lower than in untreated wine after the alcoholic fermentation (Guadalupe et al., 2007).
The anthocyanins polarity and the yeast strain can affect the wine colour; the most polar anthocyanins, as delphinidin and petunidin, are easily adsorbed on the yeast cell wall, but not the acylated anthocyanins (Medina et al., 2005).
The protective mechanism of mannoproteins is not completely clear: it seems the polysaccharides can slowdown the dimension of the tannins-anthocyanins polymers, but not their formation (Riou et al., 2002). This phenomen is due to the steric hindrance of the mannoproteins.
During the malolactic fermentation, the total polyphenols index in wine added with mannoproteins is lower than in untreated wine, due to the precipitation of mannoproteins-polyphenols complex. In this way, the wine is less astringent and it has higher roundness, but the colour is less stable and intense (Guadalupe et al., 2007).
Both the amount and the composition of the released mannoproteins affect the aroma stability: for instance, the neutral glycoproteins decrease the astringency and the acid mannoproteins increase the roundness (Vidal et al., 2004).
The interaction between tannin-anthocyanin polymers and mannoproteins is strictly correlated to the yeast strain (Escot et al, 2001) and it can modify wine colour, phenols composition and antioxidant property (Caridi et al., 2004).

1.4.2.4 Interactions with the aroma components

The aromatic quality of a wine is assessed by sensory analysis of the headspace containing the volatile aromas. The aroma equilibrium between vapor and aqueous phases is expected to be influenced by nonvolatile molecules present in wine (Dufour & Bayonove, 1999). The mannoproteins can interact with these components fortifying the aroma (Feuillat, 2003) and modifying the aromas intensity and volatility (Caridi, 2006). The interactions involve either the protein or the polysaccharide fractions (Charlier et al., 2007). The volatile properties of the aroma compounds can be modified by hydrophobic interactions with the mannoproteins, as a consequence the olfactory properties are modified, as well.
Lees contact promotes an indirect effect on the aromatic properties of wine since the clarification and stabilization treatments can decrease the mannoprotein content up to 30%. Both the adsorbed aromatic and the coloured molecules are lost by such winemaking practices with detrimental effects on the colour intensity and flavour perception. The mannoproteins addition can contribute to the aroma maintenance, in particular the notes of minerals, smoke and ageing in oak barrel are predominant. This effect was explained by the volatility decrease of floral, fruity and green aromas. Moreover, the sweet and roundness perception increases since one month after the commercial mannoprotein addition (Guadalupe et al., 2007).
Studies conducted by Lubbers et al. (1994) showed the interaction between mannoproteins and aromas occurred in presence of yeast extract ranges from 1 g L^{-1} to

10 g L^{-1}. The yeast cell-wall with higher content of mannoproteins and lipids has a higher aroma adsorption capability; therefore the yeast cell-wall composition has to be carefully evaluated in order to minimize the aromatic losses.

1.4.2.5 Stimulation of malolactic bacteria

The *S. cerevisiae* mannoproteins can improve the growth of the malolactic bacteria, decreasing the lag phase and increasing the biomass (Guillox-Benantier et al., 1993). Moreover, some bacterial enzymatic activities, as glycosidase and peptidase, are enhanced and the nutrients content of medium is increased (sugars, peptides, amino acids). In particular, *Oenococcus oeni* is stimulated on the presence of peptide fractions having molecular weight lower than 1 kDa. The bacterial growth is favored by the use of fresh yeast versus active dried yeast; the latter increases the concentration of some amino acids (i.e. glutamic acid, arginine and isoleucine), necessary for the bacteria growth (Guillox-Benatier & Chassagne, 2003).
During the alcoholic fermentation, the mannoproteins released is affected by the initial content of colloids in must. They affect the fatty acids (C_{12}-C_{18}) formation. A high colloids content in must depresses the mannoproteins released by the yeast and the content of fatty acids is higher; this can cause a slow down in malolactic fermentation (Guillox-Benantier et al., 1995). Mannoproteins can absorb the fatty acids in the medium preventing their toxic action on the malolactic bacteria (Caridi, 2006). The malolactic activity is favored by the interactions between mannoproteins and fatty acids; a high content of mannoproteins also leads to a rapid malic acid degradation (Guillox-Benantier et al. 1995).

1.4.2.6 Effects on sparkling wine

The YCWF contribute to the flocculation of the yeast employed for the sparkling wine production. The *Champenoise* method involves the re-fermentation in bottle followed by the spontaneous yeast lysis occurring along with the wine ageing. Higher content of mannoproteins can be released in wine by yeast strains capable to produce and/or release high amounts of mannoproteins or by the addition of β-1,3 glucanase. The wine obtained is characterized by more oxidized and acidic taste but also more mature in less ageing time (Feuillat, 2003; Caridi, 2006).
The amphiphilic behavior of the mannoproteins allows positive effects on the foaming properties in sparkling wines. They can increase both formation and stability of the foam (Núñez et al., 2006).

1.4.2.7 Adsorption of volatile phenols

The presence of the volatile phenols, as ethyl phenols, in wine is due to the *Brettanomyces bruxellensis* metabolism. These compounds bring to the off-flavour known as "Brett character", described as horse sweat, pharmaceutical, wet wool

(Chatonnet et al., 1992). Volatile phenols concentration higher than 600 μg L^{-1} can be detrimental for the wine flavour, strictly correlated to the wine and the individual perception capacity (Loureiro & Malfeito-Ferreira, 2003).
Mannoproteins can reduce the 4-ethylphenol content by sorption. The sorption occurs at the surface of the yeast cell wall, as the result of coupling effect of surface hydrophobicity and electron donor character. This function is strain-dependent and it can modify in relation to the environmental conditions (ethanol concentration, pH). The yeast strain, the mannoproteins extraction and drying method are the main factors affecting the volatile phenols sorption (Pradelles et al., 2008). The active dried yeast as well as the yeast lees can sorb 4-ethylguaiacol and 4-ethylphenol in synthetic medium and in wine (Chassagne et al., 2004).

1.4.2.8 Removal of reduced odours

The reduced odour related to some mercaptans (Table 1.2) can rise from the yeast metabolism, the vineyard treatments, the Strecker reaction as well as from white wine exposition to the light. These compounds can alter in particular the aroma of white wine.
Different procedures can be followed to prevent the formation of these off-flavours such as appropriate addition of sulfur dioxide in must and wine, appropriate choice of the yeast strain, must clarification, racking, aeration, readily assimilable nitrogen content in must.
In reductive conditions process, the marcaptans are easily formed. The exposure of wine to oxygen allows the oxidation of mercaptans and the formation of disulfide bridges, as represented as follows (Lavigne & Dubourdieu, 1996):

$$R_1\text{-}CH_2\text{-}SH$$
$$+ O_2 \longrightarrow R_1\text{-}CH_2\text{-}S\text{-}S\text{-}CH_2\text{-}R_2 + H_2O$$
$$R_2\text{-}CH_2\text{-}SH$$

The resulting compound has a higher molecular weight, boiling point and perception threshold.

Thiol	Compound	Perception threshold μg L-1	Descriptors
Low-boiling thiols	Sulfide hydrogen	0.8	rotten eggs
	Methanethiol	0.3	putrid
	Ethanenthiol	0.1	onion
High-boilig thiols	Dimethyl disulfide	2.5	asparagus
	2-mercaptoethanol	130	burnt rubber
	Methionol	1200	cabbage

Table 1.2: Perception thresholds and aromatic descriptors of some thiol compounds producing the reduced odour in wine (Riberau-Gayon et al., 2006).

Yeast lees contain cysteinyl thiols able to bind the soluble mercaptans and to decrease the concentration of the sulfhydryl compounds responsible of the reduced odour (Lavigne & Dubourdieu, 1996). Barrel ageing on the lees is a traditional practice which allows detrimental mercaptans to be removed from the wine by such reaction. The lees addition in *barrique* as well as the wine maintenance on the yeast lees limit the thiols off-flavours formation and accumulation. The yeast lees lose the ability to produce sulfhydryl compounds in a few days due to the inactivation of the sulfite reductase enzyme exerted by the ethanol (Riberau-Gayon et al., 2006).
The protection exerted by mannoproteins against the mercaptans in wine causes the decrease of the mercaptans content and, at the same time, the protection of the varietal aromas (Lavigne & Dubourdieu, 1996). The presence of glutathione can carry out a synergic activity with the mannoproteins in protecting the wine aroma, limiting the formation of the atypical wine ageing compounds, as sotolon and 2-aminoacetophenone.
The disulfide bridges formation between the cysteinyl group and the mercaptan thiol can be enhanced by the ion copper (Cu^{2+}) present in wine, as indicated in the reaction as follows (Vessorot et al., 2003):

$$2CH_3\text{-}SH + 2Cu^{2+} \longrightarrow CH_3\text{-}S\text{-}S\text{-}CH_3 + 2Cu^{+} + 2H^{+}$$

The mechanisms involving the ion copper in the reaction protein cysteinyl-mercaptan thiols are:

- the ion copper is directly involved in the disulfide bridge formation (Figure 1.9);
- the ion copper can form insoluble complex, as in the following reactions:

  - $Cu^{+} + nCH_3SH \rightarrow [Cu(CH_3SH)n]^{+}$
  - $CH_3SH + Cu^{+} \rightarrow CH_3SCu + H^{+}$

The ion copper could play an essential role in removing the thiols off-flavours.

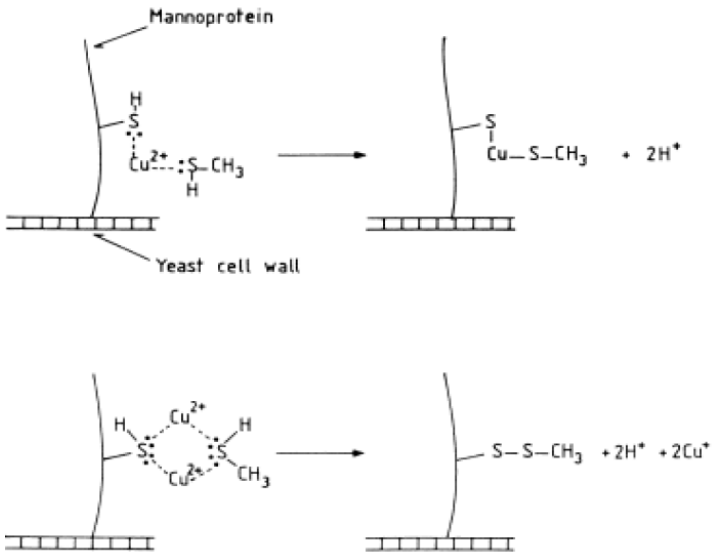

Figure 1.9: Chemical reaction proposed to explain the interactions between protein cysteinyl group and mercaptans thiol in presence of ion copper (Vesserot et al., 2003).

1.4.2.9 Adsorption of ocratoxin A

The ocratoxin A (OTA) is a mycotoxin produced by *Aspergillus* and *Penicillum* species. The must and wine contamination is mainly due to *A. carbonarius* which grow under incorrect cellar sanification and climatic conditions; it can be found particularly in the Mediterranean regions.

OTA is a cancerogenic, hepatotoxic and nephrotoxic mycotoxin; the maximum ingested amount allowed for the human health is 100 ng/kg body weigh weekly (FAO, 2001). The maximum admissible limit in wine is 2 μg L^{-1}, imposed from the vintage 2005 (UE Regulation n° 123/2005).

The OTA degradation mechanisms operated by yeast and bacteria are not well known; it is known the OTA removal is carried out either by the yeast or by the lactic bacteria and it is strain dependent. *S. cerevisiae* and *S. bayanus* can reduce the OTA concentration probably by adsorption on the cell-wall fractions (Bejaoui et al., 2004). The adsorption efficiency is correlated to the exposure of the yeast proteins to the heat which can probably increase the number of the available hydrophobic sites for the interaction with the mycotoxin as a result of the protein denaturation (Núñez et al., 2007). Besides the yeast protein addition, the OTA removal can be carried out using the bleaching carbon, through the ageing on the lees and the microfiltration. The bleaching carbon can reduce the OTA content (85-90%) and it has not negative effects on the colour and aroma content except in white wine, it adsorbs few fruity lipophlic aromas (Gambuti et al., 2005; Garcia-Moruno et al., 2005). This bleaching carbon addition is not allowed in red wine. The lees mannoproteins have absorption behavior toward the

OTA in wine (Caridi, 2006). The OTA concentration can decrease until 70% in 7 days after lees addition. The decrease is lower using lees deriving from red wine, probably for the OTA-phenols competition on the cell wall surface sites (Garcia-Moruno et al., 2005). Microfiltration is a process widely applied for the wine and it allows the concentration of OTA to decrease up to 80% (Gambuti et al., 2005). The ageing on the lees and the microfiltration could be a useful tool to remove the OTA from wine: since the mycotoxin is absorbed on the mannoproteins, it is removed with the yeast debris.

1.4.2.10 Antioxidant properties

The cysteinyl residues on the mannoproteins can exert antioxidant property (Jaehrig et al., 2007; Gallardo-Chacón et al., 2010), similarly to the antioxidant property already described for the glutathione (Paragraph 1.2.3.1).
The compounds having sulfhydryl groups in their structure can reduce the quinones, limiting the polymerization reactions and so the browning phenomena (Cheynier et al., 1990; Singleton et al., 1985). The cysteinyl thiols can bind to the oxidized caffeic acid, originating the 2-*S*-cysteinyl caffeic acid (Figure 1.10) (Bassil et al., 2005). This molecule is an antioxidant compound through the hydrogen atom liberation of the phenyl hydroxyl group. The cysteinyl sulfur reduces the dissociation energy of the hydroxyl group and the link is much weak (Bassil et al., 2005).
The mannoproteins can decrease the wine browning during the ageing and the shelf-life, the loss of grape varietal thiols and the slow down formation of sotolon, which is the major compound responsible of the atypical white wine ageing.
Nevertheless, little is known about the content of the antioxidant compounds, such as cysteinyl thiols, coming from protein, GSH in mannoproteins and YCWF commonly used, such as lysates, hulls and yeast extracts.

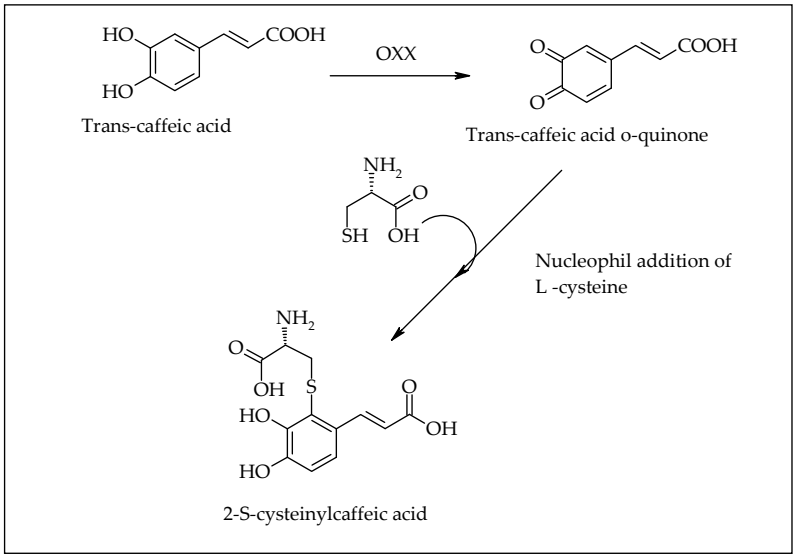

Figure 1.10: Reaction between caffeic acid and the cysteinyl group (Bassil et al., 2005).

1.4.3 Mannoprotein extraction

Yeast mannoproteins can be extracted by enzymatic treatments with glucanase, chelating agents (EDTA), dissociating and reducing agents (dithiothreitol, DTT), detergents (sodium dodecil sulphate (SDS) and β-mercaptoethanol), high temperature, mechanical treatment (French press) (Dupin et al., 2000a). The extraction methods with chemical compound are commonly use in laboratory scale, while the physical treatments are carried out to obtain these food additives industrially. The combined use of EDTA or DTT and glucanase allows a high extraction yields. The use of detergents, as hot SDS and β-mercaptoethanol, allows the mannoproteins linked to the cell wall through disulphide bridges and hydrogen bridges to be extracted (Bony et al., 1997). After the extraction, the mannoproteins are stirred, purified through affinity and ionic exchange chromatography and dried.
On industrial scale, the extraction is mainly performed by the β-1,3 glucanase enzymes which increase the cell wall degradation rate. The enzymes are from fungi (*Trichoderma harzianum*) or bacteria (*Arthrobacter luteus*). The lysis is conducted at pH values ranging from 4 to 10 and at 40-70°C for 1-24 hours (Lankhorst, 2006a; Lankhorst, 2006b). The heat treatment is also used: the yeast is treated at 138°C for 2 hours in citrate buffer at pH 7, centrifuged and the supernatant containing the mannoproteins is dried (Bertrand, 2003). The drastic heating conditions increase the Maillard reaction which can alter the colour and the structure with losses of positive oenological properties (Finot et al., 1981; Mauron, 1981). It starts with a condensation reaction between the carbonyl group of a reduced sugar and a free amino group of a protein or amino acid. The first stable molecule obtained is known as the Amadori compound. This molecule

has a negative sensorial effect described as toasted, caramelized (Finot et al., 1981; Mauron, 1981). This reaction can be continued by the Strecker degradation and the development of melanoidins responsible for the nitrogenous brown compounds. The furosine index showed to be an useful tool to evaluate the intensity of the Maillard reaction in food (Resmini et al., 1993; de Noni & Pagani, 2010). The furosine is one of the products obtained under controlled acid hydrolysis at high temperature of the Amadori compound (Finot et al., 1981; Mauron, 1981).

1.5 Analytical methods

Several analytical methods are described in literature for the thiols quantification either in proteins, from biological system and insoluble, or on grape, in must and wine. The thiols quantification carried out after a derivatization can be conducted by UV or fluorescence detector. No derivatized thiols are identified by mass spectrometry or atomic adsorption spectrometry.

1.5.1 Thiols quantification in proteins

The Ellman method is largely used for the thiol quantification in protein (Ellman, 1959). It is based on the reaction between the thiol groups with the 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). The products formed are a disulfide compound and the 5-thio-2-nitobenzoic acid (TNB^-), which is a yellow cromophor molecule adsorbing at 412 nm wavelength (Figure 1.11). This analytical method is fast and easy-to-apply. It allows the quantification of the overall concentration of the thiol groups in the solution but it is not selective and the quantification of a single thiol compound is not possible. The Ellman method found widely application for the thiols determination in biological proteins which are usually water or buffer soluble (Patsoukis & Georgiou, 2004; Rogers et al., 2006; Scampicchio et al., 2007).

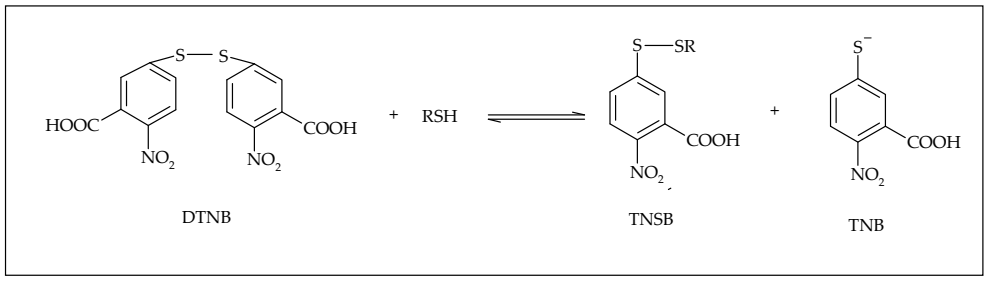

Figure 1.11: Principle of the Ellman reactant (Ellman, 1959).

The thiol quantification for insoluble proteins could be carried out as described by Kin-Yu and Wassermann (1993). The method was applied for the insoluble cereal proteins and it could find applications also for the thiols quantification in insoluble YCWF, as

hulls and lysates. The method is also based on the thiol compounds determination by the Ellman reagent, as described above. The proteins were dissolved in a buffer containing denaturing agents such as EDTA and SDS, in order to allow the reaction between the DTNB and the protein cysteinyl residues.
The protein thiols quantification can be also carried out using the monobromobimane (mBB) as derivatizing compound. After the derivatization, the thiols could be separated and quantified by high-performance liquid chromatography (HPLC). The analysis allows the identification of soluble thiols, as Cys, GSH, and protein thiols (Cotgreave & Moldéus, 1986; Fahey & Newton, 1995; Perez-Rama et al., 2005; Petrotchenko et al., 2006). This derivatizing compound was used for biological protein. Moreover, the mBB has low specificity and unknown peaks could appear in the chromatogram; its can react with other nucleophilic compounds, such as amines. This derivating compound requires the separation and the identification of all the protein fractions (Fahey & Newton, 1995). The mBB is photodegradable: reaction and HPLC analysis have to be carried out as fast as possible, otherwise the thiol content could be underestimated even of the 50% for the high instability of mmB and its reaction adducts (Radkowsky et al., 1986).

1.5.2 Glutathione quantification in must and wine

Several analytical methods are described in literature for the quantification of GSH on grape, in must and wine using different analytical techniques.
GSH was quantified after a treatment with glutathione reductase enzyme in white wine (Cassol & Adams, 1995). In grapevine tissues, the GSH was derivatized with DTNB after the enzymatic treatment and the detection was performed spectrophotometrically at 412 nm (Adams & Liyanage, 1991). The determination of total GSH could be performed after the enzymatic treatment which allows the breakdown of disulfide bridges.
The GSH could be derivatized pre-column with *o*-phthalaldehyde and quantified by HPLC; the detection was than conducted by fluorescence (Park et al., 2000b; Janeš et al., 2010).
The use of the fluorescence detector allowed the GSH quantification after derivatization with 2,3-naphthalenedialdehyde (NDA), as described by Marchand and de Revel (2010). The technique involved the pre-column derivatization and the separation by HPLC. The determination of oxidized glutathione was also allowed by enzymatic treatment with glutathione reductase.
The fluorescence detector was employed for the GSH determination after its derivatization with monobromobimane (mBB); the reaction adduct was identified by capillary electrophoresis (Lavigne et al., 2007). As described above, the mBB is photodegradable and a GSH content underestimation could occur (Radkowsky et al., 1986).

The method proposed by du Toit et al. (2007) was based on liquid chromatography coupled with a mass spectrometry detector (LC-MSMS). The determination of reduced and oxidized glutathione was allowed in the same run. The ethanol present in wine had to be removed prior the analysis and this step could cause an underestimation of the GSH content.
Recently, an analytical method for the GSH quantification was developed using the atomic adsorption spectrometry (Bramanti et al., 2008). The method is based on the reactivity of the mercury toward the thiols.
These analytical methods are hardly applicable for the routine analysis which are performed in enological laboratory either for the availability or for the cost of the instruments employed as well as for the detector. Moreover, some of these analytical techniques require highly qualified staff which is an extra cost for the cellars or the analytical laboratories.

1.6 References

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2 AIMS OF THE STUDY

This PhD research was aimed to evaluate the antioxidant properties of some commercial yeast cell wall fractions, comprehending mannoproteins, hulls, lysates and extracts. The antioxidant activity is correlated to the thiol compounds content, such as cysteine, free and linked to the proteins, and reduced glutathione. The thiols quantification was first approached by the method proposed by Ellman (1959) for the biological proteins, but the commercial yeast fractions produce yellow coloured solutions. Moreover, some of them are insoluble and the analytical response is not repeatable. Similar problems occurred with the analytical procedure proposed for the insoluble proteins (Kin-Yu Chan & Wasserman, 1993). The development of a novel analytical method applicable either for soluble or for insoluble yeast preparations was requested in order to identify and quantify the thiol compounds of some enological adjuvants. This method allowed the separation and identification of cysteine, free and linked to the proteins, and reduced glutathione by high performance liquid chromatography (HPLC) and UV detection.

Some commercial yeast cell-wall fractions were characterized through their cysteinyl thiol content. The heat damage was also evaluated and it was correlated to the antioxidant properties. It was quantified by the furosine index (Resmini et al., 1990), in order to understand the eventual influence of the industrial preparation on the thiols content of the yeast cell-wall fractions.

The same analytical approach employed for the yeast cell-wall fractions was validated for the reduced glutathione quantification in grape juice, must and wine. The preparation of grape juice sample was developed and a few devices were necessary for the must and wine sample preparation. The glutathione level was monitorated during several winemakings conducted in real condition process in order to understand what parameters and technological phases could affect its content before, during and at the end of the alcoholic fermentation.

The antioxidant properties of GSH were investigated in correlation to the sulfur dioxide, oxygen and the main phenol compounds content in white wine. The oxidation rate was evaluated to deepen the interactions between phenols, glutathione, sulfur dioxide and oxygen occurring during the white wine ageing and the shelf-life. Several researches were conducted for a better comprehension of the reaction mechanisms involving glutathione and phenols in model wine solution (Singleton, 1987; Cheynier et al., 1988; Cheynier et al., 1989; Danilewicz, 2007) and wine (Singleton et al., 1985; Singleton, 1987; Danilewicz et al., 2008), but none of them regarded the interactions between sulfur dioxide, glutathione, phenols and oxygen in white wine. For this purpose, two different experiments were carried out on Sauvignon blanc wine to evaluate the caffeic acid and phenols oxidation.

2.1 References

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3 RESULTS AND DISCUSSION

3.1 Antioxidant properties evaluation of yeast cell-wall fractions

The evaluation of the antioxidant properties of 14 commercial yeast cell-wall fractions, like mannoproteins (4), hulls (4), lysates (4) and extracts (2) were evaluated, as well as of 16 active dried yeasts. The antioxidant properties were related to the thiol compounds quantification, which were correlated with the heat damage.
A novel analytical approach was developed for the thiols determination of these preparations and an analytical method was validated, excluding the influence of some interferences, such as disulfide bridges and carbohydrate content, in the analytical response.

3.2 Materials and methods

3.2.1 Chemicals

Cysteine (Cys), 3-mercaptopropionic acid (3MPA), p-benzoquinone (pBQ) and potassium metabisulfite ($K_2S_2O_5$) were purchased from Fluka (Switzerland). Reduced glutathione (GSH), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), guanidine, sodium dodecyl sulfate (SDS), starch, caffeic acid and trifluoroacetic acid (TFA) were from Sigma-Aldrich (St. Louis, MO). Boric acid was purchased from Carlo Erba (Milan, Italy). Tris-HCl and urea were from Plus One, Pharmacia Biotech AB (Upsala, Sweden). Citric acid was purchased from J. T. Baker (Phillipsburg, NJ). HPLC grade methanol was purchased from Panreac (Barcelona, Spain), and HPLC water was obtained by Milli-Q system (Millipore Filter Corp., Bedford, MA).

3.2.2 Yeast cell wall fractions

3.2.2.1 Samples

The following commercial samples of yeast fractions for oenological use supplied by six different producers as dried products with unknown compositions were evaluated: 4 mannoproteins, 4 hulls, 4 lysates, and 2 extracts. Additionally, 16 commercial dry yeast starters employed in winemaking were evaluated: 11 strains were from Lallemand Inc. (Ontario, Canada), and 5 strains were from Dal Cin (Milan, Italy).

3.2.3 Determination of thiol compounds by the Ellman's method

The thiol compounds were quantified through the derivatization with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), as described by Ellman (1959).

3.2.3.1 Derivatization in boric acid buffer

The derivatization was carried out as follows: the sample, at concentration ranging from 20 g L^{-1} to 50 g L^{-1}, was dissolved in a boric acid buffer 0.3 M at pH 8. Two mL of sample was drawn and the derivatization was conducted adding 100 μL of DTNB 20 mM dissolved in water. The reaction mix was read by the spectrophotometer at 412 nm after one minute shaking. The blanc was prepared with 2 mL of boric acid buffer 0.3 M at pH 8 and 100 μL of DTNB 20 mM.

3.2.3.2 Derivatization in potassium phosphate buffer

The derivatization was carried out as follows: the sample, at concentration ranging from 20 g L^{-1} to 50 g L^{-1}, was dissolved in a potassium phosphate buffer 0.1 M at pH 7.4. The buffer was added with guanidine 8 M if the analyzed sample was a protein. Two mL of sample was drawn and the derivatization was conducted adding 100 μL of DTNB 20 mM dissolved in water. The reaction mix was submitted to the spectrophotometric evaluation at 412 nm after one minute shaking. The blanc was prepared with 2 mL of potassium phosphate buffer 0.1 M at pH 7.4 and 100 μL of DTNB 20 mM.

3.2.3.3 Derivation in denaturizing buffer

The method was applied for the thiols quantification in insoluble cereal proteins (Kin-Yu Chan & Wasserman, 1993). The derivatization was conducted as described as follows: the sample (50–100 g L^{-1}) was weighted in a small tube (2 mL) and it was dissolved in a buffer contained DTNB 10 mM, urea 8 M, EDTA 2 mM, Tris-HCl 0.2 mM and SDS 1% (reaction buffer). The sample was left under nitrogen flow in a dark place at room temperature for the following incubation times: 30 minutes, 45 minutes, 60 minutes and 75 minutes. The sample was centrifuged at 14000 rpm in a microcentrifuge. Two hundred microliters were drawn and diluted in 1.8 mL of a buffer prepared with urea 8 M, EDTA 2 mM, Tris-HCl 0.2 mM and SDS 1% (dilution buffer) and the spectrophotometric reading was carried out at 412 nm after shaking. The blanc was prepared with 1.8 mL of dilution buffer and 200 μL of reaction buffer.

3.2.4 Quantification of thiol compounds derivatized with *p*-benzoquinone

3.2.4.1 Sample preparation

The insoluble samples were treated as described by Tirelli et al. (2010): at least 50 mg of insoluble sample (active dried yeasts, yeast hulls, and yeast lysates) were dispersed in 50 mM of citrate buffer, pH 5.0, to obtain a 2 mL suspension with sample concentrations in the range 20-100 g L^{-1}. The dispersed sample was centrifuged at 5000 *x g* for 15 min at 15°C by a thermostatted centrifuge (Sorvall, Thermo). The supernatants were diluted 1-10 fold, and 2 mL was submitted to derivatization. The

precipitated material was re-suspended in 5 mL of 50 mM citrate buffer, pH 5.0, and submitted to the derivatization reaction. One milliliter of the derivatized suspension was centrifuged at 14000 rpm for 5 min at 25°C by a thermostatted benchtop centrifuge (Hettich), and the supernatant was submitted to HPLC separation. To recover the derivatized 3MPA potentially adsorbed on the insoluble yeast fraction, the precipitated material was rinsed with 2 mL of 0.1% hydrochloric ethanol as described by Ummarino et al. (2001), carefully re-suspended, and centrifuged at 14000 rpm for 5 minutes. The supernatant was dried under vacuum, re-dissolved in 1 mL of water, and injected into HPLC.
Mannoproteins and yeast extract samples were prepared by dissolving 100-200 mg of sample in 2 mL of 50 mM citrate buffer, pH 5.0. The solution was derivatized and then ultrafiltered using 3 kDa cutoff Microcon membranes (Millipore) at 14000 rpm for 100 min at 25°C by the thermostatted benchtop centrifuge. The permeate was submitted to HPLC separation. The retentate was added with 1 mL of 0.1% hydrochloric ethanol and centrifuged at 14000 rpm for 5 min. The supernatant was dried under vacuum, and the dried material was re-dissolved in 500 μL of water before the HPLC separation.
Each sample was analyzed at least in duplicate.

3.2.4.2 Derivatization of thiol compounds with p-benzoquinone

The derivatization of GSH and Cys was conducted as described by Tirelli et al. (2010): 2 mL of the samples were added to 100 μL of 400 μM pBQ dissolved in methanol. After 1 minute mixing, 1 mL of 500 μM 3MPA in 0.3 M citrate buffer pH 3.5 was added in order to remove the exceeding pBQ.

3.2.4.3 Calibration curve

The calibration curve were prepared diluting a solution containing Cys and GSH at concentration levels in the range 0 μM to 100 μM in citrate buffer 50 mM at pH 5. The standards derivatization was carried out as described as above (Paragraph 3.2.4.2).

3.2.4.4 Evaluation of matrix effects on the thiols determination

One sample of yeast lysate (L2) containing GSH and protein Cys was assayed with or without addition of 30 g L^{-1} or 80 g L^{-1} of amylase or caramelized amylose in order to evaluate possible interferences arising from high polysaccharide contents. The caramelization was obtained by exposing 2 g of amylose suspended in 2 mL of 0.1% HCl at 110°C for 24 h.
Interference of protein disulfides (cystine) was evaluated by submitting the L2 sample dispersed in a solution 5 g L^{-1} of lysozyme prepared in 50 mM citrate buffer at pH 5.0, to protein Cys evaluation.

3.2.4.5 Precision parameters

The repeatability of the Cys residues was assessed by assaying one sample of yeast hulls and two samples of yeast lysates to five determinations each. The response linearity of the method was evaluated for reduced protein cysteine (RPC) concentrations up to 180 μM by dispersing 27-100 g L^{-1} of a yeast hull sample containing 0.18 mmol RPC/100 g product in the 50 mM citrate buffer. The response linearity of the method for higher RPC concentrations (210-800 μM) was attained by analyzing dispersions (10-40 g L^{-1}) of a yeast hull sample containing 2.1 mmol RPC/100 g product.

3.2.4.6 High-performance liquid chromatography (HPLC) analysis

The HPLC separation was performed by a hexyl-phenyl column (250 x 4.6 mm, 110Å, Phenomenex). Eluents were water/trifluoroacetic acid 0.05% (v/v) and methanol; the concentration of the latter increased from 10% to 35% of methanol in 18 minutes in the eluting gradient (Table 3.1). The column temperature was 25°C and the injection volume was 50 μL. Detection was carried out by spectrophotometry at 303 nm. The flow rate was 1 mL/min.

Time (min)	A%	B%	Slope
0.0	90.0	10.0	6
1.0	90.0	10.0	6
18.0	65.0	35.0	6
18.5	0.0	100.0	6
21.0	0.0	100.0	6
21.5	90.0	10.0	6
36.0	90.0	10.0	6

Table 3.1: HPLC separation gradient for thiol compounds derivatized with *p*-benzoquinone. Eluent A: water/trifluoroacetic acid 0.05% (v/v); eluent B: methanol.

3.2.4.7 HPLC/Electrospray Ionization-Mass Spectrometry (ESI-MS)

For MS detection of the hydroquinone substitutes, the LCQ Deca XP spectrometer, controlled by the Excalibur software (Thermo Finnigan, San Jose, CA), was operated in positive ion mode. A post column flow splitter was used to introduce 1:15 of the HPLC flow stream into the ESI source. The ESI interface and the ion optics settings were as follows: spray potential, 5.0 kV; nebulization gas (nitrogen) relative flow value, 10; capillary temperature, 275°C; and cone voltage, 30 V. Full-scan mass spectra were acquired scanning the range 50-800 m/z. Mass accuracy was ensured by calibration with a mixture of caffeine, reserpine, and the tripeptide PFK (in methanol:water 1:1, 0.1% acetic acid) infused separately.

3.2.4.8 Quantification of thiol compounds content

Cys and GSH were quantified chromatographically by the external standard method in both the soluble and the insoluble fractions. The concentration of cysteine linked to the protein in the commercial preparations was calculated as follows:

$$[Cys_{prot}] = [pBQ] - ([Cys_{free}] + [HQ] + [GSH] + [3MPA_{derivatized}])$$

Where:
[pBQ]: concentration of pBQ used for the derivatization;
[Cys]: concentration of Cys quantified chromatographically;
[GSH]: concentration of GSH quantified chromatographically;
[3MPA-HQ]: concentration of S-3-mercaptopropionyl-hydroquinone quantified chromatographically;
[pHQ]; concentration of the underivatized hydroquinone quantified chromatographically.

3.2.5 Quantification of protein content

The quantification of the protein content was determined using the Kjeldahl method, as described in the regulation ISO 8968-1 | IDF 20-1: 2001.
The procedure is indicated as follows: 200 mg of yeast preparation were hydrolyzed in 8 mL HCl 8N at 110°C for 23 hours. Three mL of the filtered hydrolyzed sample were drawn and about 10 mg CuO (a spatula tip) as reaction catalyst, 15 mL H_2SO_4 0.1 M and a spatula tip of K_2SO_4 were added. The sample was boiled until its colour changed from dark brown, to limpid green. The ammonium ions dissolved in the solution obtained was added with concentrated NaOH up to pH 14 and stripped by steam into a 8 mL H_2SO_4 0.05 N solution and phenolftalein as reaction indicator. The acid distilled sample was titrated with NaOH 0.05 N until the colour becomes green-grey from pink-violet.
The protein content was calculated as follows:

$$Total\ protein\ (mg) = [(mL\ H_2SO_4\ \times F1) - (NaOH\ \times F2)] \times N \times 14 \times 6.25 \times {}^{8}/_{3}$$

Where:
F1: factor of H_2SO_4 solution, concentration dependent (H_2SO_4 0.05 N = 0.995);
F2: factor of NaOH solution, concentration dependent (NaOH 0.05 N = 0.997);
N: normality titrant;
14: nitrogen molecular weight;
6.25: conversion factor from nitrogen to protein;
8/3: dilution factor.

3.2.6 Quantification of furosine content

The quantification of the furosine content was determined as described in the regulation ISO 1832-9 | IDF 19-3: 2004.
The procedure is indicated as follows: 0.5 mL of filtered hydrolyzed (Paragraph 3.2.5) was purified on SPE cartridge C18 and eluted with 3 mL water.
The furosine was determined by HPLC, as described by Resmini et al. (1990). The separation was carried out on C8 furosine-dedicated column (250 x 4.6 mm, 5 μm, Grace). Eluents were water/acetic acid 0.4% (v/v) and 3 g L^{-1} potassium chloride/acetic acid 0.4% (v/v); the concentration of the latter increased from 0% to 50% of methanol in 19.5 minutes in the eluting gradient (Table 3.2). The column temperature was 30°C and the injection volume ranged from 20 μL to 50 μL. Detection was carried out by spectrophotometry at 280 nm. The flow rate was 1.2 mL/min.
The furosine index (FI) was calculated as follows:

$$FI = At \times \frac{c}{Af} \times \frac{1}{V} \times \frac{d}{F} \times \frac{M}{10} \times \frac{8}{4 \times m}$$

Where:
At: area value of the furosine peak;
c: furosine concentration in standard solution expressed in pmol;
Af: area value of standard furosine peak;
V: injection volume;
d: dilution factor for the purification;
F: recovery factor of the SPE cartridge C18;
M: furosine molecular weight (254);
m: protein content expressed in mg.

The furosine content was expressed as mg of furosine in 100 g of proteins.

Time (min)	A%	B%	Slope
0.0	100.0	0.0	6
12.5	100.0	0.0	6
19.5	50.0	50.0	6
22.0	50.0	50.0	6
24.0	100.0	0.0	6
32.0	100.0	0.0	6

Table 3.2: HPLC separation gradient for the furosine quantification. Eluent A: water/acetic acid 0.4% (v/v); eluent B: 3 g L^{-1} potassium chloride/acetic acid 0.4% (v/v)

3.2.7 Quantification of overall cysteine

The overall cysteine was quantified trough its derivatization with Dabsyl chloride (Figure 3.1) and identified by HPLC, as described by Krause et al. (1995).
The acid hydrolyzed obtained for the proteins quantification (see paragraph 3.2.5) was dried under vacuum and re-dissolved in 0.15 M sodium carbonate buffer pH 8.6. Three hundred sixty microlitres of sample was added with 400 µL dabsyl chloride 0.4% (w/v) dissolved in acetone. The sample was left at 70°C for 1 hour, shaking it after 1 minute and 10 minutes of incubation; its colour changed to orange. The sample was cooled in ice for 10 minute and it was added with 800 µL of buffer containing acetonitrile:ethanol:eluent A 2:1:1 (v/v/v). It was filtered prior the HPLC injection.

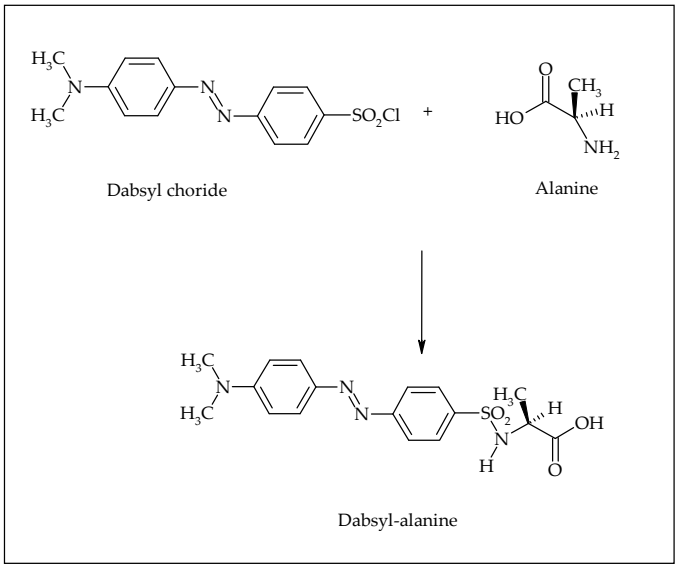

Figure 3.1: Reaction scheme of the derivatization with Dabsyl chloride.

The HPLC separation was performed by a C18 column (150 x 3.9 mm, 4 µm, Novapack, Waters). The eluents were citric acid 9 mM added with dimethylformamide 4% and triethanolamine 0.1-0.2% prepared at pH 6.55 using NaOH 1 M (eluent A), acetonitrile:water 80:20 (eluent B) and acetonitrile (eluent C). The column was maintained at 50°C and the injection volume was 20 µL. Detection was carried out by spectrophotometry at 436 nm. The flow rate was 1 mL/min and the eluting gradient is indicated in Table 3.3.

Time (min)	A%	B%	C%
0.0	70.0	30.0	0.0
3.0	70.0	30.0	0.0
15.0	60.0	40.0	0.0
44.0	0.0	100.0	0.0
45.0	0.0	100.0	0.0
45.5	0.0	0.0	100.0
50.0	0.0	0.0	100.0
51.0	0.0	100.0	0.0
52.0	70.0	30.0	0.0
60.0	70.0	30.0	0.0

Table 3.3: HPLC separation gradient for the overall cysteine derivatized with Dabsyl chloride. Eluent A: citric acid 9 mM, dimethylformamide 4%, triethanolamine 0.1-0.2%, pH 6.55; eluent B: acetonitrile/water 80/20; eluent C: acetonitrile.

3.3 Results and discussion

3.3.1 Ellman's method approach

The analytical method described by Ellman (1959) was widely employed for the thiols quantification in biological system (Patsoukis & Georgiou, 2004; Rogers et al., 2006; Scampicchio et al., 2007) because the method is both easy-to-apply and reliable for the thiols determination. The method is based on the reaction between the thiols and the 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) generating disulfide compound and the 5-thio-2-nitobenzoic acid (TNB) (Figure 1.11). The latter can be revealed at 412 nm.
As first, the method was tested for the quantification of 3-mercaptopropanoic acid (3MPA); the 3MPA was dissolved in a boric acid (HBO_3) buffer. The calibration curve for 3MPA concentration ranged from 20 μM and 200 μM gave linear response (Figure 3.2) and the method showed good repeatability (data not shown). The method was not applicable for the determination of Cys dissolved in boric acid buffer as well as for the YCWF, probably for the buffer employed. Moreover, some YCWF could originate a coloured solution and some of those preparations were insoluble. The absorbance could be overestimated due to either the natural colour or the insolubility of the commercial samples.

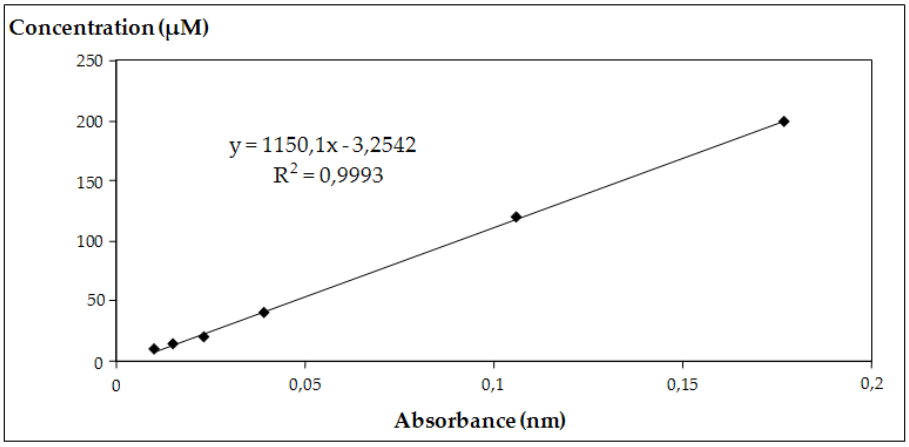

Figure 3.2: Calibration curve of 3-mercaptopropanoic acid dissolved in boric acid buffer with DTNB.

The procedure as above was modified changing the buffer: a potassium phosphate (K_2HPO_4) 0.1 M was employed at pH 7.4. The buffer was also added with guanidine 6M; it is a denaturizing agent of the protein (Aitken et al., 2002), promoting the thiol groups exposition and it helps the polysaccharides solubilization. This compound could exert an essential function for the quantification of the thiols linked to the proteins of the yeast cell-wall fractions. The calibration curve was obtained using Cys dissolved in a potassium phosphate buffer (0.1 M, pH 7.4); the Cys concentrations ranged from 5 µM to 40 µM gave a linear response (Figure 3.3).

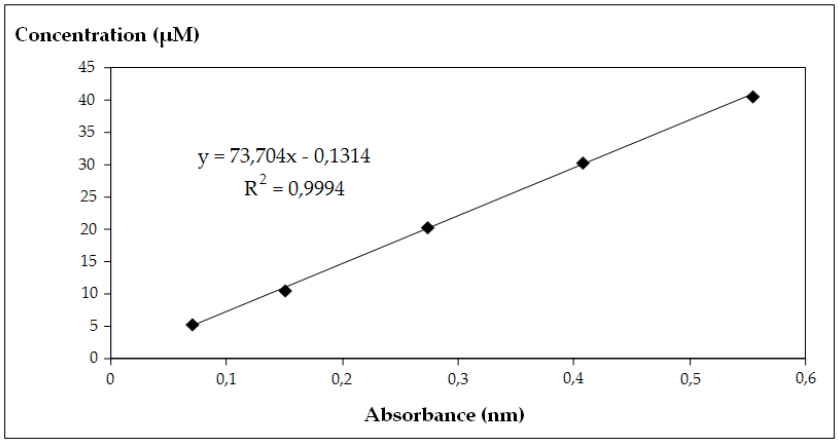

Figure 3.3: Calibration curve of cysteine dissolved in potassium phosphate buffer with DTNB.

As shown in Figure 3.2, the Ellman method was more sensitive using the potassium phosphate buffer than with the acid boric buffer and the quantification of lower amount of cysteine was allowed in potassium phosphate buffer. The maximum absorbance was reached in 5 minutes after the reaction (Aitken et al., 2002). The absorbance spectra were recorded for 30 minutes after the reaction and the maximum was not achieved in 5 minutes, but the absorbance increased over the 30 minutes registration in some mix reactions with the YCWF. The method was not repeatable in potassium phosphate buffer.
In order to resolve the quantification troubles due to the insolubility and the coloured solution, the procedure described by Kin-Yu Chan and Wasserman (1993) was followed. The method was based on the thiols quantification with DTNB in cereal proteins, insoluble. The reaction was carried out in a buffer contained Tris-HCl, EDTA, urea and SDS and the spectrophotometric measurement was carried after 30, 45, 60 and 75 minutes of incubation under nitrogen flow and in a dark place. The calibration curve gave a linear response for Cys concentration values ranged from 2.5 μM to 40 μM (Figure 3.4) and the correlation coefficient was high (R^2=0.9984).

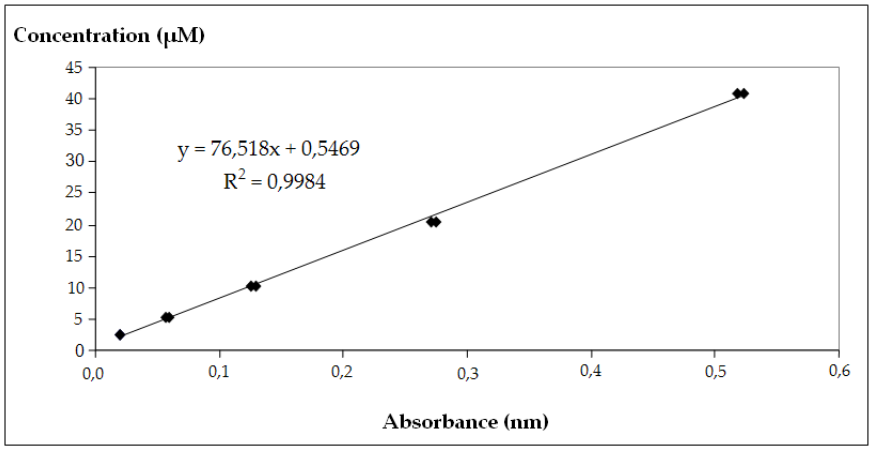

Figure 3.4: Calibration curve of cysteine according to the procedure described by Kin-Yu Chan and Wasserman (1993).

The procedure proposed by Kin-Yu Chan and Wasserman (1993) was applicable for samples containing 7% of protein, at least. In order to verify the method applicability for YCWP, the sample amount corresponded to the protein concentration indicated by the authors.
Moreover, the presence of SDS into the reaction buffer could increase the thiol compounds reactivity. A mannoprotein and some hull samples were dissolved in a buffer with and without SDS addition and the absorbances detected were higher in the buffer added with SDS as shown in Figure 3.5 and 3.6.

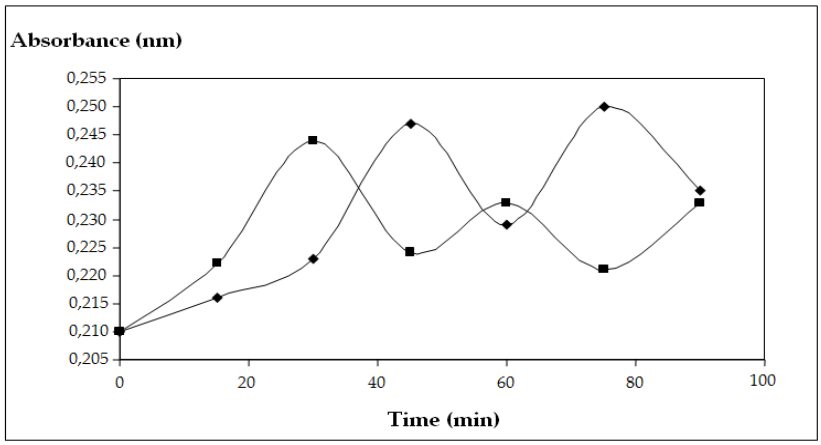

Figure 3.5: Comparison of thiols detected using a buffer added with SDS in a mannoprotein M2 (♦) and hull H2 (■) samples.

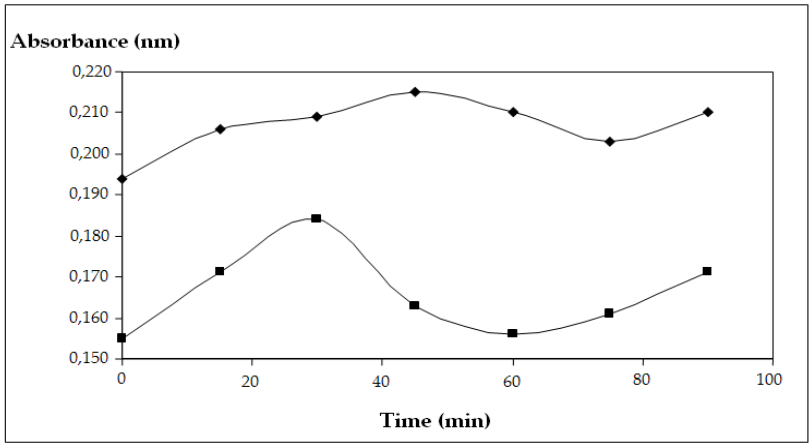

Figure 3.6: Comparison of thiols detected by a SDS free buffer in mannoprotein M2 (♦) and hull H2 (■) samples.

The sample could affect the reaction time: for the mannoprotein, the maximum absorbance was detected after 75 minutes incubation, while for the hull the maximum was reached after 30 minutes. The maximum absorbance was not repeatable and the standard conditions for thiol content determination could not be identified.

3.3.2 Analytical approach with *p*-benzoquinone

In literature the reaction between thiol group and *p*-benzoquinone (pBQ) is known: it is a nucleophilic addition to an unsatured carbonyl (Jocelyn, 1972). The analytical approach proposed was based on this reaction, as represented in Figure 3.7.

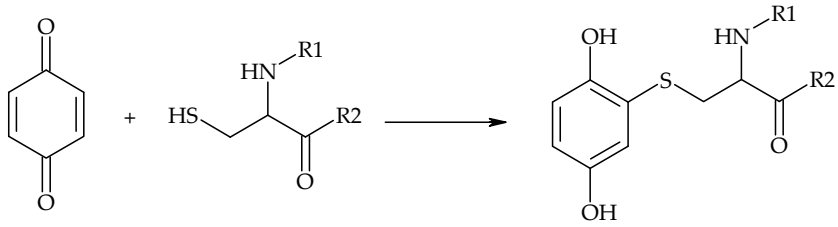

Figure 3.7: Reaction scheme between thiols and *p*-benzoquinone.

The thiol property to reduce quinones to thiol-substituted hydroquinones was adopted to assess the Cys residues in the yeast cell-wall fractions (YCWF), as it spontaneously occurs for the *o*-quinones in must and wine (Salgues et al., 1986). Cys residues include GSH, free Cys and Cys linked to the proteins. The symmetric pBQ was used as a derivatizing agent to prevent the formation of multiple hydroquinone derivatives and to obtain the single mono derivative for each thiol molecule. The molecular size of pBQ is comparable to that of the *o*-quinones detectable in must and wine, and it can react with the thiol residues actually accessible in the glycoprotein structures. The formation of thiol-substituted hydroquinones was fast and stoichiometric at room temperature and it allowed Cys and GSH to be detected spectrophotometrically as S-cysteinyl-*p*-hydroquinone (Cys-HQ) and S-glutathionyl-*p*-hydroquinone (GSH-HQ), respectively. The thiol derivatization procedure was followed by the addition of 3MPA (in excess) to remove the exceeding amount of pBQ. The latter could be reduced to pHQ by oxidation of the thiol-substituted hydroquinones to thiol-substituted *p*-quinones, which are able to bind a further thiol molecule, so forming a dithiol-substituted hydroquinone (Cilliers & Singleton, 1990). Moreover, pBQ can readily polymerize to produce brown compounds. Such thiol-substituted hydroquinones, including S-mercaptopropionyl-p-hydroquinone (3MPA-HQ), showed a maximum absorption at 303 nm wavelength (Figure 3.8).

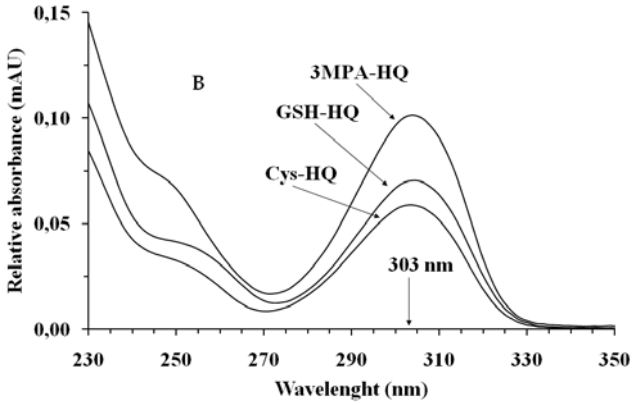

Figure 3.8: UV spectra for S-cysteinyl-*p*-hydroquinone (Cys-HQ), S-glutathionyl-*p*-hydroquinone (GSH-HQ) and S-mercaptopropionyl-*p*-hydroquinone (3MPA-HQ).

The formation of Cys-HQ and GSH-HQ in citrate buffer solution/suspension containing Cys and GSH (Figure 3.9) or yeast fractions (Figure 3.10) was confirmed by HPLC-ESI/MS (Figure 3.11). The data obtained showed the peak eluted at 6 minute was 230 g mol^{-1} and the peak eluted at 8 minute was 416 g mol^{-1}, corresponding to Cys-HQ and GSH-HQ, respectively. The 3MPA-HQ was not identified in HPLC-ESI/MS, probably because it is a non protonable molecule under the conditions adopted. The HPLC pattern of Figure 3.9 also shows the formation of both pHQ and minor amounts of dithio-substituted hydroquinones following the derivatization reaction. The latter compounds were confirmed by HPLC-ESI/MS analysis; the molecular weight of 520 g mol^{-1} and 624 g mol^{-1} were observed corresponding to dithio-substituted hydroquinones. The exceeding amount of pBQ could be effectively removed by the addition of sulfur dioxide, but the amount of the products obtained after such a reaction is affected by a number of factors (Wedzicha, 1984). On the contrary, the addition of 3MPA allows us to verify whether the amount of pBQ exceeds the content of Cys residues and to assess the level of residual pBQ after thiol derivatization. As a result of the developed analytical approach, the amount of the reduced protein cysteine (RPC) was calculated by the difference between the amount of added pBQ and the amounts of GSH, Cys, pHQ, and 3MPA-HQ chromatographically evaluated.

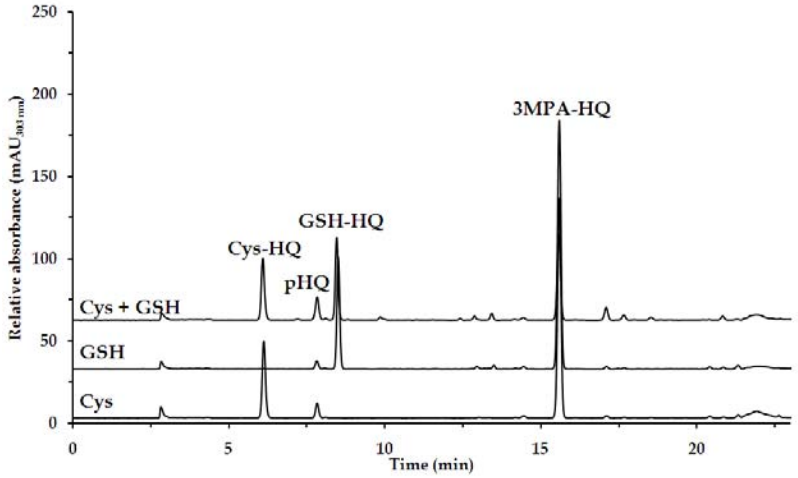

Figure 3.9: HPLC separation of Cys-HQ, GSH-HQ, 3MPA-HQ and pHQ obtained from derivatization with pBQ of standard water solutions of Cys, GSH and their mixture.

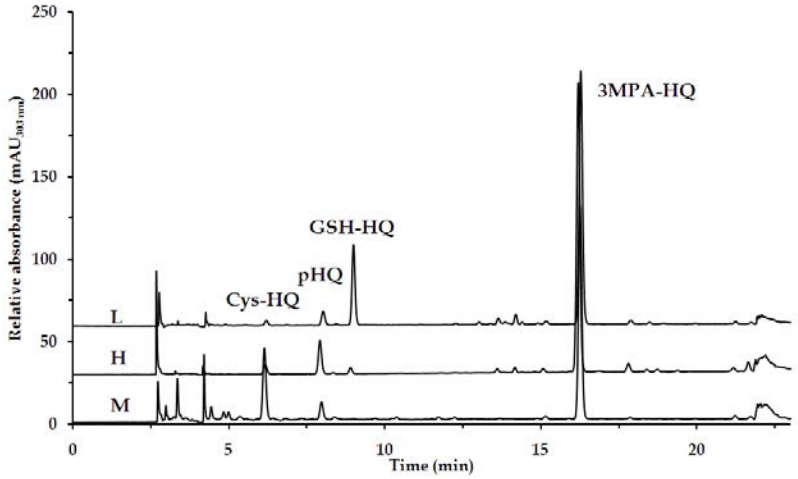

Figure 3.10: HPLC separation of Cys-HQ, GSH-HQ, 3MPA-HQ and pHQ obtained from derivatization with pBQ of mannoprotein sample added with Cys (M), yeast lysate (L), and yeast hull (H).

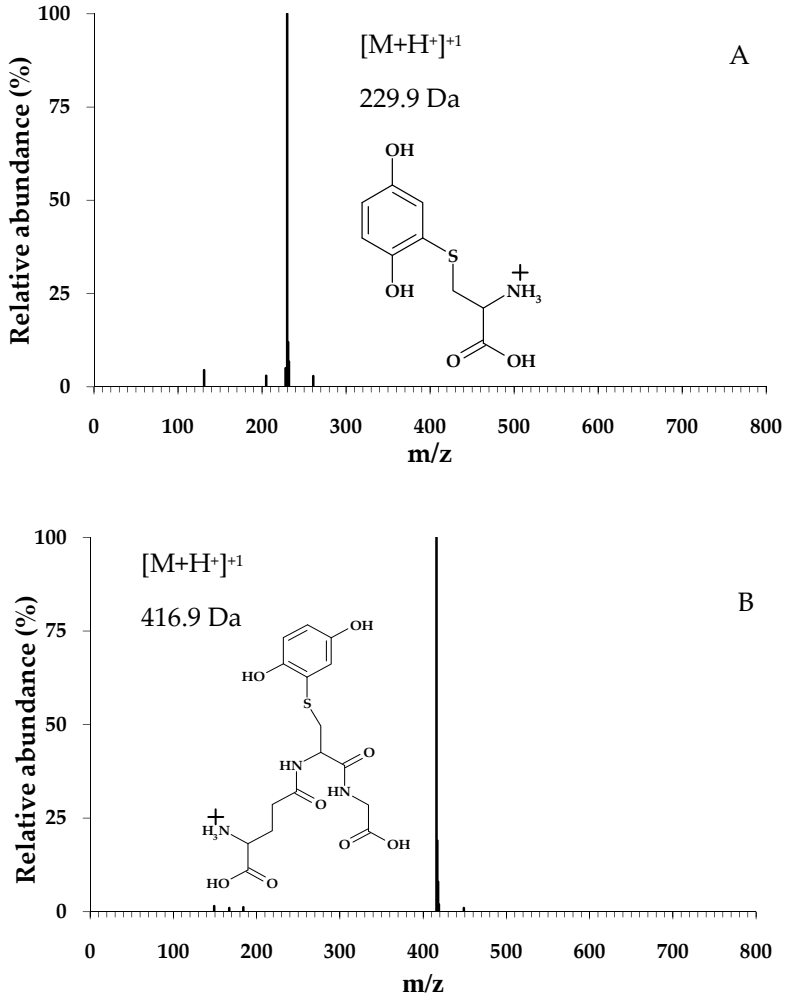

Figure 3.11: HPLC-ESI/MS spectra of Cys-HQ (A) and GSH-HQ (B).

The pH carries an important role on the derivatization efficiency. Assay were performed using citrate buffer at pH 3, 5, 7 and 9 and the maximum response was observed at pH 5 (data not shown). At the same time, the derivatization was also checked at different pH values of the 3MPA dissolved buffer, such as 3, 3.5, 4, 4.5 and 5; the maximum response was revealed at pH 3.5 (data not shown).
The HPLC separation was conducted using different separation columns:

- Nova-Pak C18, 4 μ, 3.9 x 150 mm;
- XBridge C18, 5 μ, 4.6 x 150 mm;
- PLRP-S, 300Å, 5 μ, 4.6 x 250 mm;
- C6-Phenyl, 110 Å, 4.6 x 250 mm.

he best separative performance was obtained with either with the PLRP-S column or with the C6-phenyl column; the latter was choosen due to its higher efficiency and the similarity between its stationary phase and the compounds formed with the derivatization.
Because the insoluble cell wall fractions were submitted to derivatization with pBQ after removal of the soluble Cys residues, the amount of pBQ reacted with γ-glutamyl-Cys and cysteinylglycine potentially present in yeast preparations was assumed as negligible. Such dipeptides represent about 4% of GSH in yeast (Rao et al., 2010); therefore, their contribution was not taken into account also for the MP samples where the nonproteinaceous Cys thiols were never detected. The amounts of dithiol-substituted hydroquinones were not included in the calculation since their values were close to the limit of quantification (signal-to-noise ratio >10). The precision parameters for the quantification of Cys, GSH, and RPC were assessed by analyzing one sample of yeast hull and two samples of yeast lysates (Table 3.4). An average value of 5.4% can be assumed for the relative standard deviation (RSD) of Cys thiol concentrations exceeding 10 μM.

	Cys			**GSH**			**RPC**		
	S4	L2	L4	S4	L2	L4	S4	L2	L4
	43.3	0.0	1.8	13.4	10.0	92	173	266	246
	43.0	0.0	2.1	15.0	9.7	101	171	299	241
	43.8	0.0	2.9	16.3	10.4	102	165	311	254
	44.2	0.0	2.9	13.8	11.0	103	164	286	269
	43.1	0.0	1.9	14.6	11.3	89	186	280	248
Mean	43		2.3	14.6	10.5	97	172	288	252
R.S.D.	1.2		23.4	7.7	6.4	6.9	5.1	6.0	4.2

Table 3.4: Analytical values (μM) and relative standard deviations of cysteine, glutathione and reduced protein cysteine obtained by five replicated determinations performed on one sample of yeast hull (S4) and on two samples of yeast lysates (L2 and L4).

The calibration curves of Cys and GSH (Figure 3.12A) showed linear and similar analytical responses for concentrations up to 100 μM. Because no standard material is commercially available for RPC quantification, the range of linear response was evaluated in yeast hull suspensions containing RPC levels up to 800 μM. Suspensions containing RPC amounts higher than 210 μM were prepared by dispersing 10-40 g L^{-1} of a yeast hull sample containing 2.1 mmol RPC/100 g product. Amounts higher than 50 mg were used to prepare the suspensions intended for linear response assessment,

in order to perform representative samplings. The linear response for low (< 180 μM) RPC concentrations was evaluated analyzing suspensions (27-100 g L^{-1}) of a yeast hull sample containing 0.18 mmol RPC/100 g product. The adopted conditions allowed linear responses for the entire tested range (Figure 3.12B). According to the signal-to-noise ratio, the detection limits for Cys and GSH by means of HPLC analysis were 0.30 μM and 0.26 μM, respectively. The quantification limits were 1.0 μM and 0.85 μM, respectively for Cys and GSH.

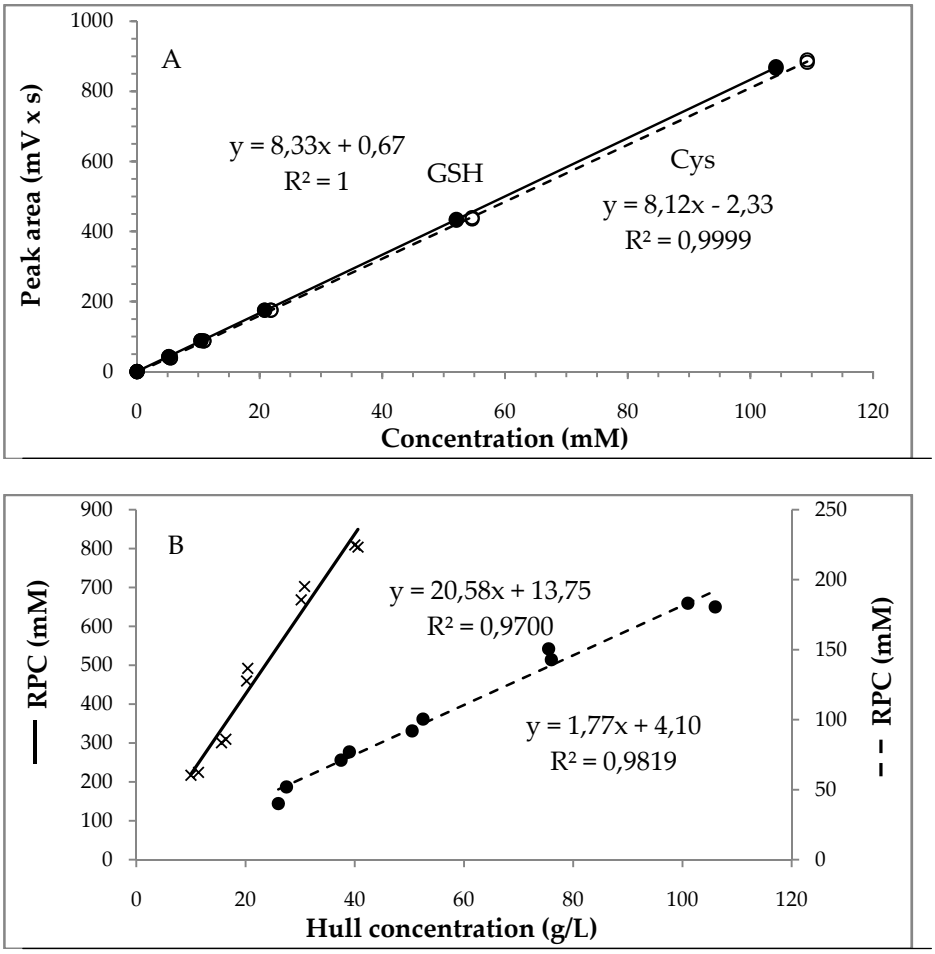

Figure 3.12: Calibration curves obtained from (A) standard solutions of Cys and GSH and (B) analytical response obtained dispersing increasing concentrations of a yeast hull sample containing 2 mmol RPC/100 g product. Data of duplicated determinations are reported.

The influence of the matrix effects arising from polysaccharides and disulfide bonds on RPC quantification was also evaluated. The RPC determination was performed after the addition of increasing amounts of amylose or caramelized amylose to a yeast

lysate. No significant difference ($p < 0.05$) was found for RPC and GSH quantification in the presence of either amylose or caramelized amylose (Table 3.5). Similarly, no interference was observed due to the addition of 5 g L^{-1} lysozyme, a protein containing four disulfide bonds, was added to water solutions containing GSH or Cys (data not shown).

Polysaccharide added (g L^{-1})	*Amount (mmol / 100 g sample)*			
	Amylose		**Caramelized amylose**	
	GSH	RPC	GSH	RPC
0	0. 22 – 0.21	1.59 – 1.44	0.23 – 0.23	1.38 – 1.35
30	0.22 – 0.22	1.52 – 1.56	0.25 – 0.26	1.35 – 1.33
80	0.21 – 0.22	1.57 – 1.60	0.27 – 0.28	1.38 – 1.35

Table 3.5: Effect of amylose and caramelized amylose on the quantification of the Cys residues in a yeast lysate sample. Results of duplicated determinations are reported.

3.3.3 Yeast cell-wall fractions

The analytical approach was first applied to the characterization of 16 commercial samples of oenological active dry yeasts to evaluate their natural thiols content. Because the yeast samples tested were aimed to both effective propagation and alcoholic fermentation, a very low intensity of Maillard reaction was expected. Indeed, the furosine values were lower than 8 mg/100 g protein (Table 3.6) according to the values detected in other unprocessed biological material (Ruiz et al., 2004; Cattaneo et al., 2008; Morales et al., 2009), although no data are reported in the literature for active dry yeasts. Amounts of reduced protein cysteine (RPC) in the range 0.76-1.28 mmol/100 g were detected in yeast samples as well as GSH levels up to 0.92 mmol/100 g (Table 3.6). GSH is a cytoplasmatic metabolite and it likely arises from the lysis of yeast cells following to preparation procedures. None of the starter yeast samples contained free Cys. The mannoprotein fractions represent about 10% of the yeast cell-wall and they are mainly linked to the outer layer of the glucan backbone (Lesage & Bussey, 2006), oenological glycoprotein fractions with a higher content of Cys residues can be potentially obtained.

Sample	Protein	Furosine	Reducing Cys		Overall Cys
	(g/100 g)	*(mg/100 g protein)*	GSH	RPC	*(mmol/100 g)*
			(mmol/100 g)		
1	11.3	2.7	0.92	0.76	7.1
2	11.0		0.45	0.80	
3	10.5		0.71	0.86	
4	12.0	3.6	0.39	0.88	6.8
5	12.0		0.73	0.89	
6	10.3	7.6	0.63	0.90	6.9
7	12.0		0.77	0.91	
8	12.0		0.60	0.91	
9	11.2		0.83	0.91	
10	13.9	7.8	0.55	0.95	4.5
11	11.0		0.45	0.96	
12	9.2		0.54	0.97	
13	16.9	2.3	0.58	0.99	7.8
14	13.6	6.4	0.82	1.02	7.8
15	14.9		0.58	1.05	
16	11.2		0.63	1.28	
Average	12.1	5.1	0.64	0.94	6.8

Table 3.6: Characterization of 16 samples of oenological dried *Saccharomyces cerevisiae* strains according to their content of Cys forms (free and protein linked Cys and GSH), protein and intensity of the Maillard reaction expressed as furosine level. Overall Cys refers to Cys + 2 × cystine.

The commercial mannoproteins (M) samples showed levels of RPC very different from each other. Samples M1 and M2 had the lowest Cys levels (Table 3.7). Sample M2 had the highest furosine level, thus suggesting a strong heat damage, which agreed with the dark brown color and the burnt odour characterizing the sample. The RPC level of sample M4 was close to the values found in the active dry yeast samples. Overall, the RPC levels were very lower than those needed to obtain effective antioxidant activity if the usual amounts of mannoproteins added to wine (200-500 mg L^{-1}) are taken into account. Neither GSH nor Cys were detected in the commercial mannoproteins samples. Surprisingly, the amount of 3MPA-HQ increased when Cys was added to samples before derivatization. Such a behavior occurred with most of the samples not containing free Cys and GSH, and it was likely due to the presence of oxidized phenol aminoacids in the protein structure (Jeahrig et al., 2007). The sensorial properties of wines containing odour-related thiols can be detrimentally affected by the addition of mannoproteins capable of binding high amounts of thiols, like sample M4. In this

regard, 200-400 mg L^{-1} of such mannoproteins could deplete up to 250-500 μM of thiols from wine. Contrarily, such mannoproteins could be usefully added to wine as an early treatment for removing the reduced odours since they can link mercaptans, so avoiding wine exposure to oxygen or the addition of copper sulfate. The high furosine values of mannoprotein samples indicate a strong extent of the Maillard reaction. These commercial mannoproteins are capable of rapidly increasing the formation of atypical ageing-related compounds in wine. Therefore, their addition to wine to improve colloidal and tartaric stabilities or to modify the astringency and the viscosity can also deplete wine odours and decrease wine shelf life.

Yeast hulls (H) having a high RPC content could protect wine during barrel ageing from the oxidative effect of micro-oxygenation. The analyzed commercial samples presented RPC levels close to the values detected for mannoproteins as well as similar capability to bind free Cys. The amounts of GSH and free Cys revealed in sample H4 were likely due to GSH and Cys addition during manufacturing to increase the reducing properties of the product.

Amounts of RPC having an antioxidant effect were detected in the yeast lysates (L) L2 and L4. The latter also contained high level of GSH. The amounts of GSH detected in samples L2 and L3 accounted for 0.8-1.2% of yeast dry weight as reported in the literature (Rauhut, 2009). Nevertheless, their overall Cys content was about twice higher than the level found in the dry yeast samples, and likely, an addiction of exogenous GSH occurred. Because the cytoplasmic GSH represents about 0.5-1% of yeast dry weight, high amounts of GSH were expected to be found in yeast extracts. Moreover, in spite of the high value of total Cys (about 30 mmol/100 g sample), no GSH was detected in the two extract samples, and up to 0.50 mmol Cys/100 g sample was combined after the addition of Cys to the same samples. Such data suggest an intense oxidation of the yeast extracts likely due to the chemical/heat damage arising from the industrial production as supported by the high furosine values observed. A wide range of oenological yeast fractions are commercially available, and yeast hulls, lysates, and mannoproteins effective against wine oxidation and wine atypical aging can be potentially obtained.

Sample	Protein	Furosine	Unrecovered	Cys			Overall
				Free	GSH	RPC	
	(g/100 g)	*(mg/100 g*		*(mmol/100 g)*			
M1	2.86	28	<0.01	0	0	0.03	0.55
M2	10.51	254	<0.01	0	0	0	0.54
M3	9.02	62	0.04	0	0	0.03	2.3
M4	8.77	67	0.41	0	0	0.51	3.1
H1	7.48	17	0.04	0	0	0.08	3.3
H2	8.75	12	0.03	0	0	0.07	4.4
H3	8.87	6	0.04	0	0	0.14	3.3
H4	11.88	12	0	2.6	0.85	0.86	10.6
L1	9.96	3	0	0	0.45	0.73	6.9
L2	17.94	5	0	0	0.33	1.4	15.8
L3	16.84	38	0	0.32	4.6	0.81	12.0
L4	14.33	3	0	0.07	2.8	1.3	8.1
E1	21.68	154	0.46	0	0	1.1	37.4
E2	23.87	20	0.29	0	0	0.76	25.4

Table 3.7: Characterization of commercial yeast cell-wall fractions and yeast extracts according to their contents of free Cys, GSH, RPC and overall Cys (Cys + 2 x cystine). The protein content and the intensity of the Maillard reaction (expressed as furosine level) are reported. The unrecovered Cys refers to the amount of analytically missing Cys after addition of known amounts of Cys to samples lacking non-proteinaceous Cys residues (M: mannoprotein, H: hull, L: lysate, E: extract).

3.4 Conclusion

The data reported show that a number of such oenological samples do not have useful reducing properties. Moreover, the technologies applied for their production are not suitable for preserving the RPC content of the yeast fractions. On these bases, both odour and antioxidant properties of wine could be potentially endangered by using most of the studied samples, which cannot be considered profitable in winemaking. The RPC content of yeasts suggests that more useful fractions could be obtained to protect the odour-related thiols and to increase the shelf life of wine. In this regard, the adoption of specific culture media to increase GSH and RPC contents in yeasts, the selection of yeast strains with improved mannoprotein release properties, and the use of specific enzymatic procedures could greatly enhance the oenological properties of yeast cell-wall fractions.

3.5 References

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3.6 Glutathione quantification in grape juice, must and wine

The reduced glutathione (GSH) carries out several activities in grape juice, must and wine. It is able to reduce the *o*-quinones and to prevent their polymeritazion with browning effects (Salgues et al., 1986). It also decreases the loss of thiol-related aromas as well as the formation of off-flavours, such as sotolon (3-hydroxy-4,5-dimethyl-2(5H)furanone)) and 2-aminoacetophenone (Lavigne & Dubordieu, 2004).
A fast analytical method was developed to assay GSH concentration in grape juice, must and wine using the traditional chromatographic apparatus (HPLC and UV detector) in order to evaluate the GSH levels along with several winemakings, from the grape juice to the end of the alcoholic fermentation and during the ageing. Moreover, the identification and quantification of 2-*S*-glutathionyl caftaric acid (Grape Reaction Product, GRP) was allowed.
The analytical approach used was recently proposed and applied for the antioxidant properties evaluation of yeast cell-wall fractions (Tirelli et al., 2010). It showed a sensitive and reliable quantification of GSH and cysteine, free and linked to the proteins, by a derivatization with a *p*-benzoquinone (pBQ) and the spectrophotometric detection at 303 nm.

3.7 Material and methods

3.7.1 Chemicals

3-Mercaptopropionic acid (3MPA), pBQ and potassium metabisulfite ($K_2S_2O_5$), polyvinylpolypirrolidone (PVPP) were purchased from Fluka (Switzerland). Glutathione (GSH), cysteine (Cys), sodium fluoride (NaF), EDTA, trifluoroacetic acid (TFA) were from Sigma-Aldrich (St. Louis, MO). Citric acid was purchased from J. T. Baker (Phillipsburg, NJ). HPLC grade methanol was purchased from Panreac (Barcelona, Spain), and HPLC water was obtained by Milli-Q system (Millipore Filter Corp., Bedford, MA).

3.7.2 Samples

Six grape samples from different cultivars and production area (South Africa, Chile, Argentina), 13 red wine samples, 5 white wine samples and 1 sparkling wine sample, produced from different grape cultivars, vintages (to 2006 from 2008) and winery, were collected at the market.
The trend of GSH level was monitored in some winemakings in real condition process during the vintages 2009 and 2010 performed in three Italian regions (Lombardia, Tuscany and Sardinia) (Table 3.8). For the vintage 2009, must and wine samples were obtained from 8 winemaking processes performed in 4 different wineries with 3 grape cultivars (Chardonnay, Sauvignon blanc and Trebbiano) and 7 *S. cerevisiae* strains. For the vintage 2010, must and wine samples were obtained from 10 winemaking

processes performed in 5 different wineries with 3 grape cultivars (Chardonnay (2 clones), Trebbiano and Vermentino) and 5 *S. cerevisiae* strains. The GSH trend was followed for 24 days at least.

Winery	Cultivar	Region	Winemakings	
			Vintage 2009	Vintage 2010
1	Chardonnay	Lombardia	2	3
2	Trebbiano	Lombardia	2	2
3	Chardonnay	Lombardia	2	0
4	Sauvignon Blanc	Sardinia	1	0
5	Chardonnay	Lombardia	0	2
6	Vermentino	Tuscany	0	3

Table 3.8: Description of samples drawn during the winemakings in real condition process on vintages 2009 and 2010.

3.7.3 Preparation of grape samples

The GSH quantification was carried out on about 250-300 g of grape berries which were put in a plastic bag. The berries were kept under vacuum after the addition of 2 mL 0.12 M NaF and 0.5 mL 0.75 M EDTA. The grape was hand crushed and the juice obtained was stored at room temperature for 60 minutes. The juice was removed from the plastic bag, transferred in a beaker and then maintained under nitrogen flow for 2-3 minutes. The derivatization was conducted on 2 mL juice after centrifugation at 5000 *x g* for 5 minutes (Paragraph 3.7.6); the reaction mix was filtered by 0.22 μm pore size disposable PTFE filters prior the HPLC separation.

3.7.4 Must and wine sample preparation

Must and white wine were stirred at 5000 *x g* for 5 minutes. Twenty mL of red wine were added with PVPP 15 g L^{-1}, stirred for 5 minutes and centrifuged at 5000 *x g* for 5 minutes. The supernatant was added with 100 μL ethanal 320 mM, it was left for 15 minutes at room temperature and then the pBQ was added, as described for the derivatization (Paragraph 3.7.6).

3.7.5 Preparation of 2-*S*-glutathionyl caffeic acid

The 2-*S*-glutathionyl caffeic acid was prepared in a citric buffer 50 mM at pH 3.5 added with GSH and caffeic acid in molar ratio 4:1 (GSH:caffeid acid), as described by Cilliers and Singleton (1990). The solution contained GSH 350 μM (110 mg L^{-1}) and caffeic acid 90 μM (16 mg L^{-1}). The polyphenol oxidase enzyme (PPO) was obtained as described as follows: few grape berries were hand crushed and the juice obtained was centrifuged at 14000 rpm for 5 minutes at 15°C in a benchtop thermostatted centrifuge.

The supernatant was wasted and the pellet was re-suspended in the buffer containing GSH and caffeic acid. The solution was stirred for 5 minutes, 2 mL were drawn, derivatized with pBQ (Paragraph 3.7.6) and injected by HPLC. The solution was stirred for 1 hour more and a further amount of PPO was added in order to complete the reaction versus the 2-*S*-glutathionyl caffeic acid formation. Two mL were derivatized (Paragraph 3.7.6), filtered and injected into the HPLC.

3.7.6 Derivatization of thiol compounds with p-benzoquinone

The derivatization of GSH and Cys was conducted as described by Tirelli et al. (2010): 2 mL of the samples were added to 100 μL of 400 μM p-benzoquinone (pBQ) dissolved in methanol. After 1 minute mixing, 1 mL of 500 μM 3MPA in 0.3 M citrate buffer pH 3.5 was added in order to remove the exceeding pBQ.

3.7.6.1 Calibration curves

The calibration curves were obtained spiking grape juice, white wine or red wine with amounts of Cys and GSH in the range from 0 mg L^{-1} to 30 mg L^{-1}. The analytical response was compared with a calibration curve achieved in citrate buffer 50 mM at pH 5. The determinations were performed in duplicate at least.
The 2-*S*-glutathionyl caftaric acid was quantified as caffeic acid. The calibration curve was obtained from caffeic acid solutions ranging from 0 mg L^{-1} to 50 mg L^{-1}.

3.7.6.2 Precision parameter

Three different concentrations of GSH (1.5 mg L^{-1}, 15.4 mg L^{-1} and 30.7 mg L^{-1}) were added to white wine. All the reaction mixes were injected 5 times in order to assess the repeatability parameters and the relative standard deviation.

3.7.6.3 High-performance liquid chromatography (HPLC) analysis

The HPLC separation was performed by a hexyl-phenyl column (250 x 4.6 mm, 110Å, Phenomenex). Eluents were water/trifluoroacetic acid 0.05% (v/v) and methanol; the concentration of the latter increased from 10% to 35% of methanol in 18 minutes in the eluting gradient (Table 3.9). The column temperature was 25°C and the injection volume was 50 μL. Detection was carried out by spectrophotometry at 303 nm. The flow rate was 1 mL/min.

Time (min)	A%	B%	Slope
0.0	90.0	10.0	6
1.0	90.0	10.0	6
18.0	65.0	35.0	6
18.5	0.0	100.0	6
21.0	0.0	100.0	6
21.5	90.0	10.0	6
36.0	90.0	10.0	6

Table 3.9: HPLC separation gradient for thiols compounds derivatized with *p*-benzoquinone. Eluent A: water/trifluoroacetic acid 0.05% (v/v); eluent B: methanol.

3.7.6.4 HPLC/Electrospray Ionization-Mass Spectrometry (ESI-MS)

For MS detection of the 2-*S*-glutathionyl caffeic acid and 2-*S*-glutathionyl caftaric acid, the LCQ Deca XP spectrometer, controlled by the Excalibur software (Thermo Finnigann Jose, CA), was operated in positive ion mode. A post column flow splitter was used to introduce 1:15 of the HPLC flow stream into the ESI source. The ESI interface and the ion optics settings were as follows: spray potential, 5.0 kV; nebulization gas (nitrogen) relative flow value, 10; capillary temperature, 275°C; and cone voltage, 30 V. Full-scan mass spectra were acquired scanning the range 50-800 m/z. Mass accuracy was ensured by calibration with a mixture of caffeine, reserpine, and the tripeptide PFK (in methanol:water 1:1, 0.1% acetic acid) infused separately.

3.7.7 Quantification of readily assimilable nitrogen

The readily assimilable nitrogen was quantified in must samples before the yeast strain addition in all the winemakings considered. Twenty-five mL of must were drawn, the pH was moved to 8.3 with NaOH 1 N and 2 mL $BaCl_2$ 1 g L^{-1} were added. The must was centrifuged at 5000 *x g* for 10 minutes; the pH of supernatant was moved to 8.3 with NaOH 0.1 N and 20 mL formaldehyde 40% at pH 8.3 were added. The sample obtained was titrated with NaOH 0.1 N.
The readily assimilable nitrogen was quantified as follows:

$$N = mL\,NaOH \times 1.4 \times 40$$

Where:
mL NaOH: mL NaOH 0.1 N used to move the pH to 8.3 after the formaldehyde addition;
1.4: conversion factor;
40: dilution factor.

The readily assimilable nitrogen was expressed in mg L^{-1}.

3.8 Results and discussion

3.8.1 Analytical method development

The analytical conditions adopted allow the separation of GSH, Cys and GRP in grape juice, must and both red wine and white wine (Figure 3.13).
For the grape juice sample preparation, the addition of NaF and EDTA prevents the oxidations of juice due to tyrosinase activity. In particular, NaF can inhibit the oxidase enzymes binding the copper, which is essential for their catalytic action (Janovitz-Klapp et al., 1990; Richard et al., 1991). Experiments were also conducted with potassium metabisulfide 2 mM ($K_2S_2O_5$) as antioxidant agent, but it had less protective effect on the oxidase enzyme inhibition than NaF (decrease of spiked GSH concentration) and the analytical response was not repeatable (data not shown).
Assays conducted quantifying GSH every 30 minutes, from 30 minutes to 150 minutes incubation at room temperature after grape crushing, showed the maximum concentration of GSH was detectable from 30 minutes to 90 minutes of storage (Table 3.10).
The nitrogen flow allowed the homogenisation of the juice avoiding the air contact.

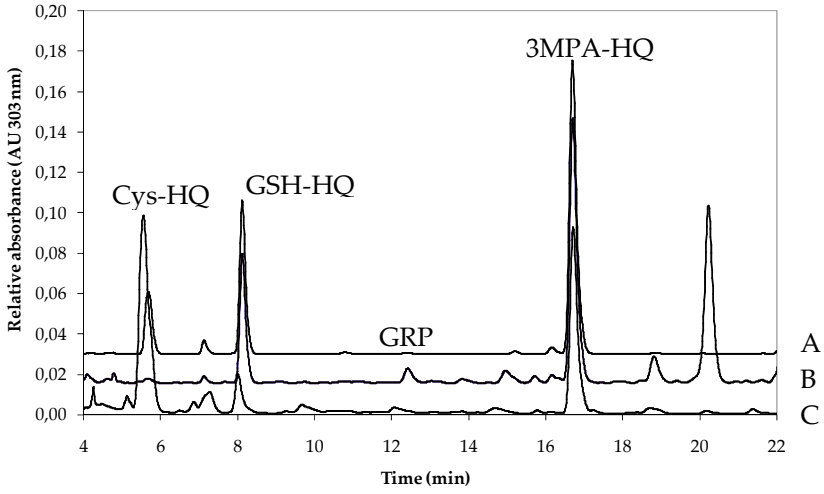

Figure 3.13: HPLC separation of Cys, GSH, GPR and 3MPA in a standard solution (A), white wine (B) and red wine (C).

Sample	Time incubation	GSH
	minutes	*mg L^{-1}*
0	0	13.2
1	30	17.8
2	60	18.4
3	90	18.1
4	120	16.9
5	150	13.2

Table 3.10: GSH quantification in grape at variable incubation times at room temperature.

GSH quantification was not affected by temperature: experiments conducted maintaining juice at controlled temperature of 20°C did not show differences than assays conducted at not controlled room temperature (data not shown).
In red wine, the use of PVPP allowed a lower content of phenols, so preventing interferences in the HPLC separation. The centrifugation removed not only PVPP, but also the phenols particles adherent to it.
The analytical method had linear response for GSH ranging from 0 mg L^{-1} to 30 mg L^{-1}; the calibration curve for Cys was obtained only in grape juice and the analytical response was linear for Cys concentration ranged from 0 mg L^{-1} to 30 mg L^{-1}. In grape juice, the analytical response did not differ significantly than a citrate buffer, as shown by the calibration curves (Figure 3.14), either for GSH or Cys. In red and white wine, the analytical response differed than a citrate buffer of 20%, as indicated by their slope (Figure 3.15). This difference has to be considered for the GSH quantification.

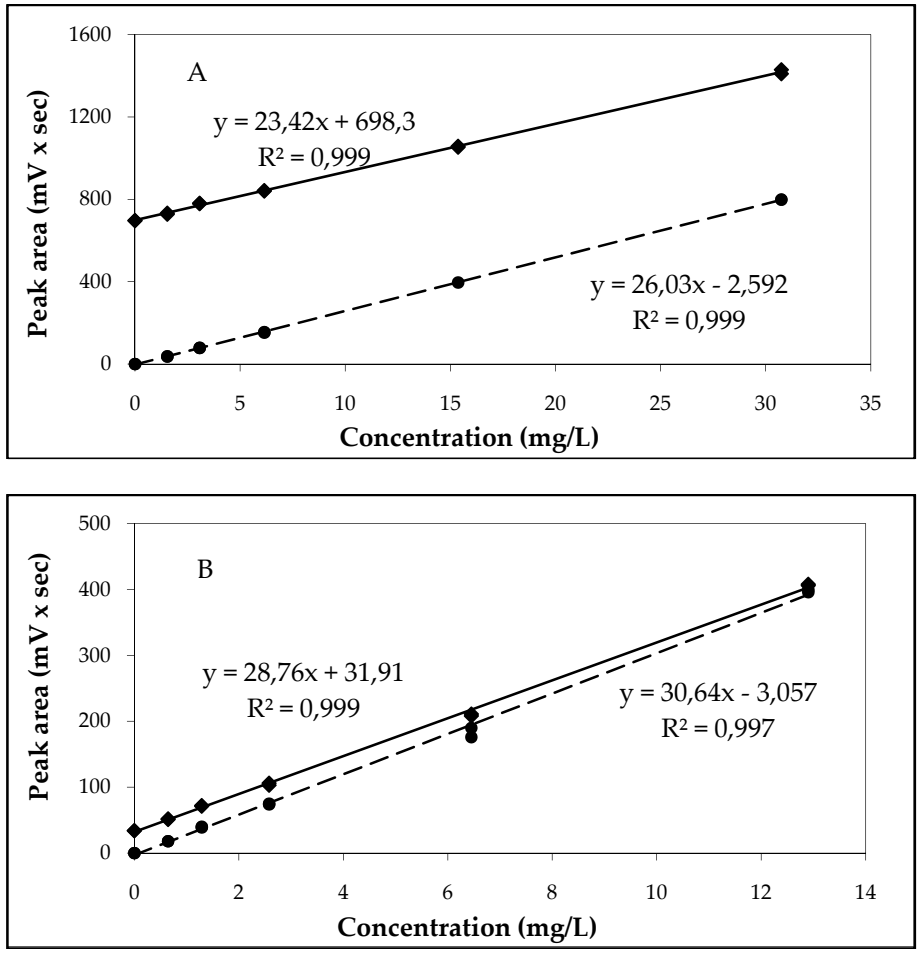

Figure 3.14: Calibration curve in grape juice from known addition of A) GSH: citrate buffer (•) and GSH in juice (♦); and B) Cys: citrate buffer (•) and Cys in juice (♦).

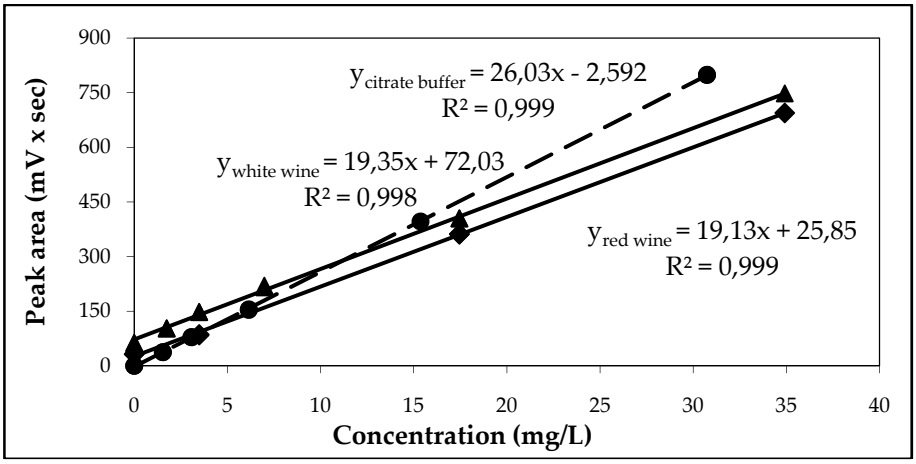

Figure 3.15: Calibration curve in grape juice spiking known concentration of GSH: citrate buffer (•), red wine (▲) and white wine (♦).

The repeatability was calculated by spiking known and increased concentrations of GSH to white wine (Table 3.11). The relative standard deviation (%RDS) was 3.1% for GSH concentration ranged 5-30 mg L^{-1}.
The detection limit (signal to-noise ratio > 3) for GSH and Cys were 0.13 mg L^{-1} and 0.07 mg L^{-1} and the quantification limit (signal to-noise ratio > 10) were 0.43 mg L^{-1} and 0.21 mg L^{-1}, respectively. These concentrations were detected without sample treatments. The GSH level of 0.5 mg L^{-1} is widely lower than a GSH content exerting an appropriate antioxidant activity (Lavigne & Dubourdieu, 2004).

Concentration	Chromatographic Area (mAU x sec)			SD	RDS
mg L^{-1}	min	max	average	mg L^{-1}	%
1.5	34.5	39.0	36.8	1.8	5.0
15.4	378	395	386	6.7	1.7
30.7	658	708	686	18.1	2.6

Table 3.11: Repeatability parameters (n=5) for the GSH determination.

3.8.1.1 Identification of 2-*S*-glutathionyl caffeic acid

The preparation of 2-*S*-glutathionyl caffeic acid was carried out in order to recognize this compound and provide the elution time of 2-*S*-glutathionyl caftaric acid (GRP).
As expected, the reaction rate of 2-*S*-glutathionyl caffeic acid formation was very fast (Singleton et al., 1985; Riberau-Gayon et al., 2006). The caffeic acid dissolved in the buffer solution reacted completely with GSH with the first addition of PPO, as shown by the HPLC analysis (Figure 3.16): no caffeic acid was detected. The derivatization

was conducted to check the GSH concentration before and after the addition of the PPO. The HPLC analysis separated one peak more at 15.5 minutes; the MS detection confirmed that peak was 2-*S*-glutathionyl caffeic (Figure 3.17).

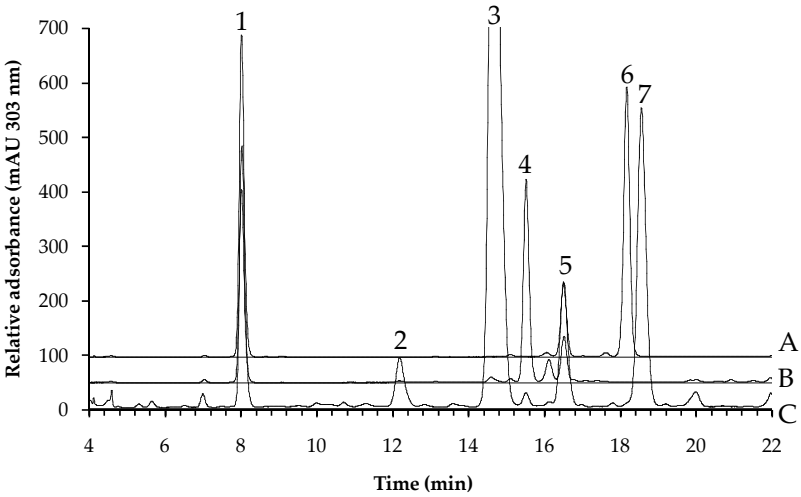

Figure 3.16: HPLC separation of GSH (1), GRP (2), 2-S-glutathionyl caffeic acid (4) and derivatized MPA (5) in a solution of GSH and caffeic acid (A), solution of GSH and caffeic acid after PPO addition (B), grape juice. The peaks 3 and 7 detected in grape juice are unknown.

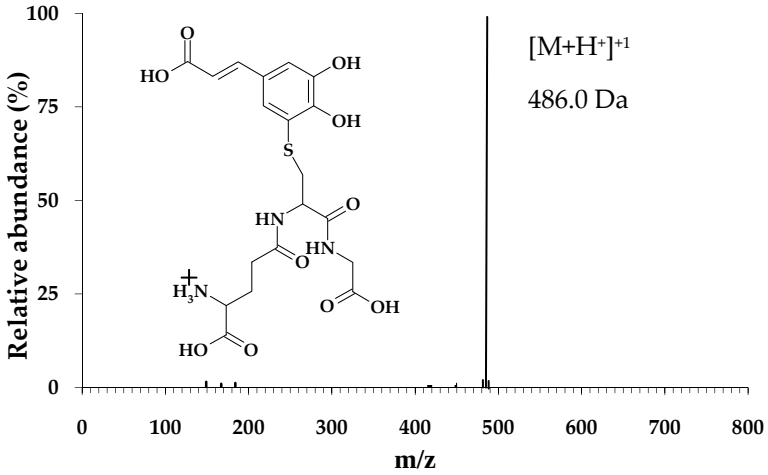

Figure 3.17: MS detection of 2-*S*-glutathionyl caffeic acid.

3.8.1.2 Identification of 2-*S*-glutathionyl caftaric acid

The 2-S-glutathionyl caftaric acid, commonly known as GRP, is the compound arising from the reaction between the GSH and the *o*-quinone of caftaric acid in the presence of oxygen and PPO (Singleton et al., 1984). Its formation during in the running must has been clearly described (Singleton et al, 1984; Singleton et al., 1985; Cheynier et al., 1986) as well as its stability in the presence of PPO. In this way the formation of brown polymers is hindered.

The HPLC pattern obtained from the analysis of grape and wine samples (Figure 3.13 and Figure 3.16) showed a peak eluting at 12.2 minutes having the maximum absorbance at 326 (Figure 3.18), a value close to the absorbance of GRP reported in literature (Cheynier et al., 1986). The small difference (less of 2 nm) is probably due to the HPLC eluent.

The HPLC separation coupled to the MS detection confirmed that peak was GRP, as shows the MS spectra in Figure 3.19.

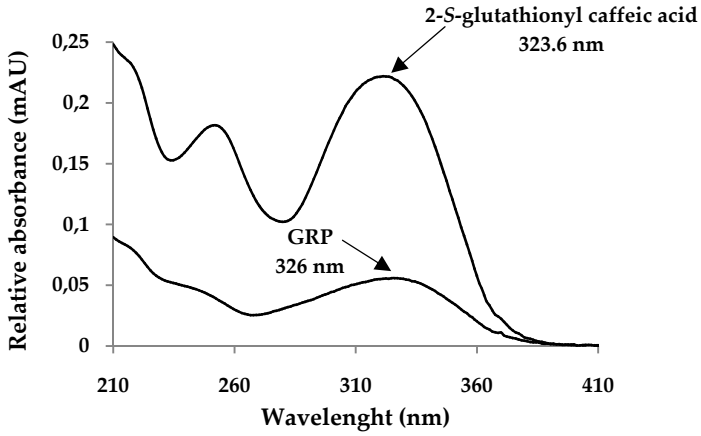

Figure 3.18: UV-spectra for GRP and 2-*S*-glutathionyl caffeic acid.

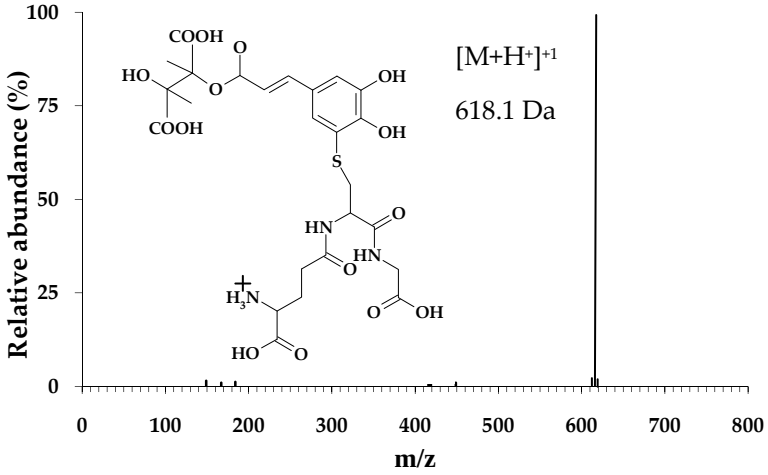

Figure 3.19: MS detection of 2-*S*-glutathionyl caftaric acid.

The GRP trend was monitored during the winemakings conducted in real condition process in the vintages 2009 and 2010.

3.8.2 Quantification of GSH in grapes and wines

Six different cultivars of grape were analyzed and GSH detected was ranged from 40 mg L^{-1} to 77 mg L^{-1} (Table 3.12), according as reported by Cheynier et al. (1989), Okuda & Yokotsuka (1999) and Janěs et al. (2010). Low amounts of Cys were also detected (0.5-3.4 mg L^{-1}), contrarily as indicated in literature (Park et al., 2000a; Park et al., 2000b).

Cultivar	Type	GSH	Cys
		mg L^{-1}	mg L^{-1}
Aledo 1	white	50.3	1.0
Aledo 2	white	40.8	0.6
Crimson	red	73.3	0.5
Moscatella	red	44.5	n. d.
Red Globe	red	39.9	3.4
Thompson	white	75.5	0.8

Table 3.12: GSH and Cys quantification in 6 grape cultivars (n. d.: not detected).

GSH was detected only in white wines and sparkling wine, in concentrations ranging from 1 mg L^{-1} to 3 mg L^{-1} (Table 3.13), similarly as reported in literature (Cassol & Adams, 1995; Lavigne & Dubourdieu, 2004; du Toit et al., 2007; Bramanti et al., 2008; Marchand & de Revel, 2010). None of the red wine samples contained GSH, probably

due to the interactions between thiols and quinones during winemaking (Salgues et al., 1986). Cys was not detected in any samples of analyzed wines.

Wine	Type	Vintage	GSH
			mg L^{-1}
Barbera 1	red	2008	n. d.
Barbera 2	red	2008	n. d.
Cabernet Sauvignon 1	red	2008	n. d.
Cabernet Sauvignon 2	red	2008	n. d.
Cortese	white	2008	1.5
Chianti	red	2008	n. d.
Gutturnio 1	red	2008	n. d.
Gutturnio 2	red	2008	n. d.
Merlot 1	red	2008	n. d.
Merlot 2	red	2008	n. d.
Muller	sparkling	2008	1.2
Novello	red	2008	n. d.
Sauvignon Blanc 1	white	2008	3.1
Sauvignon Blanc 2	white	2008	1.5
Trebbiano	white	2008	2.5
Traminer	white	2007	2.8
Aglianico	red	2007	n. d.
Shiraz 1	red	2006	n. d.
Shiraz 2	red	2006	n. d.

Table 3.13: GSH quantification in 19 Italian commercial wine samples (n. d.: not detected).

The exposure to air of must and pomace during the alcoholic fermentation could cause a phenols oxidation and a complete depletion of GSH in the red wines.
Moreover, the GSH content was low also in the white wines analyzed and probably, it was not able to perform an effective protection against the oxidation during the shelf-life (Lavigne & Dubourdieu, 2004).

3.8.3 Modification of glutathione levels in winemaking

The GSH trend was monitored in several winemakings for the 2009 and 2010 vintages.

3.8.3.1 Vintage 2009

Eight winemaking processes performed in 4 different cellars were followed.

The GSH trend in winemakings performed in winery 1 are represented in Figure 3.20; these productions were carried out on Chardonnay grape. For the winemaking A (Figure 3.20A), the must was added with oxygen for an amount equal to two saturation before the clarification treatment. In the winemaking B (figure 3.20B), the sulfur dioxide was already added during the pressing and the fermentation tank, where the must was transferred, was inertized with nitrogen. The initial concentration of GSH was lower than 1 mg L^{-1}. The maximum GSH concentration was 9.2 mg L^{-1} in both the winemakings, independently from the yeast strain, oxygen exposition and fermentation rate which was slower in winemaking B. The initial low level of GSH did not seem to affect the amount of GSH produced by the *S. cerevisiae*. In both the winemakings, the GSH concentration increased during the alcoholic fermentation, but with some differences. In oxidative conditions winemaking (Figure 3.20B), the highest amount of GSH was reached with the racking and it was halved after 10 day. In reductive condition winemaking (Figure 3.20A), the GSH level increased during the fermentation and it was higher than 7 mg L^{1} for two weeks after the racking. The rapid decrease of GSH in winemaking B could be explained by residual amounts of *o*-quinones in the must.
The initial GRP content was 4 folds higher in the winemaking conducted in reductive condition and it remained stable; in winemaking performed in oxidative condition its content became double at the end of the fermentation and then it decreased slowly. This decrease could be due to a partial GRP degradation occurring after fermentation, but no data are available in literature concerning the GRP trend level during the winemaking. It is known the GRP could be degraded during the wine storage (Cheynier et al., 1986); its decrease could be due to a degradation phenomena. The formation of 2,5-diglutathionyl caftaric acid (GRP2) could not be achieved in so short time. It is formed in presence of high concentration of GSH and oxygen through the laccase activity (Dietrich & Pour-Nikfardjam, 2009), and the GRP chemical oxidation to GRP2 could need more time.

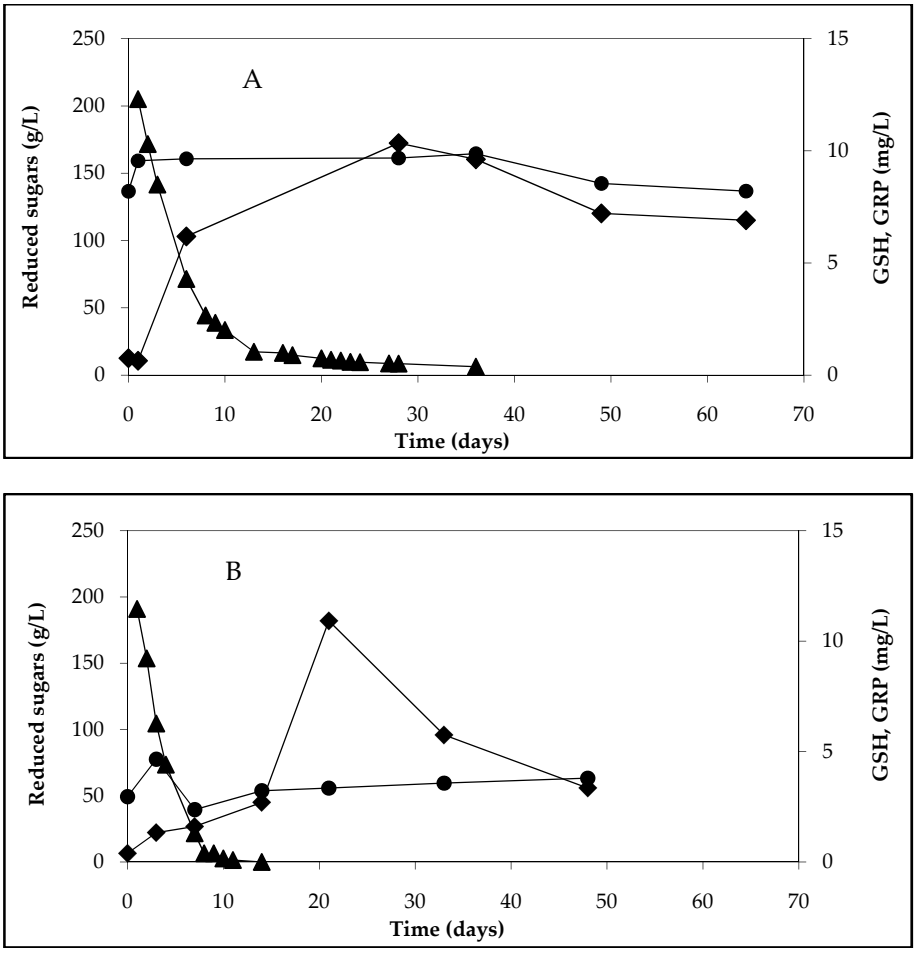

Figure 3.20: GHS (♦), GRP (•) and reduced sugars (▲) trend in winemakings conducted by the winery 1 on Chardonnay grape in reductive condition (A) and oxidative condition (B) during the vintage 2009.

The important role carried by the exposition of must to the oxygen was confirmed by the GSH and GRP contents shown by the winemaking carried out in winery 2 with the running must arising from either the low pressure pressing (Figure 3.21A) or the high pressure (Figure 3.21B). Grape pressing was conducted through a press inertized with nitrogen. Two different *S. cerevisiae* strains were employed in fermentation. The initial GSH content was low in both the winemakings; the GSH increased during the fermentation up to 19 mg L^{-1} and 15 mg L^{-1} for winemaking A and B, respectively, likely due to the aeration of the must. This operation can enhance the *S. cerevisiae* growth and, consequently, the GSH biosynthesis. Nevertheless, the winemaking B was carried out using the high pressure fraction of the juice and the initial delay of GSH formation could be due to a higher concentration of the PPO and *o*-quinones. The latter

can derive from the skin laceration and seeds fragmentation which can promote the quinones formation and their presence in fermentation. For both the winemakings, the GSH decreased in a similar way and its concentration 1 month after the racking was 3.4 mg L^{-1}.

The initial GRP content was comparable in both the winemakings and exceeded 40 mg L^{-1}. It high content could be probably due to oxidative mechanisms occurring into the grape. During the fermentation its content decreased and it is lower in winemaking of the first fraction juice (28 mg L^{-1}) than the second fraction (35 mg L^{-1}). The higher GRP content in the second pressing juice was likely due to the skin laceration and seeds fragmentation of the grape which cause a higher development of oxidative mechanisms.

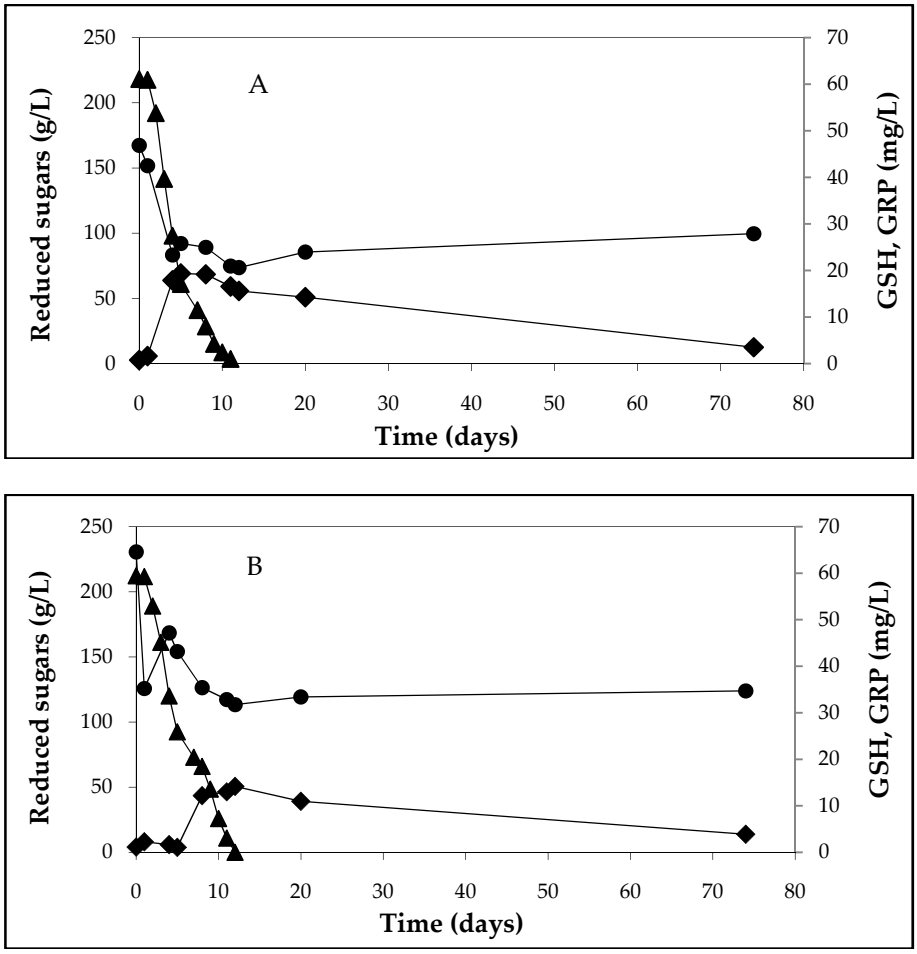

Figure 3.21: GSH (♦), GRP (•) and reduced sugars (▲) trend in winemakings conducted by winery 2 on Trebbiano (Lugana) grape; first pressing grape juice (A) and second pressing grape juice (B) during the vintage 2009.

The yeast strain employed in fermentation seems to have a weak effect on the GSH concentration trend (Figure 3.22). The winemakings were carried out with the same grape cultivar (Chardonnay) in winery 3 and the must was fermented by 3 different yeast strains. Both the fermentation kinetics and the GSH release showed only minor differences for all the yeast strains employed.
The GRP trend was similar between the three winemakings considered, as well.

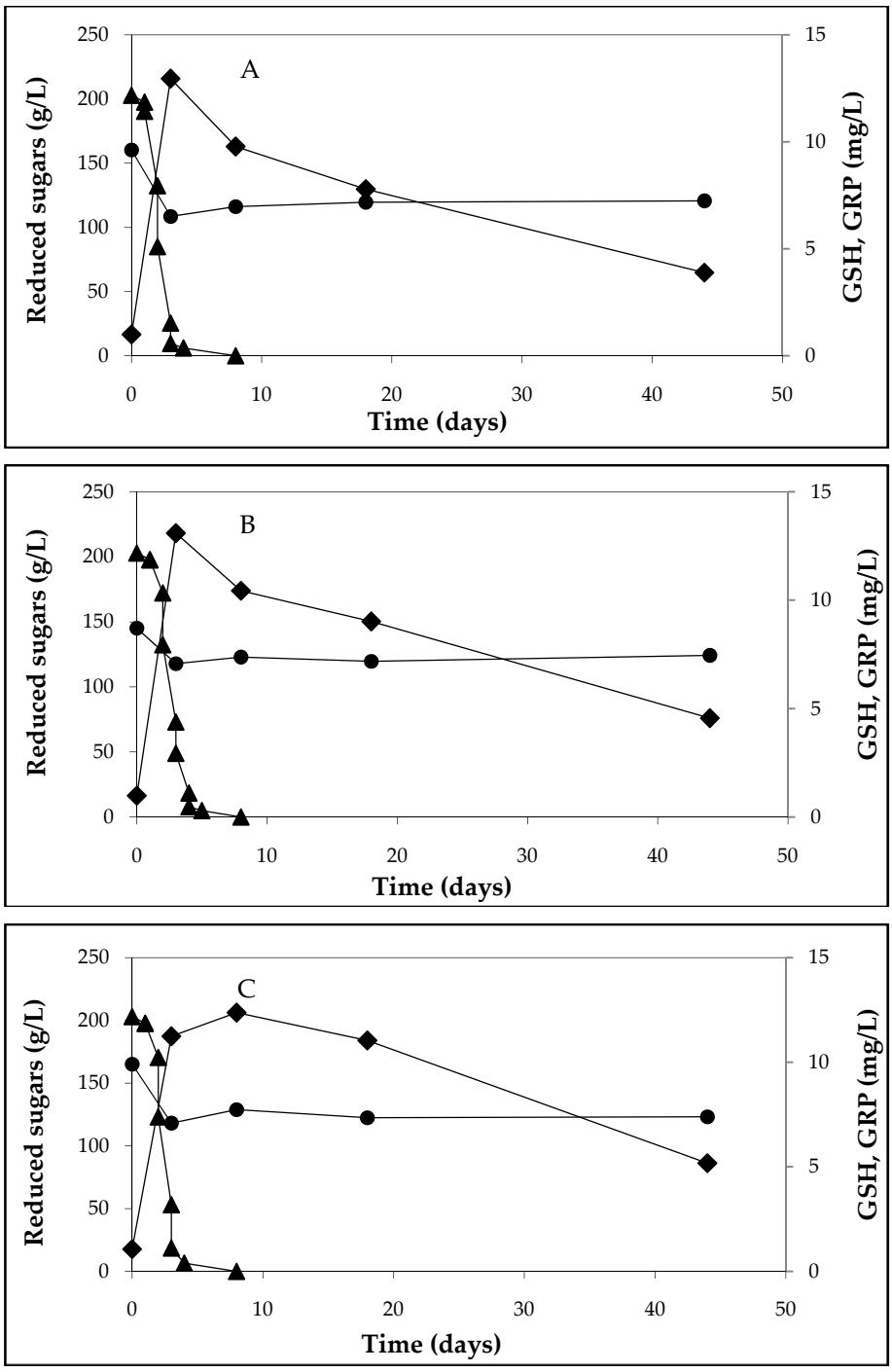

Figure 3.22: GHS (♦), GRP (•) and reduced sugars (▲) trend in winemakings carried out in winery 3 with Chardonnay grape employing 3 different yeast strains (A-C) during the vintage 2009.

The content of readily assimilable nitrogen could represent a limiting parameter on the *S. cerevisiae* GSH biosynthesis. The GSH content in winemaking conducted by winery 4 on Sauvignon blanc grape cultured in a hot-dry climate was negligible after pressing and it was not released during the fermentation. The readily assimilable nitrogen content in this must was 129 mg L^{-1}, while its concentration ranged from 250 mg L^{-1} to 350 mg L^{-1} in the other winemakings above described. These data are in agreement with Lavigne and Dubourdieu (2004) which suggested 200 mg L^{-1} of readily assimilable nitrogen is needed to allow the release of GSH by the yeast.

The GSH levels detected in must after pressing were low in comparison to the GSH concentration usually measured in grape, as described in literature (Cheynier et al. 1989; Okuda & Yokotsuka, 1999; Janěs et al., 2010). The GSH data in grape were not available for these considered winemakings.
The GRP concentration could not explain the GSH decrease, especially for a vintage, with positive climatic conditions and safety of grape, as the vintage 2009 has been.

3.8.3.2 Vintage 2010

Ten winemaking processes performed in 4 different wineries were monitorated.
For vintage 2010, the copper concentration was also evaluated in order to understand if the low initial GSH concentration could be correlated to the amount of this metal ion. Moreover, the GSH and GPR content were quantified in grape employed in winemakings and the results are represented in Table 3.14.

Winery	Winemaking	GSH	GRP
		mg L^{-1}	mg L^{-1}
1	A	1.8	1.3
1	B-C	3.7	1.8
2	A-B	84.3	4.6
5	A-B	35.3	1.5
6	A-C	2.8	0.9

Table 3.14: Quantification of GSH and GRP in the grape cultivars employed in winemakings during the vintage 2010.

The winemaking kinetics represented in Figure 3.23 were obtained from Chardonnay grape (2 clones) in winery 1 and the pressing was performed trough a pneumatic press. Both winemaking A and B were carried out in oxidative condition; the first running must was aerated up to two oxygen saturation (iperoxigenation) and racked in *barriques* where it was inoculated and fermented (Figure 3.23A). The pressing of winemaking B was performed in oxidative conditions and the malolactic bacteria was inoculated after the alcoholic fermentation (Figure 3.23B). In the process represented in

Figure 3.23C, the addition of sulfur dioxide was performed after pressing and the fermentation tank was saturated with carbon dioxide. The winemaking A was performed with a different Chardonnay grape clone than winemakings B and C.
The initial GSH concentration was lower than 2 mg L^{-1} in all the winemakings, according to the low GSH level detected in grape (Table 3.14). Probably the initial GSH content could be also correlated to a high concentration of copper ion in must which was 6.6 mg L^{-1}, 5.6 mg L^{-1} and 5.6 mg L^{-1} for winemaking A, B and C, respectively. The Cu^{2+} could be involved in disulfide bridge formation between two molecules of GSH, as it happens for the removal of thiols-related reduced defects (Monk, 1986; Riberau-Gayon et al., 2006; Bartowsky & Pretorious, 2009). As already observed in vintage 2009 for the winey 1 (Figure 3.20), the higher GSH content was reached faster in reductive conditions (6 days fermentation) than in oxidative condition (12 days fermentation). Contrarily to the previous vintage (Figure 3.20A-B) in which the GSH released during fermentation was comparable in oxidative and reductive conditions, for vintage 2010 the GSH released in oxidative condition (Figure 3.23A-B) was 5 mg L^{-1} lower than in reductive condition (Figure 3.23C). Moreover, the iperoxidative condition and the alcoholic fermentation performed in barrel (Figure 3.23A) could further decrease the GSH level than the fermentative process carried out in tank (Figure 3.23B). After 45 days of ageing, the GSH content was halved in all the winemakings.
Several parameters changed during these winemakings performed in winery 1, such as grape cultivar clones, red-ox pressing conditions, yeast strains, co-inoculation of malolactic bacteria, productive steps. Nevertheless, the GSH detected was similar in all the winemakings. It was not allowed to identify what condition affected mainly the GSH content which trend could be affected by more than one of the productive conditions applied.
The higher initial content of GRP was observed in reductive conditions winemaking after pressing (7.6 mg L^{-1}) (data not shown in Figure 3.23C) probably due to the higher grape break during the pressing. Its concentration decreased to 0.8 mg L^{-1} after racking and it was slightly higher than in winemaking A and B (0.3 mg L^{-1} for both productions). The GRP trend was similar for all the winemakings and after 55 days it was lower than 0.7 mg L^{-1}. This fact could be linked to the hydroxycinnamic acids content in grape, caftaric acid in particular: when their concentration is low, GRP could not be formed.

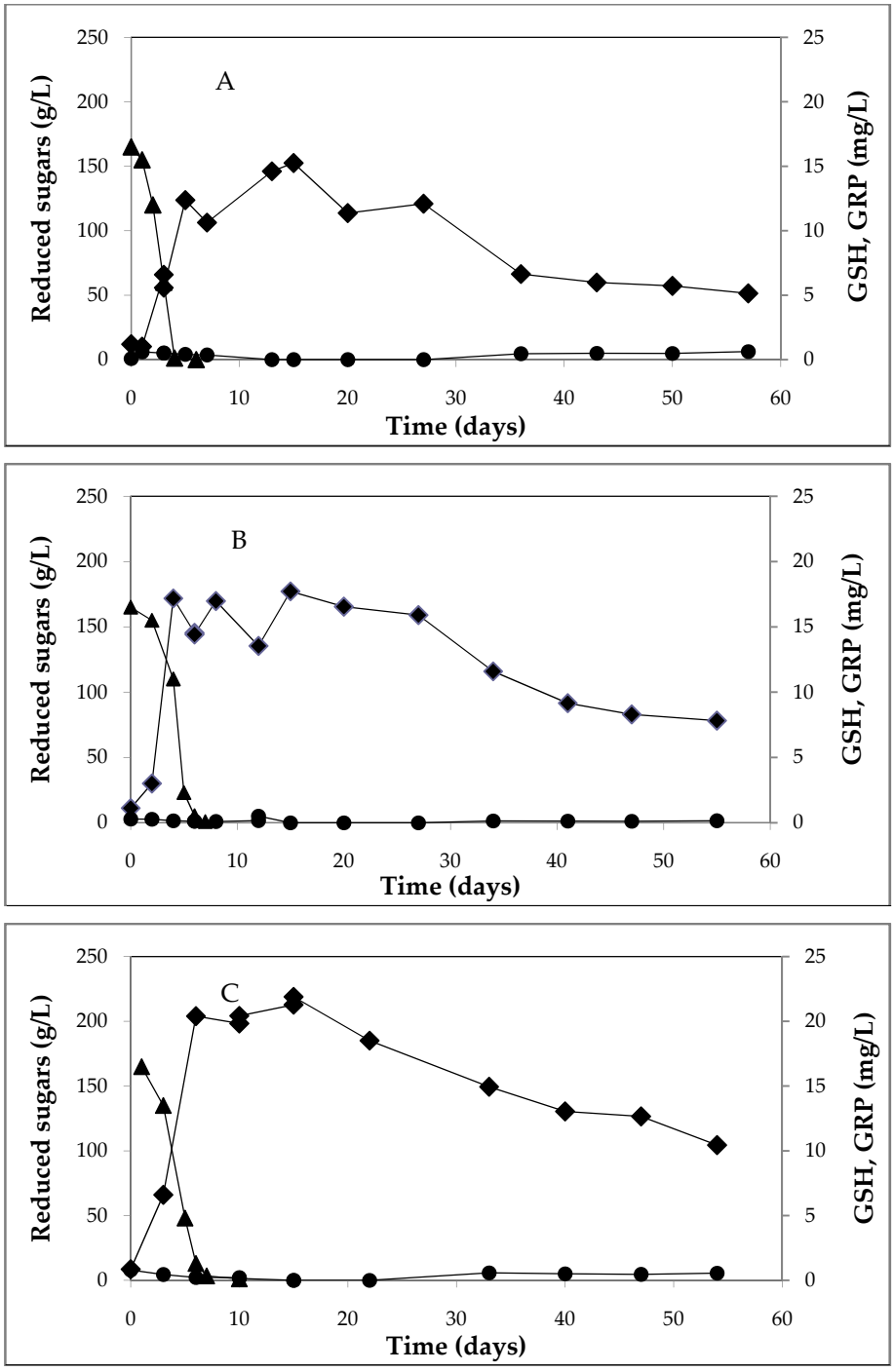

Figure 3.23: GHS (♦), GRP (•) and reduced sugars (▲) trends in winemakings performed by the winery 1 with Chardonnay grape in iper-oxidative conditions (A), oxidative condition (B) and reductive condition (C) during the vintage 2010.

High GSH levels can be preserved during the pressing conducted in winery 5, as shown in Figure 3.24 which was carried out through a pneumatic press and the initial GSH was higher than 5 mg L^{-1}. The GSH content in grape was up than 35 mg L^{-1} and more than 25 mg L^{-1} were lost, even using an inertized press. The free run juice showed a high GSH amount (22.8 mg L^{-1}), but it is not usually fermented for its high content of solid particles which can increase the must oxidation. Differently from the winemakings described as above for winery 1 in vintage 2010 (Figure 3.23), the Cu^{2+} content was ten times lower in both of the musts (0.47 mg L^{-1} and 0.33 mg L^{-1} for must A and must B, respectively); its concentration can affect the GSH released during the production. The winemakings were conducted on Chardonnay grape and the fermentation was conducted by the same yeast strain. When the pressing without oxygen removal was performed, the initial GSH content was lower than in reductive conditions and a faster beginning of the alcoholic fermentation was observed. In this condition, up to 22 mg L^{-1} GSH was quickly released, probably due to an aeration step before the fermentation. Afterwards, the fermentation rate slowed down and then increased again and more GSH was detected (27.5 mg L^{-1}). It is not allowed to know what caused an increase of fermentative rate; the must could be aerated or a further yeast starter addition could be carried out. In reductive conditions, the maximum GSH level was 14.4 mg L^{-1}, lower than in oxidative condition. The GSH concentration decreased in both oxidative and reductive conditions; its level was higher in oxidative condition at the 24th day. It means the GSH released through the *S. cerevisiae* employed in fermentation could be affected by the availability of oxygen before and, probably, also during the fermentation, it is a response to the oxidative stress conditions (Rauhut, 2009). The oxygen concentration, as well as the aeration can increase the release of GSH which was higher during the fermentation and until the last trend day (24 days after the inoculum) in oxidative condition. In this way, the GSH can mostly prevent the wine oxidation during the fermentative process and ageing.

The initial GRP content was higher when the reductive conditions were adopted and it decreased at the beginning of the alcoholic fermentation. The GRP trend was comparable in both the winemakings and it was lower than 3 mg L^{-1} at the end of the fermentation. The oxidative conditions could not affect the GRP concentration during the fermentation.

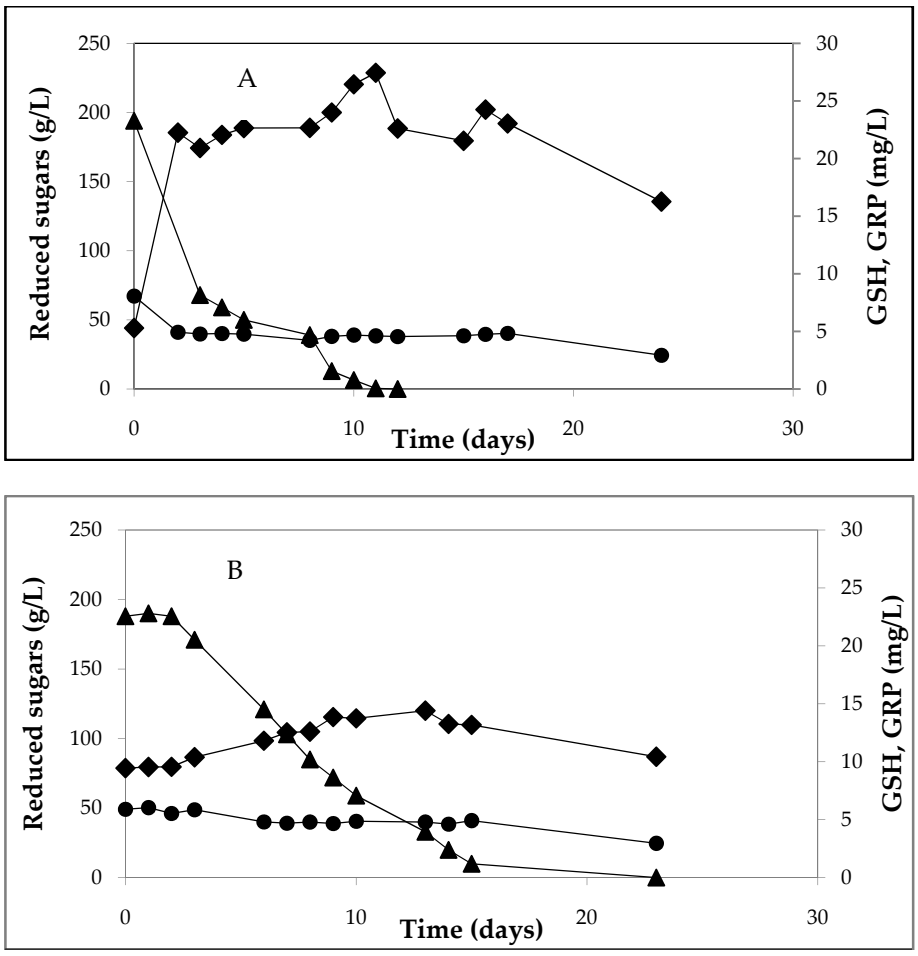

Figure 3.24: GHS (♦), GRP (•) and reduced sugars (▲) trend in winemakings conducted by winery 5 on Chardonnay grape in oxidative condition (A) and in reductive condition (B) during the vintage 2010.

The effect of Cu^{2+} on the GSH content is showed in Figure 3.25. These winemakings were conducted with Trebbiano (Lugana) grape in winery 2, using the same *S. cerevisiae* strain; the winemaking A was performed using the running must from the low pressure pressing. The second part of the must, obtained with the higher pressure (0.5 – 0.8 bars), was used for the winemaking B. The grape showed a content of GSH up to 80 mg L^{-1} and most of it was lost during the pre-fermenting operations. The initial GSH content was similar (up to 4 mg L^{-1}) for both the winemakings, nevertheless the Cu^{2+} was 6.5 mg L^{-1} for the first fraction and 2.3 mg L^{1} for the second fraction. Even though the fermentation course was similar, the GSH was released faster and in higher amount in the second fraction winemaking. It is probably due to the lower Cu^{2+} content detected in this must. GSH decreased after the wine racking and its

concentration was lower in the first fraction winemaking (12.8 mg L^{-1}) than in the second fraction winemaking (15.2 mg L^{-1}).
The pressing pressure could affect the initial content of GRP which was higher in the second fraction. The GRP trend was similar during the winemaking and after 22 days the GRP concentration was lower in the first fraction (5 mg L^{-1}) than in the second fraction (7.7 mg L^{-1}). It is probably due to the higher degree of the grape skin laceration occurred during the second pressing.

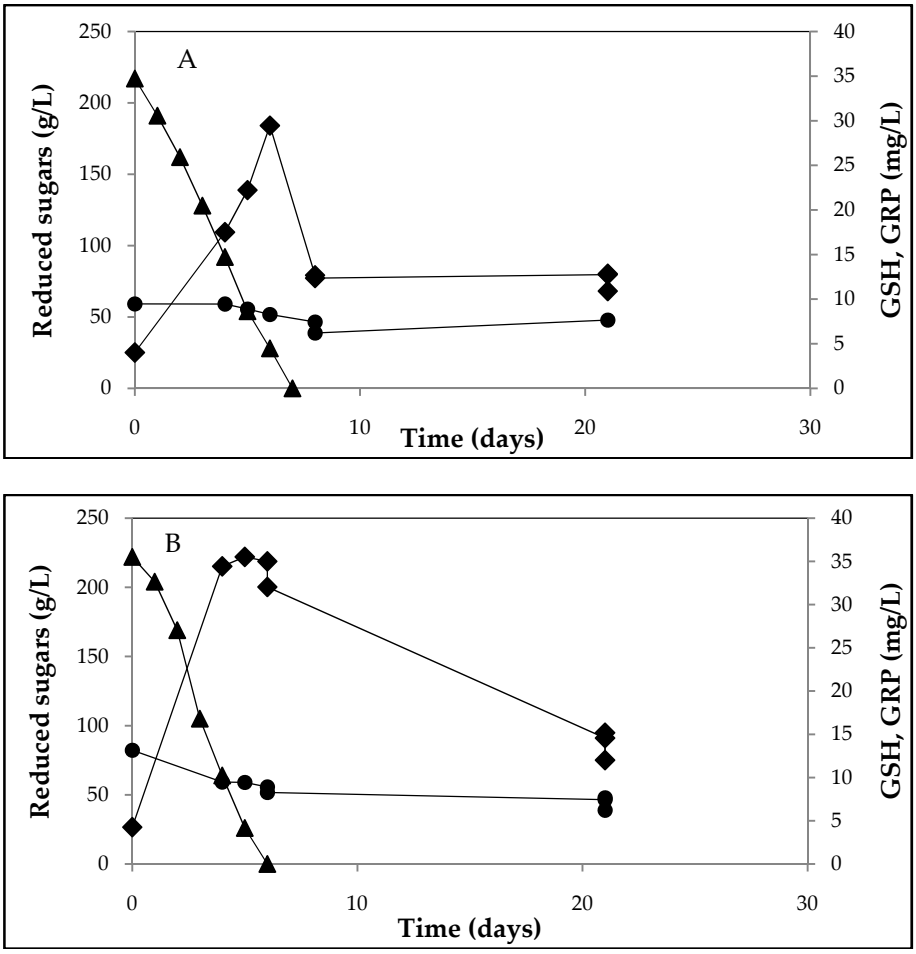

Figure 3.25: GHS (♦), GRP (•) and reduced sugars (▲) trend in winemakings conducted in winery 2 on Trebbiano (Lugana) grape on the first pressed fraction (A) and the second pressed fraction (B) during vintage 2010.

The copper content could affect the winemakings, as shown in Figure 3.26. These small scale wine productions were conducted in winery 6 on Vermentino grape and the same yeast strains was used. No treatment was carried out on grape for winemaking

A. In winemaking B and C, the grape was rinsed, in order to remove the copper residues on the grape skin, and then dried before pressing. Moreover, a Bordeaux mixture powder was added after pressing in winemaking B. The Bordeaux mixture powder is an adjuvant containing copper sulfate, copper hydroxide and calcium sulfate; the Cu^{2+} concentration is ranged from 2% to 5% (Villavecchia & Eigenmann, 1976). The GSH content was higher in the winemaking where the grape was rinsed (Figure 3.26C) either in juice after pressing or in must during the alcoholic fermentation. Its level was 4.4 mg L^{-1}, 4.7 mg L^{-1} and 5.0 mg L^{-1} after 33 days for wine from winemaking A, B and C, respectively. The GSH level could be correlated to the copper content, even though it could be affected by other mechanisms not involving the copper, as shown the highest GSH decrease happened in winemaking C. However, for these winemakings, the low GSH level could be correlated to the yeast which probably was a low GSH producer strain, since the readily assimilable nitrogen was up to 200 mg L^{-1}. The GSH content was probably affected by the copper concentration, but it was so low to make it difficult to estimate the influence of this metal.
The GRP concentration was higher in juice after pressing in the production A (5.5 mg L^{-1}) and it decreased to 3.6 mg L^{-1} after 33 days. A lower amount was detected for the winemakings B and C (4.9 mg L^{-1} and 2.8 mg L^{-1}) and it decreased in both of the productions showing closer levels (2.4 mg L^{-1} and 2.3 mg L^{-1}).

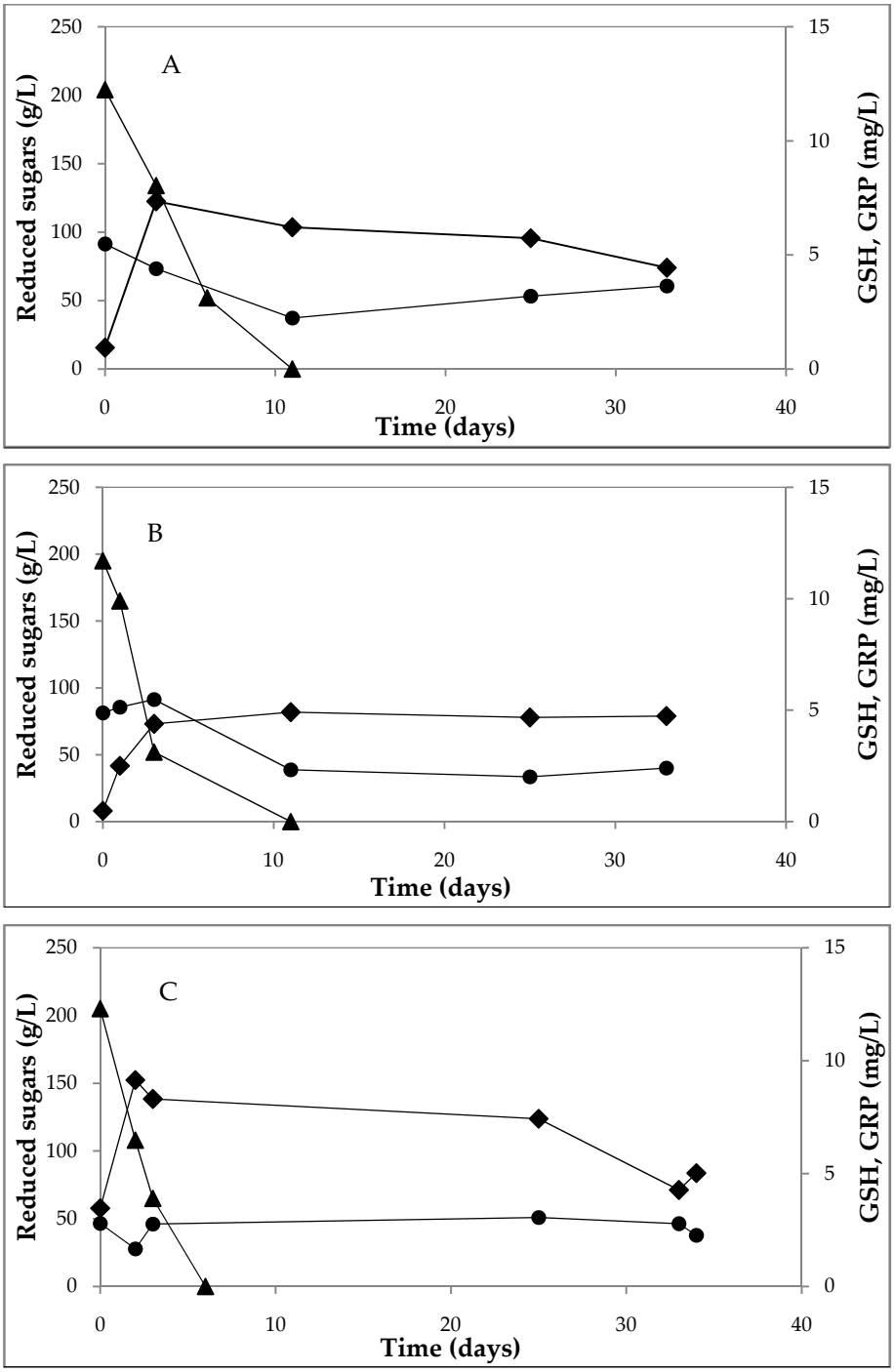

Figure 3.26: GHS (♦), GRP (•) and reduced sugars (▲) trend in winemaking conducted by winery 6 on Vermentino grape in the following condition: no rinsed grape (A); rinsed grape and addition of Bordeaux mixture powder (B); rinsed grape (C) during the vintage 2010.

The GSH content was evaluated in the must fractions obtained from Lugana grape pressed by a pneumatic press under inertized and not inertized conditions. As shown in table 3.15, the GSH content was stable in the 6 fractions analyzed with a not inertized press, while it increased using the inertized press, except for the 6th fraction. The GSH concentration was different between the two pressing conditions probably due to the presence of the oxygen which could cause the GSH oxidation. The increasing GSH content using the inertized press was probably due to the copper washout effect occurring during the pressing: the upcoming pressing was left with a lower content of copper in juice. For the same reason, in the the last fraction, the GSH content was low it was completely taken by the previous fractions.
As expected, the GRP content was higher using the not inertized press for each pressed fraction analyzed, except for the 6th fraction which shown a GSH level comparable to the inertized pressed fraction. The higher GRP content did not explain the differences in GSH level between inertized and not inertized press.

Fraction	**Air**		**Nitrogen**	
	GSH	*GRP*	*GSH*	*GRP*
	mg L^{-1}			
1° fraction	3.8	10.5	3.6	7.8
2° fraction	3.8	16.4	4.9	10.6
3° fraction	3.8	19.3	13.9	12.8
4° fraction	4.6	15.0	19.6	14.4
5° fraction	3.8	19.3	23.1	14.0
6° fraction	4.9	18.0	5.3	19.6

Table 3.15: Effect of different pressing pressure and oxygen on GSH and GRP content.

3.9 Conclusions

The data obtained from the winemakings showed the GSH concentration could be correlated to the yeast strain activity. The aeration can affect the GSH level during or after the fermentation: it can increase the GSH released by the yeast but, at the same time, it can decrease the GSH concentration faster. Indeed, the *S. cerevisiae* growth up faster by aerating the must releasing more GSH. At the end of fermentation, a higher level of dissolved oxygen, deriving from aeration treatment, could increase the loss of GSH.
The GSH deriving from the grape was almost completely lost in the must. The pressing does negatively affect its concentration: most of the winemaking considered showed low concentration of GSH after pressing even thought it was conducted in reductive conditions. This fact could be explained by several not oxidative mechanisms which should be deepened.

The copper concentration has to be considered for a better comprehension of the parameters affecting the GSH level during the winemaking. Among its effect, it has to be correlated to the fermentation course as well as to the dissolved oxygen.
These data confirms the readily assimilable nitrogen affects the GSH content during the winemaking, as previously described in literature (Lavigne & Dubourdieu, 2004).
The level of GSH detected after the fermentation could be enough to protect the wine against the oxidation during the tank or barrel ageing (Lavigne & Dubourdieu, 2004).
Previous researches showed the yeast can metabolize the GSH which decreased during the alcoholic fermentation (Lavigne & Dubourdieu, 2004). This data were not in accordance to the followed winemakings in real condition process. In all of these productions, the GSH concentration decreased after pressing, for reasons not directly linked to the yeast, and it increased back during the fermentation. The GSH content in wine could be probably dependent firstly from the yeast strain employed in fermentation and, secondly, from the copper concentration dissolved in must.
The GRP concentration could be correlated to several factors, such as oxygen, winemaking process, grape cultivar. The latter seems to strongly affect the GRP level and it is linked to environmental conditions and the agronomic practices. For most of the winemaking analyzed, the GRP content can decrease during the fermentation as an effect of its hydrolysis occurring during the ageing (Cheynier et al., 1986).
Further experiments can be necessary in order to better understand the role of copper during the winemaking and its influence on the GSH concentration in grape, must and wine.

3.10 References

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3.11 Evaluation of oxidation rate in white wine

The oxidation rate of selected South African Sauvignon blanc wines from the 2010 vintage was evaluated.
The experiments were carried out in order to understand the effect of sulfur dioxide and glutathione on the oxidation of phenols which can occur during the ageing of white wines. The first aim was to investigate the oxidation of caffeic acid in wine as well as in a synthetic wine solution. The second aim was to assess the oxidation rate and the factors affecting it in 13 commercial wines. In order to increase the oxidation rate, the samples were left at an elevated temperature. The content of iron and copper were also quantified whereas these metals could affect the wine oxidation, as already described in literature (Danilewicz, 2003; Danilewicz, 2007; Danilewicz et al., 2008). Methods developed as part of this study to quantify certain compounds such as glutathione and caffeic acid was further used in this investigation.

3.12 Materials and methods

3.12.1 Chemicals

3-Mercaptopropionic acid (3MPA), p-benzoquinone (pBQ) and potassium metabisulfite ($K_2S_2O_5$), $CuSO_4.5\ H_2O$ and $FeSO_4.7\ H_2O$ were purchased from Fluka (Switzerland). Reduced glutathione (GSH), oxidized glutathione (GSSG), caffeic acid, catechin and trifluoroacetic acid (TFA) were from Sigma-Aldrich (St. Louis, MO). HPLC grade methanol was purchased from Panreac (Barcelona, Spain), and HPLC water was obtained by Milli-Q system (Millipore Filter Corp., Bedford, MA).

3.12.2 Samples

For the analytical method validation, GSH, catechin and caffeic acid determination was carried out on 12 South African white juice samples consisting of 9 Sauvignon Blanc juices, 2 Semillon juices and 1 Chenin Blanc juice. Additionally, 43 South African white wine samples, of which 32 wines were sampled from stainless steel tanks and 11 commercial bottled wines, produced from 5 different grape cultivars (Chardonnay, Chenin Blanc, Sauvignon Blanc, Petillant Blanc and Semillon) from the 2004 - 2010 vintages were analyzed.
For the experiment described in Paragraph 3.12.3, Sauvignon blanc wine (vintage 2010) was treated with bentonite (0.1 g L^{-1}) and active carbon (0.4 g L^{-1}) in order to remove proteins and phenols, respectively. It was drawn from a tank directly from the producer. A synthetic wine solution containing 12% ethanol and 2.5 g L^{-1} tartaric acid was also made up and the pH adjusted to 3.5 with sodium hydroxide pellets.
For the experiment described in Paragraph 3.12.4, 13 young South African Sauvignon Blanc wines (vintage 2010) were drawn from a storage tank directly at the producer and analyzed.

3.12.3 Evaluation of caffeic acid oxidation

The GSH and phenolic concentrations of the wine were measured and established to be very low. GSH and caffeic acid additions were 0.22 mM where required. The natural occurring total SO_2 concentrations of the wine was 17 mg L^{-1} and 30 mg L^{-1} SO_2 was added to increase it to around 50 mg L^{-1}. In the synthetic wine solution where SO_2 additions were required, 50 mg L^{-1} SO_2 was added, to keep it in line with that of the wine. The iron and copper concentrations of both the wine and synthetic wine solution were increased to 5 mg L^{-1} and 0.12 mg L^{-1} by adding $FeSO_4.7\ H_2O$ and $CuSO_4.5\ H_2O$, respectively. Wine and synthetic wine solution were transferred in a 100 mL bottles and the bottles were sealed hermetically. All experiments were performed in triplicate and stored in the dark at 37°C for 60 days.

The treatments conducted on wine and synthetic wine solution were thus the following:

1) wine added with caffeic acid;
2) wine added with caffeic acid and GSH;
3) wine added with caffeic acid and SO_2;
4) wine added with caffeic acid, SO_2 and GSH;
5) synthetic wine solution added with caffeic acid and SO_2 (20 mg L^{-1});
6) synthetic wine solution added with caffeic acid, SO_2 (20 mg L^{-1}) and GSH;
7) synthetic wine solution added with caffeic acid and SO_2 (50 mg L^{-1});
8) synthetic wine solution added with caffeic acid, SO_2 (50 mg L^{-1}) and GSH;
9) not added wine;
10) synthetic wine solution added with SO_2 (20 mg L^{-1}).

The oxygen concentration was periodically monitored (2-3 times daily) with the NomaSense oxygen meter from Nomacorc. Sensor dots attached inside the bottle are activated by a blue light and transmitted through a fiber optic wand. The sensor dot responds by emitting a red light, which indicates the relative presence of dissolved oxygen in the wine.

A resume of the compounds analyzed is represented in Table 3.16. For GSH, caffeic acid and free and total SO_2 quantification in both wine and synthetic wine solution bottles were opened either and the analytes determined 5 times for the 60 days duration of the experiment. These assays were thus performed at days 0 (beginning), 5, 23, 42 and 60 (end).

GSH was derivatized and quantified by ultra-pressure liquid chromatography (UPLC) (Paragraph 3.12.6) at these 5 times, as well as caffeic acid was directly determined by UPLC.

The free and total SO_2 concentrations were determined by a titration with KI solution (Paragraph 3.12.7) at the 5 days indicated above.

Cis-caftaric acid, trans-caftaric acid, 2-*S*-glutathionyl caftaric acid (GRP), cis-coutaric acid, trans-coutaric acid, cis-fertaric acid, p-coumaric acid and ferulic acid were quantified by HPLC (Paragraph 3.12.8) at days 0, 23 and 60 for the experiment where

GSH was added, while at days 0 and 60 for the experiment where no GSH addition was not expected. GSSG was quantified by liquid chromatography coupled to mass spectrometry (LC-MSMS) (Paragraph 3.12.9) at days 0, 23 and 60 for the experiment where GSH was added, while at days 0 and 60 for the experiment carried out in wine which was not expected GSH addition. GSSG was not quantified in the synthetic wine solution assay where no GSH was added.
Copper and iron were quantified by atomic adsorption spectrometry (Paragraph 3.12.10) at the beginning and at the end times. Color changes were also monitored at wavelength 280 nm, 420 nm and 440 nm with a spectrophotometer for the 5 sampling times.

Compound	**Sampling time (day)**				
	0	5	23	42	60
GSH	X	X	X	X	X
Caffeic acid	X	X	X	X	X
Free and total SO_2	X	X	X	X	X
Cis-caftaric acid	X		X*		X
Trans-caftaric acid	X		X*		X
GRP	X		X*		X
Cis-coutaric acid	X		X*		X
Trans-coutaric acid	X		X*		X
Cis-fertaric acid	X		X*		X
p-coumaric acid	X		X*		X
Ferulic acid	X		X*		X
GSSG	X**		X*		X**
Copper	X				X
Iron	X				X
Colour changes	X	X	X	X	X

Table 3.16: Analysis carried out for the experiment concerning the evaluation of caffeic acid oxidation (Paragraph 3.12.3). X*: analysis performed only for the treatments with addition of GSH. X**:analysis carried out on wine and in synthetic wine with added GSH.

3.12.4 Evaluation of oxidation rate in different commercial Sauvignon blanc wines

Thirteen different Sauvignon blanc wines were drawn from storage tanks under nitrogen atmosphere just after the completion of alcoholic fermentation, before any SO_2 additions were made by the winemakers. Each wine was divided into two aliquots and no SO2 was added in one of them, whereas SO_2 30 mg L^{-1} were added in the second one. One hour after the SO_2 addition, the wines were stirred to saturate them with oxygen. The wines were then transferred in 100 mL bottles, sealed hermitically and stored in the dark at 37 °C for 60 days.

The treatments carried out on each wine were thus as follows:

1) no addition;
2) addition of sulfur dioxide (30 mg L^{-1}).

The oxygen concentration was periodically monitored (2-3 times daily) with the NomaSense oxygen meter, as described in Paragraph 3.12.3. Wines were analyzed at the beginning and at the end of the experiment. The investigated parameters were: GSH, catechin and caffeic acid quantified by UPLC (Paragraph 3.12.6), GSSG by LC-MSMS (Paragraph 3.12.9), SO_2 (free and total) by titration (Paragraph 3.12.7). Cis-caftaric acid, trans-caftaric acid, GRP, cis-coutaric acid, trans-coutaric acid, cis-fertaric acid, p-coumaric acid and ferulic acid were determined by HPLC (Paragraph 3.12.8), copper and iron by atomic adsorption spectrometry (Paragraph 3.12.10), ascorbic acid by an enzymatic kit (Paragraph 3.12.11). Color changes were also monitored at wavelength 280 nm, 420 nm and 440 nm.

3.12.5 Juice and wine sample preparation

Juice and white wine were stirred at 14000 rpm for 5 minutes. The supernatant was added with 100 μL ethanal 320 mM, left for 15 minutes at room temperature, derivatized with pBQ as described in Paragraph 3.12.6 and then microfiltered (0.22 μm).

3.12.6 Derivatization of glutathione with p-benzoquinone

The derivatization of GSH and Cys was conducted as described by Tirelli et al. (2010): 2 mL of the sample were added with 100 μL pBQ 400 μM dissolved in methanol. After 1 minute mixing, 1 mL of 500 μM 3MPA in 0.3 M citrate buffer pH 3.5 was added in order to remove the exceeding pBQ.

3.12.6.1 Calibration curves

Calibration curves were obtained by spiking known amounts of GSH (0.3 mg L^{-1} – 100 mg L^{-1}), catechin (0.5 mg L^{-1} – 80 mg L^{-1}) and caffeic acid (0.5 mg L^{-1} – 50 mg L^{-1}) to juice, synthetic wine solution and white wine.

3.12.6.2 Precision parameters

The analytical method response was evaluated by spiking increasing concentrations of GSH, caffeic acid and catechin in juice and white wine. The added amount of caffeic acid and catechin were 4 mg L^{-1}, 10 mg L^{-1} and 22 mg L^{-1} both in juice and white wine. The addition of GSH was carried out at 6.7 mg L^{-1}, 21.2 mg L^{-1} and 45.5 mg L^{-1} in juice and at 1.2 mg L^{-1}, 3 mg L^{-1} and 6.7 mg L^{-1} in white wine. Samples were analyzed in triplicate.

3.12.6.3 Recovery

The recovery was calculated by comparing six replicate determinations of spiked and unspiked samples in the matrices for all considered compounds. For caffeic acid and catechin in juice and white wine three different concentrations were spiked, 4 mg L^{-1}, 10 mg L^{-1} and 22 mg L^{-1}. GSH recovery was evaluated at 6.7 mg L^{-1}, 21.2 mg L^{-1}, 45.5 mg L^{-1} in the juice and 1.2 mg L^{-1}, 3 mg L^{-1}, 6.7 mg L^{-1} in white wine.

3.12.6.4 Limit of detection and of quantification

The LOQ and the LOD were determined on the bases of signal to noise ratio 10:1 and 3:1, respectively. Baseline noise was calculated considering peak-to-peak measurement for 3 minutes in two parts of the chromatogram.

3.12.6.5 Ultra-pressure liquid chromatography

The GSH derivatized with pBQ, catechin and caffeic acid were determined by UPLC.
The UPLC separation was performed by a BEH-C18 column (100 x 1.7 mm, 1.7 μm, Waters). Eluents were water/trifluoroacetic acid (0.05% v/v) and methanol; the concentration of the latter increased from 10% to 35% in 8.5 minutes in the eluting gradient (Table 3.17). Detection was carried out by spectrophotometry at 303 nm, 280 nm and 320 nm wavelength for GSH, catechin and caffeic acid, respectively. The injection volume was 2 μL and the column was thermostatted at 25°C.

Time (min)	A%	B%	Slope
0.0	90.0	10.0	6
5.0	83.0	17.0	6
7.5	70.0	30.0	7
8.5	65.0	35.0	6
8.8	0.0	100.0	6
9.3	90.0	10.0	6
11.0	90.0	10.0	6

Table 3.17: UPLC separation gradient for GSH derivatized with *p*-benzoquinone and caffeic acid. Eluent A: water/trifluoroacetic acid (0.05% v/v); eluent B: methanol.

3.12.7 Quantification of free and total sulfur dioxide

The free SO_2 was determined as follows: 25 mL of wine were drawn and 15 mL H_2SO_4 at 9% concentration were added. The SO_2 was titrated with a KI/KIO_3 solution 1/128 after the addition of iodine indicator until the purple colour was stable at least for 30 seconds.

For the total SO_2 quantification, 25 mL of wine were added to 20 mL of NaOH 1N and left at room temperature for 20 minutes. The determination was then carried out as for the free SO_2.
The SO_2 concentration was calculated as follows:

$$SO_2 = mL\ KI \times 10$$

The SO_2 content was expressed in mg L^{-1}.

3.12.8 Quantification of hydroxycinnamic acids

The content of cis-caftaric acid, trans-caftaric acid, cis-coutaric acid, trans-coutaric acid, cis-fertaric acid, p-coumaric acid and ferulic acid were quantified as described by Vanzo et al. (2007).
The sample was microfiltered prior the HPLC injection.
Separation was performed using a ODS Hypersil C18 column (5 μm, 250 x 2.1 mm, Agilent Technologies) with a ODS Hypersil guard column (5 μm, 20 x 2 .1 mm, Agilent Technologies). The mobile phase consisted of water acidified with 0.5% formic acid 2% formic acid in methanol. Separation was carried out at 40 °C for 33 min and then the column was equilibrated for 10 minutes; the gradient conditions are shown in Table 3.18. The flow rate was 0.4 mL/min and the injection volume was 70 μL. The detection was carried out by a diode array detector (Agilent Technologies) and the UV/Vis spectra were recorded from 220 to 700 nm, with detection at 320 nm.
The identity of each hydroxycinnamates was based on their UV/Vis spectra and retention times which were compared to the respective standards. Quantification of compounds was based on peak areas at 320 nm wavelength and the respective concentrations in samples were expressed as trans-caftaric acid equivalents.

Time (min)	A%	B%	Slope
0.0	84.0	16.0	6
15.0	75.0	25.0	6
28.0	57.0	43.0	6
28.1	0.0	100.0	6
33.0	0.0	100.0	6
33.1	84.0	16.0	6
43.0	84.0	16.0	6

Table 3.18: HPLC separation gradient for hydroxycinnamic acids. Eluent A: water/formic acid 0.5%; B: methanol/formic acid 2%.

3.12.9 Quantification of oxidized glutathione

The quantification of GSSG was conducted as described by du Toit et al. (2007). A microtriple quadropole mass spectrometer was coupled with HPLC. Separation was carried out with a C18 column (3 μm, 150 x 2.1 mm, Waters). The eluents were water acidified with formic acid 0.1% and acetonitrile; the concentration of the latter increased to 80% in 6.5 minutes in the eluting gradient (Table 3.19). The MS method consisted of a reaction monitoring (MRM) function with electrospray ionization in the positive mode, a capillary voltage of 3.5 kV, and argon as the collision gas. The GSSG was monitored with at m/z 613.1 > 355.1 transition at a collision energy of 20 eV and cone voltage of 30 V.
Ethanol was removed from the wine samples under reduced pressure at 40 °C by a rotary evaporator and wine samples were re-dissolved to the initial volume with deionized water. The samples were filtered through a 0.45 um syringe filter prior to the LC-MSMS analysis.

Time (min)	A%	B%	Slope
0.0	100.0	0.0	6
0.5	75.0	25.0	6
6.5	57.0	43.0	6
6.6	0.0	100.0	6
10.9	0.0	100.0	6
11.0	100.0	0.0	6
18.0	100.0	0.0	6

Table 3.19: HPLC separation gradient for oxidized glutathione. Eluent A: water/formic acid 0.1%; B: acetonitrile.

3.12.10 Quantification of iron and copper

Iron and copper were quantified by atomic absorption spectrometry, as described in the OIV method such as MA-F-AS322-05-FER for iron and MA-F-AS322-06-CUIVRE for copper by Vinlab, an ISO 9000 accredited laboratory that does routine wine analyses for the South African wine industry. For the former, ethanol evaporation is needed prior to the analysis, while for the second one no preparation steps before the analysis except the eventual dilution.

3.12.11 Quantification of ascorbic acid

The ascorbic acid was determined through the enzymatic method of Thermo Fischer (Thermo Scientific) by Vinlab.

3.12.12 Statistical analysis

The data concerning the experiments focused on the caffeic acid (Paragraph 3.13.3) and phenols oxidation (Paragraph 13.1.4) described as above were statistically analyzed with two way ANOVA analysis using STATISTICA software (version 8).

3.13 Results and discussion

3.13.1 UPLC method development

Derivatized GSH, catechin and caffeic acid were separated using the analytical conditions developed. The retention times were 4.2 min, 7.5 min and 9.2 min for derivatized GSH, catechin and caffeic acid, respectively, with the derivatized MPA eluting at 8.2 min. The GSH derivatization with pBQ was fast, accurate and easy-to-apply; no purification step, other than centrifugation, for either juice or white wine samples was necessary before the derivatization reaction. The short run time and the quick sample preparation allowed the compounds to be quantified in less than 20 minutes. All of the compounds investigated were separated as interference-free chromatographic peaks (Figure 3.27).

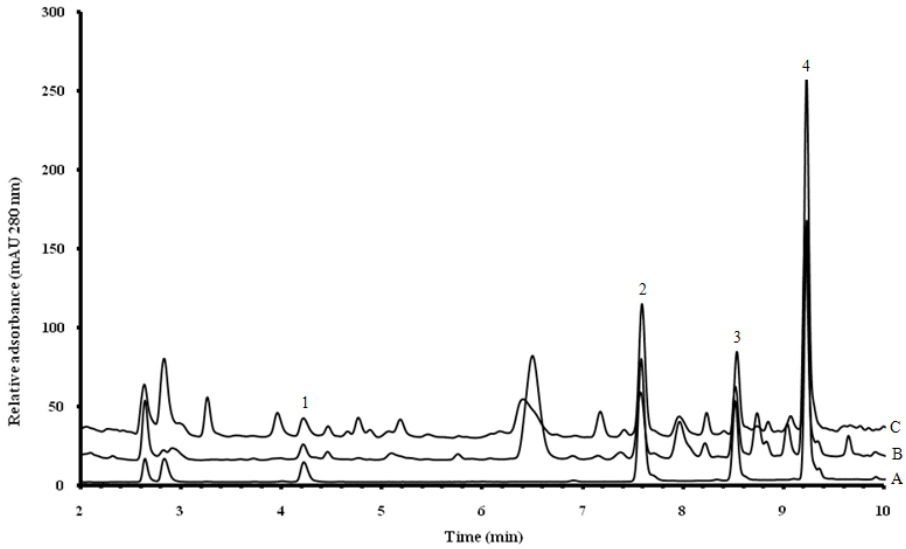

Figure 3.27: UPLC separation at 280 nm in a model wine solution (A), must (B) and white wine (C). Peaks: 1: GSH, 2: catechin, 3: derivatized MPA, 4: caffeic acid.

The linearity was verified by spiking juice, synthetic wine and white wine with the compounds considered at six concentration levels in. All of the samples were prepared and injected at least in duplicate for juice, synthetic wine solution and white wine and

for each concentration. The analytical method showed a linear response for added concentration of GSH ranging from 0 mg L^{-1} to 100 mg L^{-1}, catechin ranging from 0 mg L^{-1} to 80 mg L^{-1} and caffeic acid ranging from 0 mg L^{-1} to 50 mg L^{-1}, in accordance with the concentrations indicated in literature (Margalit, 2004; Janěs et al., 2010; Makhotkina & Kilmartin, 2010). Shown in Figure 3.28 are the calibration curves for juice, synthetic wine and white wine, respectively. For the quantification of GSH, catechin and caffeic acid, no significant differences were found in the response between the synthetic wine solution and the white wine. The calibration curves slopes were compared through the F Test (p = 95%). As a high value of the intercept shows, higher absolute values were detected because of the native GSH amount in juice and catechin and caffeic acid content in white wine.

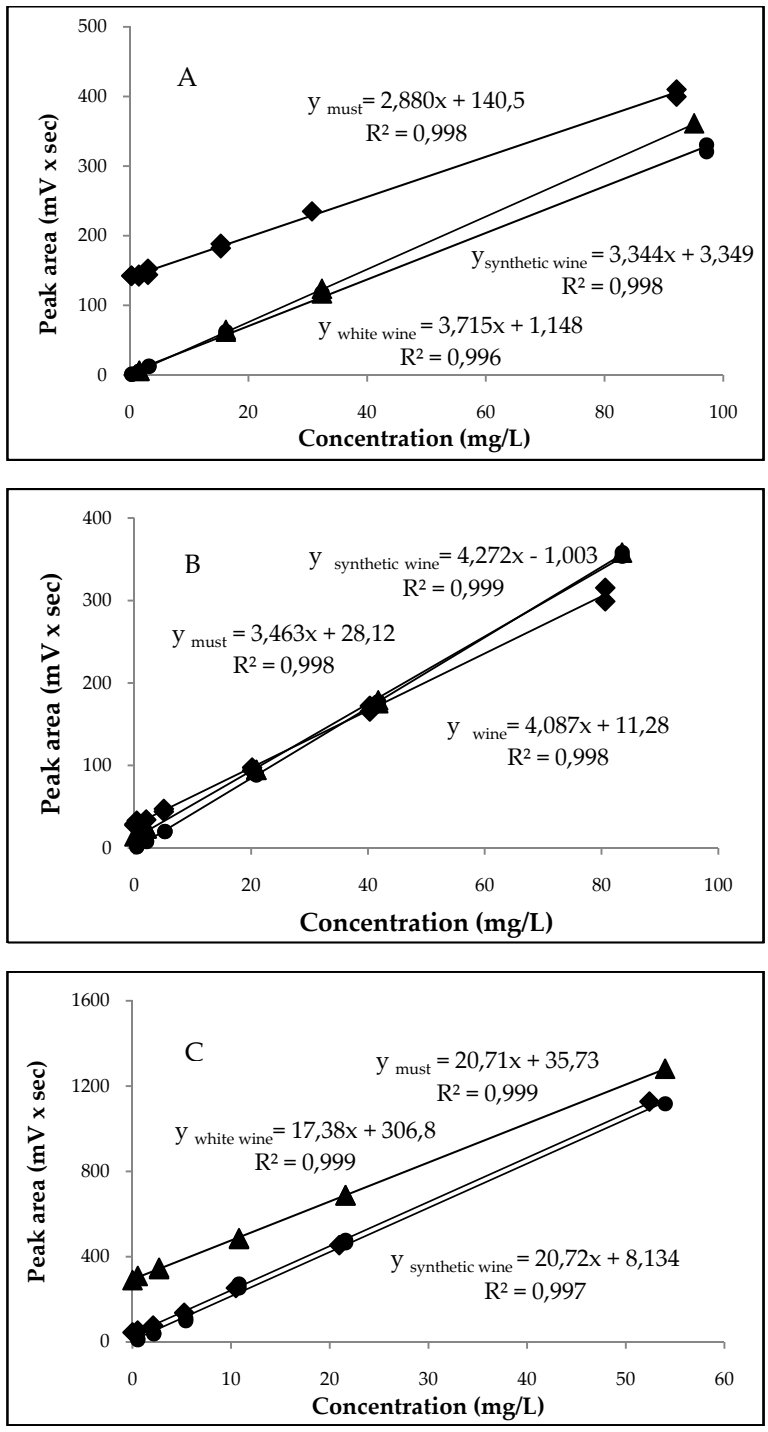

Figure 3.28: Calibration curves for GSH (A), catechin (B) and caffeic acid (C) in juice (♦), white wine (▲) and synthetic wine solution (•).

3.13.1.1 UPLC method validation

The repeatability and the intermediate repeatability were determined as described in Paragraph 3.12.6.2. Higher GSH levels were spiked in juice than wine due to higher GSH concentrations occurring in juice than in white wine (Cheynier et al., 1989; du Toit et al., 2007; Janěs et al., 2010). The values presented in Table 3.20 and 3.21 thus correspond to the sum of the native content and those spiked for each compound. As can be seen in Table 3.20 and Table 3.21 for both juice and white wine, good repeatability were obtained.

Compound	**GSH**			**Catechin**			**Caffeic acid**		
Concentration level	38.1	58.3	117.4	30.6	41.7	53.1	27.2	37.5	49.6
SD repeatability (mg L^{-1})	2.6	1.6	3.9	0.27	0.75	0.79	0.26	0.44	0.22
SD intermediate repeatability (mg L^{-1})	3.3	2.5	8.6	1.4	0.77	1.2	0.61	0.63	1.4
Repeatability limit (mg L^{-1})	5.1	3.1	7.6	0.53	1.5	1.6	0.52	0.87	0.43
Intermediate repeatability limit (mg L^{-1})	6.5	5.4	12.1	2.7	1.5	2.4	1.2	1.2	2.8
RSD (%)	6.2	7.7	8.2	4.7	7.0	5.3	2.5	8.1	7.9
Average RSD (%)	7.4			5.7			6.2		

Table 3.20: Standard deviation of repeatability and intermediate repeatability in juice; SD: standard deviation, expressed in mg L^{-1}; RSD: residual standard deviation, expressed in percentage.

Compound	**GSH**			**Catechin**			**Caffeic acid**		
Concentration level	1.2	3.1	6.8	5.4	12.6	24.6	0.21	4.7	12.8
SD repeatability (mg L^{-1})	0.0084	0.18	0.24	0.19	0.033	0.15	0.00078	0.11	0.17
SD intermediate repeatability (mg L^{-1})	0.13	0.58	0.54	0.30	0.43	0.39	0.0031	0.30	0.34
Repeatability limit (mg L^{-1})	0.16	0.36	0.46	0.37	0.065	0.29	0.0015	0.21	0.34
Intermediate repeatability limit (mg L^{-1})	0.26	1.1	1.1	0.60	0.83	0.77	0.0061	0.59	0.67
RSD (%)	3.8	6.3	2.6	8.6	2.6	1.1	1.5	6.7	2.7
Average RSD (%)	4.2			4.1			3.6		

Table 3.21: Standard deviation of repeatability and intermediate repeatability in white wine; SD: standard deviation, expressed in mg L^{-1}; RSD: residual standard deviation, expressed in percentage.

3.13.1.2 Limit of detection and quantification

The LOD in both juice and wine was 0.017 mg L^{-1}, 0.014 mg L^{-1}, and 0.0026 mg L^{-1} for GSH, catechin and caffeic acid, respectively The LOQ in both juice and wine was 0.057 mg L^{-1}, 0.048 mg L^{-1} and 0.0088 mg L^{-1} for GSH, catechin and caffeic acid respectively. For GSH, LOD and LOQ were lower than those previously reported in the literature when HPLC equipped with fluorescence detector was used. Values of 0.06 mg L^{-1} and 0.03 mg L^{-1} for LOD for juice and white wine and LOQ values of 0.2 mg L^{-1} and 0.1 mg L^{-1} for juice and wine were reported (Janěs et al., 2010; Marchand & de Revel, 2010). The LOD concentration for both catechin and caffeic acid in white wine were also lower than previously found (0.11 mg L^{-1} and 0.02 mg L^{-1}, respectively) (Castellari et al., 2002).

3.13.1.3 Recovery

The recovery was performed and calculated by standard addition of the analytes of interest to juice and white wine as indicated in Paragraph 3.12.6.3.
For GSH, the recovery in juice was 100.3% compared to 88.4% in white wine. This is not surprising, as juice is known to have significant higher levels of GSH than wine and confirms previous findings (du Toit et al., 2007; Janěs et al., 2010). The recovery for catechin was 98.7% and 111.3% in juice and white wine respectively, while that of caffeic acid was 111.3% and 85.8% in juice and wine. These recoveries achieved during evaluation of the method were in accordance with those reported in white wine for both catechin and caffeic acid when quantified by HPLC (Castellari et al., 2002; Russo et al., 2008).

3.13.1.4 Quantification in South African juices and white wines

In order to evaluate if the method is suitable for use as a routine analytical tool, 12 grape juice samples (Table 3.22) and 43 white wine samples (Table 3.23) were analyzed. As seen in Table 3.22, GSH concentrations in juice ranged from 1.1 mg L^{-1} to 42.3 mg L^{-1}, which correlates well with values previous reported (Janěs et al., 2010; Maggu et al., 2007). The investigated phenols ranged from 0.73 – 8.7 mg L^{-1} and 0.50 – 3.7 mg L^{-1} for catechin and caffeic acid, respectively.
In wine, as can be seen in Table 3.23, the highest GSH concentration detected was 27.4 mg L^{-1}, which was in general higher in the white wines of the 2010 vintage. During wine ageing, GSH concentrations are known to decrease, leading to lower concentrations of this compound in older wines (Lavigne et al., 2007) which could explain the higher levels we observed in the 2010 wine in general. The amounts detected were also in agreement with previous findings (Janěs et al., 2010; Woraratphoka et al., 2007). The average GSH levels of 2010 Sauvignon blanc tank samples (10 mg L^{-1}) was higher than those of the 2010 Chenin blanc tanks samples (5 mg L^{-1}). Sauvignon blanc juice is often treated more reductively in South Africa, with

the addition of N_2, CO_2 and higher levels of SO_2 to the juice, leading to an elevated preservation of GSH levels in the juice and resulting wines (du Toit et al., 2007; Marais, 1998). The catechin concentration ranged from 1.20 mg L^{-1} to 23.01 mg L^{-1} and caffeic acid between 0.16 mg L^{-1} and 3.69 mg L^{-1}, once again correlating well with values reported in the literature (Landrault et al., 2001; Margalit, 2004).
The low concentrations of these *o*-diphenols observed in the white wines analyzed could have a positive influence during ageing, as high levels of these compounds contribute to non-enzymatic browning of white wine (Li et al., 2008).

Juice	Cultivar	GSH	Catechin	Caffeic acid
		mg L^{-1}	mg L^{-1}	mg L^{-1}
1	Sauvignon blanc	40.0	5.9	0.78
2	Sauvignon blanc	39.6	6.3	0.83
3	Sauvignon blanc	10.6	6.6	0.51
4	Sauvignon blanc	41.4	4.5	0.41
5	Sauvignon blanc	41.8	8.7	0.59
6	Sauvignon blanc	37.2	7.7	3.7
7	Sauvignon blanc	31.6	7.0	1.0
8	Sauvignon blanc	42.3	7.0	1.0
9	Sauvignon blanc	36.5	6.6	1.0
10	Semillon	10.1	4.4	0.82
11	Semillon	20.7	3.4	0.50
12	Chenin blanc	1.1	0.73	0.51

Table 3.22: Description of juices analyzed and their quantification of GSH, catechin and caffeic acid.

Wine	Cultivar	Vintage	Description	GSH	Catechin	Caffeic acid
				mg L^{-1}	mg L^{-1}	mg L^{-1}
1	Sauvignon blanc	2010	tank sample	9.8	23.0	0.64
2	Sauvignon blanc	2010	tank sample	7.0	1.5	0.16
3	Sauvignon blanc	2010	tank sample	8.3	2.5	6.2
4	Sauvignon blanc	2010	tank sample	6.8	8.0	19.4
5	Sauvignon blanc	2010	tank sample	1,8	9.0	6.6
6	Sauvignon blanc	2010	tank sample	9.1	12.8	0.87
7	Sauvignon blanc	2010	tank sample	6.6	3.1	0.89
8	Sauvignon blanc	2010	tank sample	12.6	2.3	1.2
9	Sauvignon blanc	2010	tank sample	4.2	2.7	0.66
10	Sauvignon blanc	2010	tank sample	27.4	2.7	1.2
11	Sauvignon blanc	2010	tank sample	5.9	9.7	0.29
12	Sauvignon blanc	2010	tank sample	11.8	2.0	1.0
13	Sauvignon blanc	2010	tank sample	11.5	1.2	1.0
14	Chenin blanc	2010	tank sample	10.4	9.7	18.5
15	Chenin blanc	2010	tank sample	4.3	3.2	0.51
16	Chenin blanc	2010	tank sample	8.3	1.7	0.28
17	Chenin blanc	2010	tank sample	5.8	1.8	0.84
18	Chenin blanc	2010	tank sample	4.4	1.4	0.87
19	Chenin blanc	2010	tank sample	3.5	2.9	1.2
20	Chenin blanc	2010	tank sample	13.0	2.5	11.7
21	Chenin blanc	2010	tank sample	10.8	1.8	7.1
22	Chenin blanc	2010	tank sample	7.7	3.2	6.1
23	Chenin blanc	2010	tank sample	0.39	2.0	0.89
24	Chenin blanc	2010	tank sample	0.27	3.9	7.2
25	Chenin blanc	2010	tank sample	0.34	1.4	3.7
26	Chenin blanc	2010	tank sample	5.9	2.0	0.65
27	Chenin blanc	2010	tank sample	n.d.	2.1	3.1
28	Chenin blanc	2010	tank sample	n.d.	2.1	2.9
29	Chenin blanc	2010	tank sample	0.19	1.9	2.8
30	Chenin blanc	2010	tank sample	0.23	4.8	7.1
31	Chenin blanc	2010	tank sample	7.2	4.0	1.0
32	Chenin blanc	2010	tank sample	4.9	3.6	0.72
33	Sauvignon blanc	2009	commercial wine	n.d.	5.3	5.5
34	Sauvignon blanc	2009	commercial wine	n.d.	4.6	2.7
35	Petillant blanc	2009	commercial wine	0.50	11.7	5.0
36	Semillon	2009	commercial wine	0.27	5.9	2.8
37	Sauvignon blanc	2008	commercial wine	n.d.	5.0	8.0
38	Sauvignon blanc	2008	commercial wine	0.27	5.9	2.8
39	Sauvignon blanc	2008	commercial wine	0.13	1.6	6.4
40	Sauvignon blanc	2008	commercial wine	n.d.	4.4	2.9
41	Sauvignon blanc	2008	commercial wine	n.d.	2.5	3.9
42	Chardonnay	2007	commercial wine	n.d.	3.9	5.3
43	Sauvignon blanc	2004	commercial wine	n.d.	2.2	5.3

Table 3.23: Description of white wines analyzed and their quantification of GSH, catechin and caffeic acid.

3.13.2 Evaluation of caffeic acid oxidation

This experiment was carried out in order to understand the effects of GSH and SO_2 on the rate of oxygen disappearance and caffeic acid oxidation, which was compared in wine and synthetic wine solution.
Caffeic acid, GSH and oxygen were added at the same molar concentration (0.22 mM). The caffeic acid was added at concentration normally detected in white wine (Laundrault et al., 2001; Castellari et al., 2002).
The presence of metal ions, such as iron and copper, can increase the oxidation rate (Danilewicz, 2003; Danilewicz, 2007).

3.13.2.1 Wine

The bentonite and active carbon treatments in the Sauvignon blanc wine were effective in terms of protein and phenols removal; the latter was reduced to 18 mg L^{-1}, expressed as total phenols. The wine contained 5.5 mg L^{-1} free SO_2 and 17.2 mg L^{-1} total SO_2. Since no SO_2 was added to wine, it could have been produced by the yeast strain employed during fermentation. The concentration of Fe and Cu in the tested wine was 0.21 mg L^{-1} and 0.12 mg L^{-1}, respectively. The final concentration of Fe was 5 mg L^{-1}, while no extra amount of Cu was added. The levels of these metals were in accordance to those used by Danilewicz (2007) when investigating oxidation of phenolics in a wine medium.
The oxygen consumption (Figure 3.29) increased in the presence of higher SO_2 concentrations. This trend was similar in wines containing higher SO_2 (50 mg L^{-1}) concentrations, irrespective if GSH was added or not. On the other hand, in wine with no added SO_2, the oxygen consumption was a bit slower in the presence of GSH, as it showed a lower slope of the model curve representing the trend. Moreover, the oxygen was also monitored for wine to which no caffeic acid, GSH and SO_2 were added; its trend was comparable with the oxygen consumption observed for wine added with caffeic acid. This could mean the oxygen consumption was more affected by the low levels of phenols and SO_2 still present in the wine, than the added caffeic acid. In fact, the content of dissociated caffeic acid affects the reaction rate between oxygen and caffeic acid and it is low in the wine conditions (Cilliers & Singleton, 1989; Cilliers & Singleton, 1991). The content of tartaric acid can compete for the iron coordination and can also affect the caffeic acid oxidation (Danilewicz, 2003).
The GSH content decreased during the experiment, faster where no SO_2 was added, and no GSH was detected at the end time. The SO_2 slowed down, as well, almost disappearing (final concentration lower than 14 mg L^{-1}; decrease up to 30 mg L^{-1}). In the treatments where GSH was added its content decreased slowly and the free SO_2 concentration was higher. The presence of GSH and SO_2 could affect the rate oxidation each other.
A lower content of caffeic acid was observed during the experiment and its decrease was double in the treatments enriched with GSH. It confirmed the interactions between caffeic acid and the thiol compounds (Cilliers & Singleton, 1990).

No increased GRP concentration was detected in these experimental conditions. This was probably due to the continue equilibrium shifts between GRP and caftaric acid already known in wine which did not allow to observe an increase of GRP content (Riberau-Gayon 2006). The presence of hydroxycinnamic acids could affect the GRP formation, as well. Indeed, the wine contained some hydroxycinnamic acids, such as caftaric acid, coutaric acid, fertaric acid, coumaric acid, ferulic acid, which level was investigated, although the wine was treated with active carbon.
No changes in the content of caftaric acid and cis-coutaric acid were observed. The trans-coutaric acid, cis-fertaric acid and trans-fertaric acid decreased in all the treatments proportionally correlated to the antioxidant compounds level. The concentration of coumaric and ferulic acid increased during the experiment.
For the total phenols content, a decrease was observed inversely correlated to the antioxidant compounds level: when the GSH and SO_2 were higher, the total phenols drop was lower. The SO_2 showed a stronger effect on phenols protection than GSH.
Changes in colour were observed through the absorbance measures at 420 nm and 440 nm which were increased for treatments where GSH added. An increase in the absorbance means an oxidative colouration happened in white wine (Skouroumounis et al., 2005). In these experimental conditions, the data did not confirm the positive effect of GSH on the wine colour, as described in literature (Lavigne & Dubourdieu, 2004; Hosry et al., 2009). No change was detected at 280 nm.

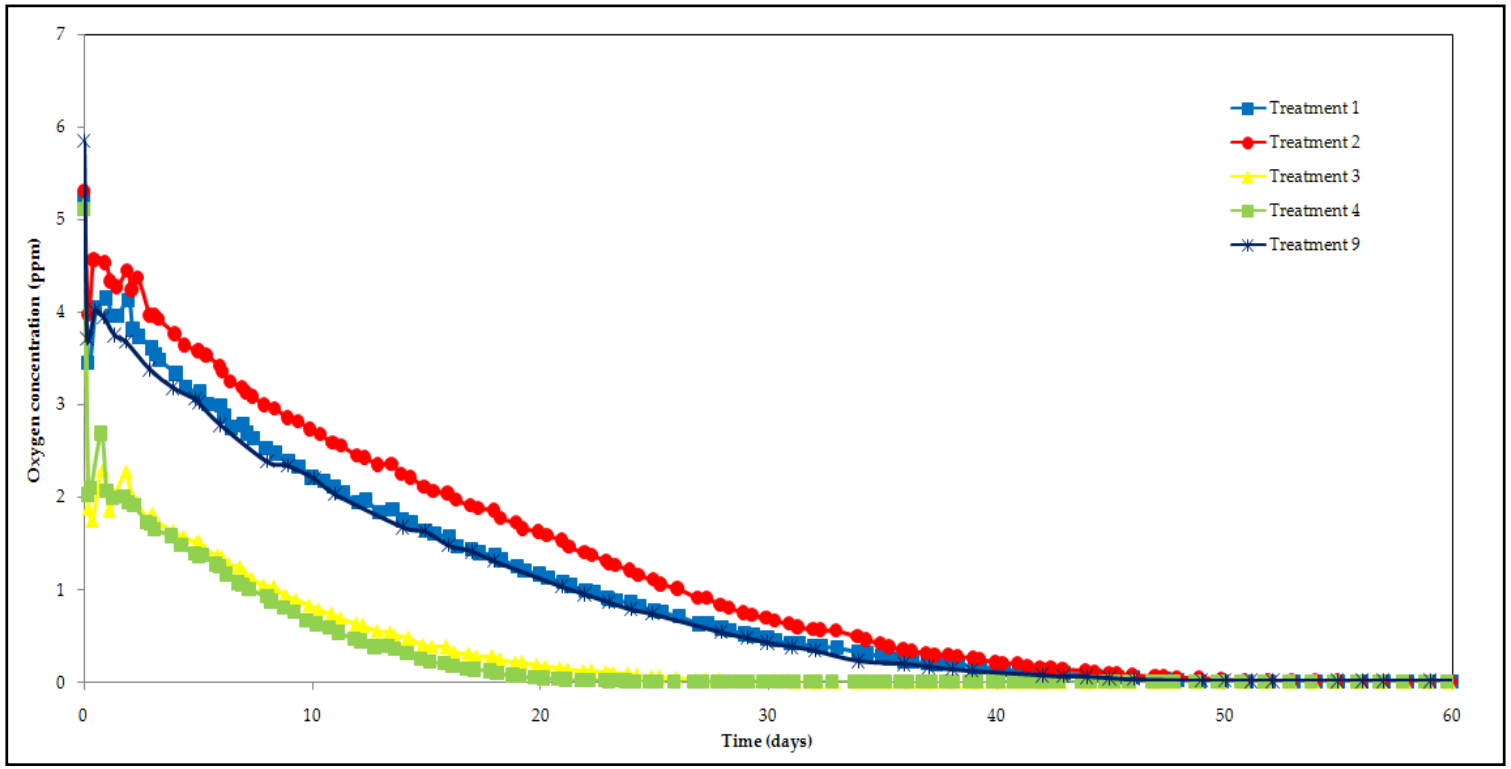

Figure 3.29: Evolution of oxygen concentrations in white wine with different treatments. Treatments: 1: caffeic acid addition; 2: caffeic acid and GSH addition; 3: caffeic acid and SO_2 addition; 4: caffeic acid, GSH and SO_2 addition; 9: no addition.

Two different parameters were considered for the statistical analysis: the first was an indication of the oxygen concentration at the end of the experiment (b0) (Figure 3.30). The second parameter was corresponding to the rate of oxygen decrease (slope of curve representing the oxygen trend) during the assay (b2) (Figure 3.31).

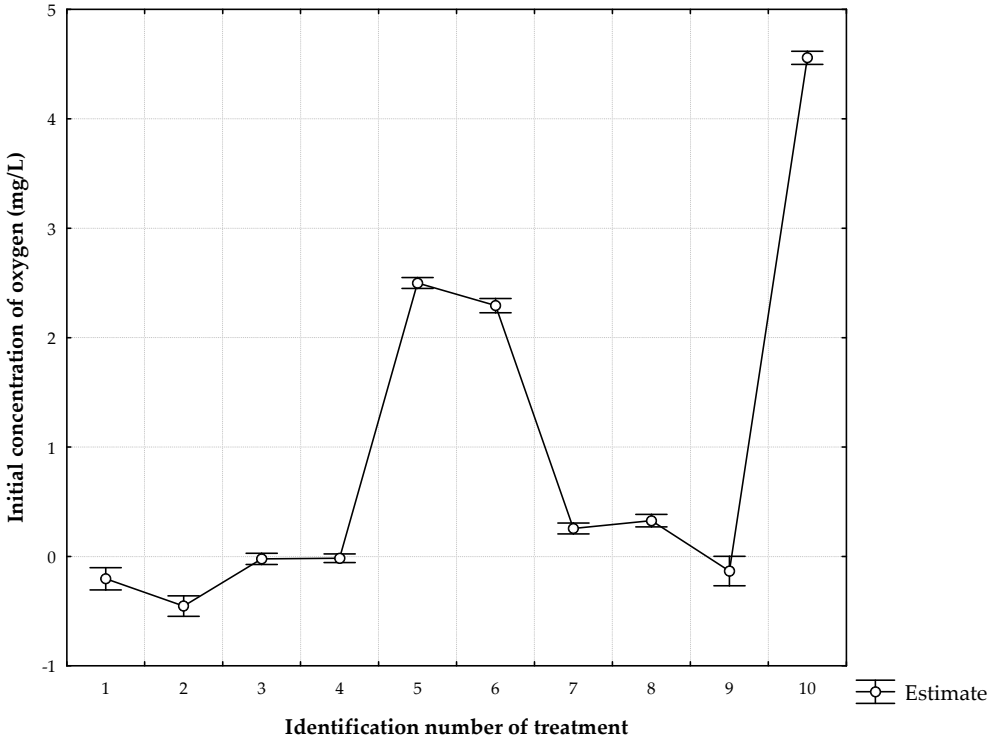

Figure 3.30: Range plot of estimate for final oxygen concentration after 60 days incubation for the different treatments (parameter b0). The treatments description is indicated in Paragraph 3.12.3.

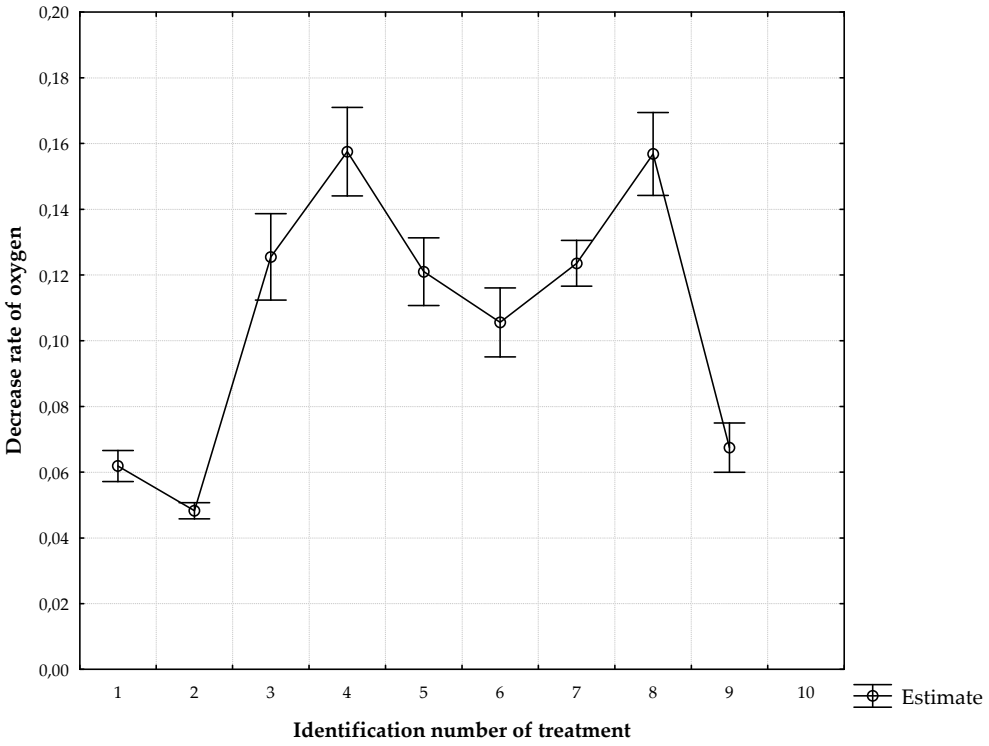

Figure 3.31: Range plot of estimate for the rate of oxygen decrease for the different treatments (parameter b2). The treatments description is indicated in Paragraph 3.12.3.

No significant differences were evaluated between wine with no additions and wine enriched with caffeic acid for all the parameter considered; it means the higher caffeic acid content did neither affect the oxygen concentration after 60 days incubation nor the oxygen decrease rate (numbers 1 and 9 in Figure 3.5 and 3.6). This was probably due to low concentration of dissociated caffeic acid in wine condition, as described in literature (Cilliers & Singleton, 1989; Cilliers & Singleton, 1991).
The oxygen concentration after 60 days of incubation showed that only the addition of GSH as an antioxidant compound yielded not significant differences from the wines to which SO_2 was added (Figure 3.5). Wine with no additions and the addition of caffeic acid were not different; among the samples where GSH was added, the treatments showed significant differences from those enriched with SO_2. This could mean that the final oxygen concentration is affected by the presence of antioxidant compounds, SO_2 in particular. The combination of higher SO_2 and GSH increased the oxygen disappearance. This data is partially in accordance to previous research, which showed that the presence of GSH increased the net oxidation rate (Cheynier & Van Hulst, 1988). It was confirmed only for low SO_2 concentration, but for high level of SO_2 the oxygen decrease was not affected by an high GSH content. After 60 days incubation the oxygen level was lower in presence of GSH, even though the oxygen consumption rate represented in Figure 3.29

and Figure 3.31 was slower where GSH was added. However, it is clear that the addition of SO_2 drastically increased the rate of oxygen consumption (Figures 3.29 and 3.31). Its content had a much larger effect than GSH addition for both level of SO_2 added. This is in accordance with results found by Danilewicz et al. 2008, who also found the rate of oxidation in red wine to increase in the presence of SO_2.
The oxygen decrease rate during the experiment was thus related to the additions GSH, SO_2 or a combination of both, showing significant differences from those where no additions were made or only caffeic acid. The oxygen decrease rate was however, slightly lower in wine to which only GSH was added than in wine enriched with only caffeic acid. This was in accordance to the study carried out by Cilliers and Singleton (1990), which observed no effect exerted by the Cys on the oxygen consumption. On the contrary, the study conducted by Danilewicz et al. (2008) showed an accelerating effect of the Cys on the oxidation, probably due to its capability to form complex with copper readily reacting with oxygen.

3.13.2.2 Synthetic wine solution

The Fe and Cu at the concentration of the synthetic wine solution was increased to 5 mg L^{-1} and 0.12 mg L^{-1}, respectively (Danilewicz, 2007). SO_2 was added at 20 mg L^{-1} and 50 mg L^{-1} to the synthetic wine solution in order to bring it on par with that of the white wine.
The oxygen consumption (Figure 3.32) was comparable between GSH and no GSH additions where only 20 mg L^{-1} of SO_2 were added, but the GSH addition led to the curve model slope being lower, again being in accordance to literature (Cheynier & Van Hulst, 1988). However, again the oxygen concentration decreased faster with the addition of SO_2 50 mg L^{-1}.
The GSH content decreased during the experiment and no GSH was detected at the end time. The SO_2 slowed down, as well, until disappearing. In the treatments where GSH was added its content decreased faster.
As already observed for the wine treatments, a lower content of caffeic acid was observed during the experiment and its decrease was double in the treatments enriched with GSH. It confirmed the interactions between caffeic acid and the thiol compounds in synthetic wine solution, as well (Cilliers & Singleton, 1990).
The GRP was formed in synthetic wine solution and its concentration was double where only GSH added. It could be due with no SO_2 more *o*-quinones could be available to react with GSH.
Cis-fertaric acid and trans-fertaric acid were detected and the concentration of the trans isomer was higher. No data are described in literature concerning an increased concentration of fertaric acid in synthetic wine solution enriched with caffeic acid, as well as in wine.
For the total phenols content, a decrease was observed inversely correlated to the antioxidant compounds level: when the GSH and SO_2 were higher, the total phenols drop was lower. The SO_2 showed a stronger effect on phenols protection than GSH.

Increased values of the absorbance were observed at 280 nm correlated neither with the GSH nor with the SO_2 addition.

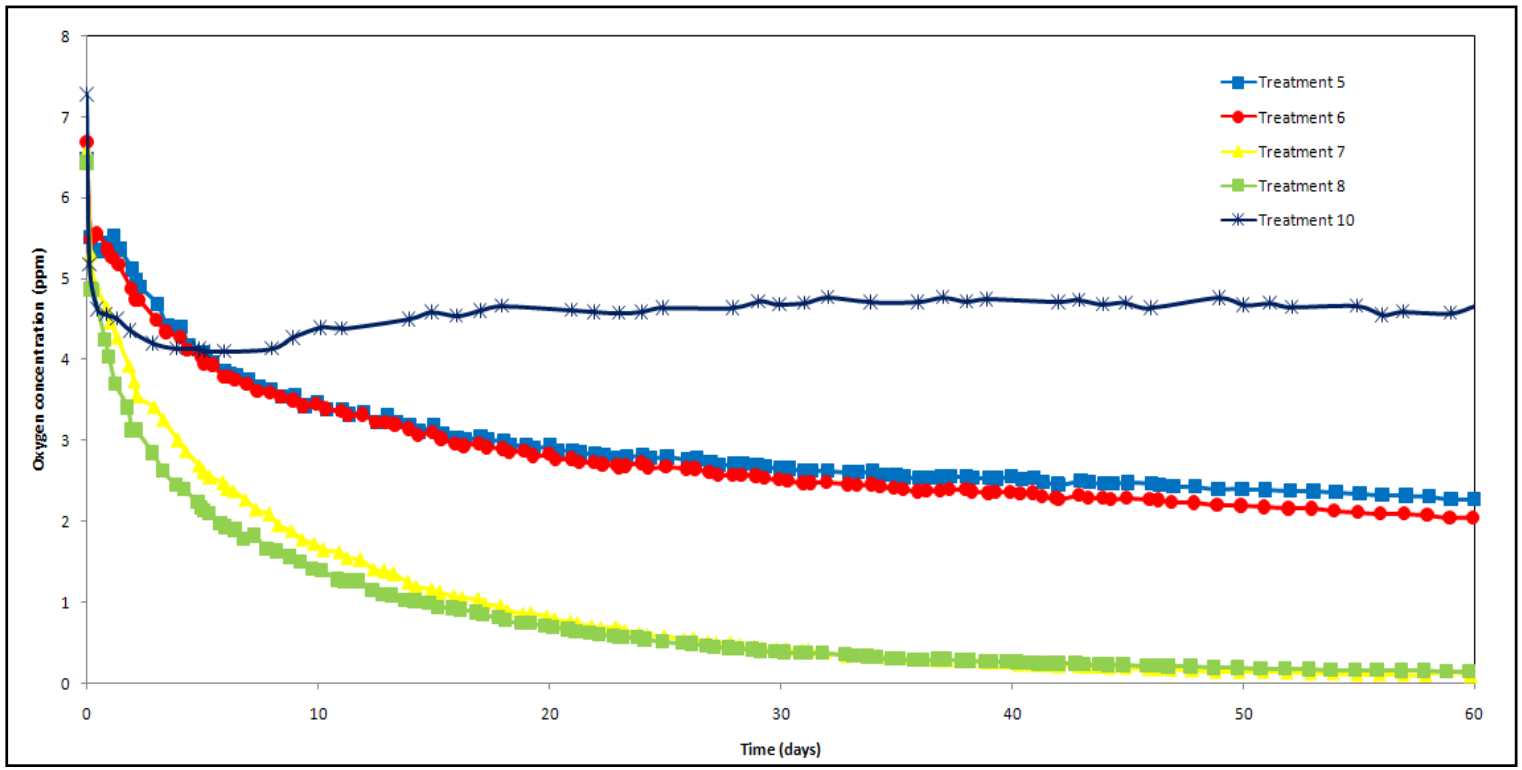

Figure 3.32: Evolution of oxygen concentrations in synthetic wine with different treatments. Treatments: 5: caffeic acid and SO_2 20 mg L^{-1} addition; 6: caffeic acid, GSH and SO_2 20 mg L^{-1} addition; 7: caffeic acid and SO_2 50 mg L^{-1} addition; 8: caffeic acid, GSH and SO_2 50 mg L^{-1} addition; 10: SO_2 20 mg L^{-1} addition.

The parameters considered for the wine treatments were evaluated also for the synthetic wine solution, as well.

Regarding the oxygen concentration at the end of the experiment, the synthetic wine solution without any additions, to which only caffeic acid and only caffeic acid and GSH was added to, was significant different to all the treatments applied (Figure 3.30). This parameter was not significant different where higher SO_2 additions were made. However, the addition of GSH did not lead to significant differences at the end of the experiment between the samples containing higher concentrations of SO_2 (Figure 3.30). This means that caffeic acid on its own affected the oxygen level after 60 days incubation, as well as the GSH addition. However, the latter only had an effect on the final oxygen amount only at the lower SO_2 content. This indicates that the SO_2 at higher levels could be mainly correlated to the final concentration of oxygen in synthetic wine solution and the GSH did not play a relevant role in these samples. This is probably due to the stronger nucleophilic behavior of SO_2 at high concentration (Danilewicz et al., 2008).

The rate of oxygen decrease was affected by higher amounts of SO_2. Indeed, no significant differences were observed between synthetic wine solution where only caffeic acid, caffeic acid and GSH, and caffeic acid and 50 mg L^{-1} SO_2 were added, while for the addition of higher concentrations of GSH and SO_2 the parameter was significant different. This indicates that the synergic effect of both the antioxidant compounds at a high enough concentration could faster decrease the oxygen level in a model system. This correlates with our findings obtained with the real wine, where a combination of high SO_2 and GSH additions also led to a quicker decrease in the oxygen content (Paragraph 3.13.3.1). This data confirmed a previous research showing the GSH could increase the net oxidation rate (Cheynier & Van Hulst, 1988).

3.13.2.3 Comparison

In wine, the enrichment of caffeic acid did not affect the oxygen consumption in the same dramatic manner as when added to the synthetic wine solution. This data confirmed the antioxidant protection exerted by the caffeic acid, as observed in synthetic wine solution where it was the only phenol compound dissolved (Waterhouse, 2002; Li et al., 2008). In presence of several phenols, as happens in wine, the antioxidant activity is exerted by all of them, even though the wine was depleted of the phenols content. The caffeic acid activity is in competition with the other phenols. A higher caffeic acid concentration increases oxygen consumption due to the level of dissociated caffeic acid, but this could be low under wine conditions (Cilliers & Singleton, 1989; Cilliers & Singleton, 1991).

The concentration of oxygen at the end of the experiment was affected by the GSH concentration either in wine or in synthetic wine solution where lower concentrations of SO_2 were present (Figure 3.30). This similar behavior can confirm the GSH influenced this parameter in both the matrices considered. However, the lower oxygen concentration at the end of the experiment in the synthetic wine was mainly induced by the SO_2 present at higher levels.

Moreover, the treatments carried out in the same conditions for wine and synthetic wine showed significant differences. This was probably due to some compounds, such as phenols, still being present in the wine which was not completely removed by the bentonite and active carbon finings. These compounds can affect the oxidation of wine and then modify the oxygen level after 60 days incubation. This data could confirm the antioxidant potential of the *o*-phenols (Waterhouse, 2002; Li et al., 2008).

Oxygen concentrations in wine and synthetic wine solutions, showed a similar rate of decrease of the oxygen during the 60 days incubation (Figure 3.31). In this case, in wine different concentrations and antioxidant compounds can affect the decrease rate, which was only significantly influenced by higher GSH and SO_2 concentrations. This probably means that the more complex interactions happening in wine could have a stronger effect on the level and behavior of antioxidant compounds (Waterhouse, 2002). Moreover, the rate of oxygen consumption was lower in the presence of GSH in both wine and synthetic wine solution. For low SO_2 concentrations, the GSH did not increase the net oxidation rate, which was instead affected by high SO_2 levels, confirming only partially the data described in literature (Cheynier & Van Hulst, 1988).

The rate of oxygen consumption was not significant different between synthetic wine solution at low level of SO_2 with and without GSH added, and wine and synthetic wine solution enriched with SO_2, as well as between wine and synthetic wine solution with the higher content of GSH and SO_2. For higher SO_2 concentrations, the wine and synthetic wine were similar, while it was not the case in the lower SO_2 concentrations. The reason for this is probably that the SO_2 played a large role in the oxygen consumption at higher concentrations. However, this effect was reduced at lower SO_2 concentrations, leading to other compounds, of which their concentrations and reactivity differed between real wine and synthetic wines, playing a more significant role.

The treatments performed in wine and in synthetic wine solution did not show differences on the GSH, caffeic acid and the total phenols content. An increased concentration of GRP was detected only in synthetic wine solution, probably due to the absence of several hydroxycinnamic acids, as the wine contained even after the active carbon addition. The decrease of the absorbance at 280 nm occurring only in synthetic wine solution could be observed for the same reason, as well. An increased content of fertaric acid was detected only in synthetic wine solution, but no data are reported in literature. The SO_2 decreased faster when GSH was added to synthetic wine, contrarily as what happened in wine. Probably, the SO_2 could be oxidized faster than the GSH in presence of lower concentration of phenols, as in the synthetic wine solution. Moreover, it decreased no more than 30 mg L^{-1} as total SO_2 in wine and it disappeared in synthetic wine solution (50 mg L^{-1}), even where GSH was added. It can mean the phenols content could affect the SO_2 oxidation, as well as the equilibrium SO_2/HSO_3 could have an influence on its decrease.

3.13.3 Evaluation of oxidation rate in different commercial Sauvignon blanc wines

Thirteen commercial 2010 South African Sauvignon Blanc were used in this study and drawn just after alcoholic fermentation from steel tanks. No ascorbic acid was detected in any of the wine.
The oxygen consumption was different among the different wines analyzed; for most of them no oxygen was measured after 40 days of incubation (Figure 3.33). However, in the case of wines 1, 4 and 7, oxygen was still detected until the end of the experiment.
As expected, the addition of SO_2 increased the oxygen consumption, as can be seen in Figure 3.34. No oxygen was measured after 35 days incubation in all wines, even though in most cases this was happening after only 15 days. This indicates that SO_2 can affect the oxygen consumption during the ageing of white wines.

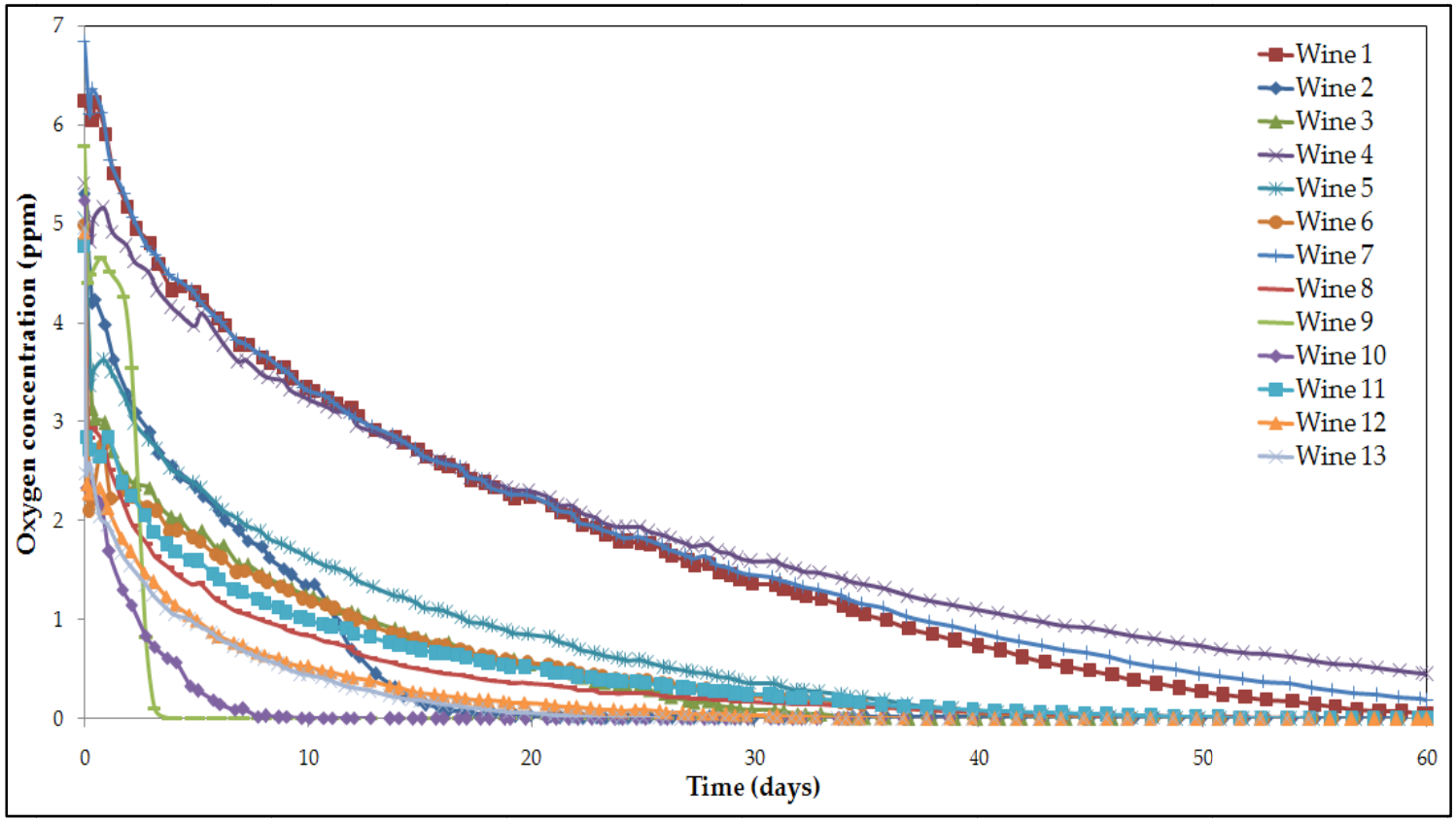

Figure 3.33: Evolution of oxygen concentration in some commercial Sauvignon blanc wines to which no SO_2 was added.

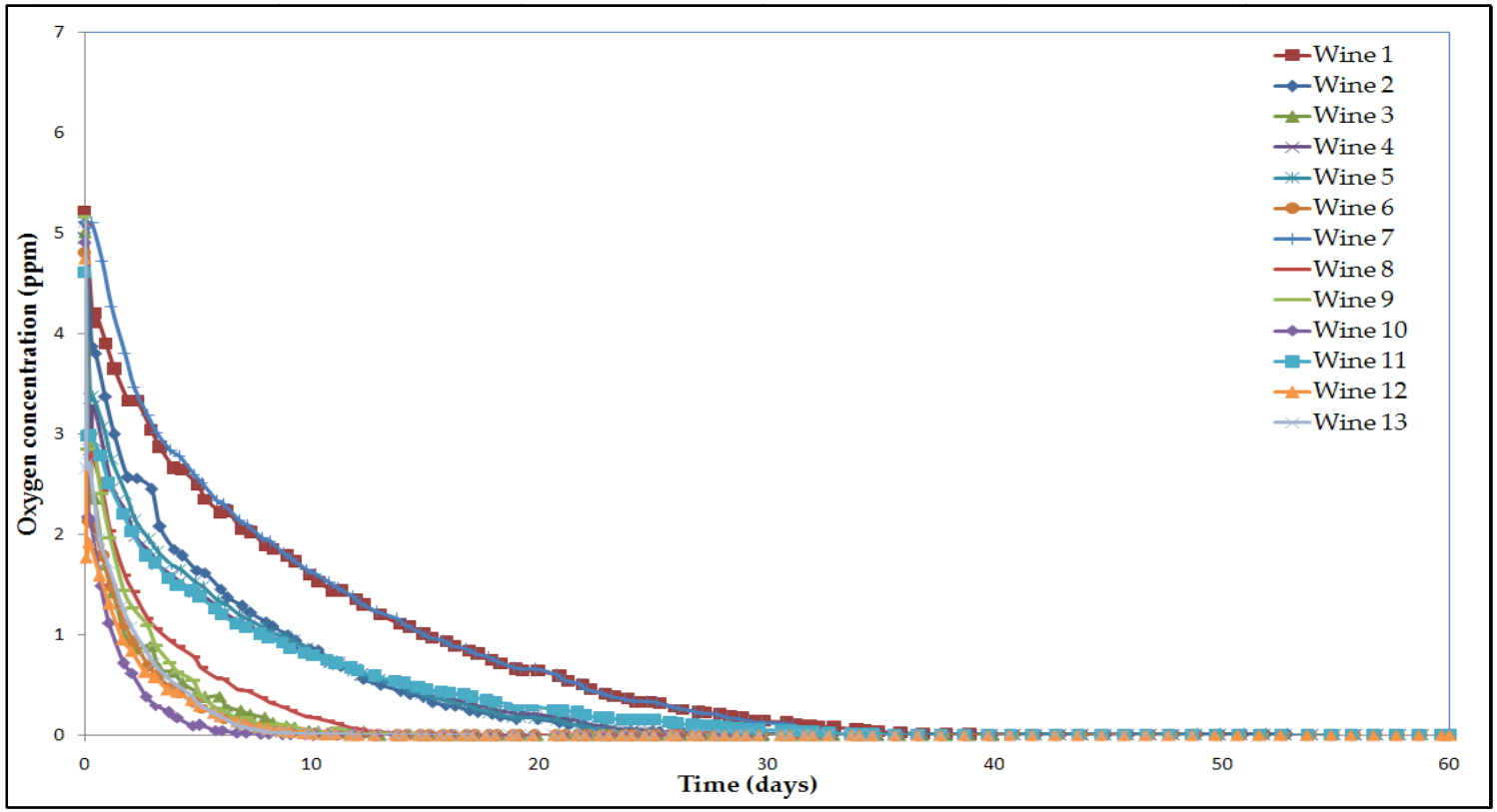

Figure 3.34: Evolution of oxygen concentration in some commercial Sauvignon blanc wines to which 30 mg L^{-1} SO_2 was added

No GSH was detected in any analyzed wines after 60 days incubation neither in wine added nor not added with SO_2.
The GRP level increased and no correlation seems to exist with its formation and the higher SO_2 content.
The SO_2 concentration halved at least after 60 days incubation, decreasing of 30 mg L^{-1} and more.
The GSSG content exceeded twice in all the wines considered; its increase was lower when the SO_2 was added meaning the SO_2 could slow down the GSH oxidation.
The level of caftaric acid (cis and trans), coutaric acid (cis and trans), cis-feratric acid, caffeic acid, coumaric acid and ferulic acid were stable during the 60 days incubation. The trans-fertaric acid increased in all the wines, highly when the SO_2 adding.
The catechin concentration decreased in all the wines and it does not seem correlated to the higher amount of SO_2.
The colour changes were monitored at 420 nm and 440 nm: higher absorbance values were detected and the increase was related to SO_2 addition which carried less changes.
The absorbance measures increased at 280 nm for all the wines, independently to the higher SO_2 concentration.
The SO_2 addition showed an influence on the oxygen consumption rate, the trans-fertatic acid content and the colour changes in the experimental conditions considered.
Two different parameters were considered for the statistical analysis, as described above (Paragraph 3.13.2.1): the first was an indication on the oxygen concentration at the end of the experiment. The second parameter corresponded to the decrease rate of oxygen during the assay. The final concentration of oxygen was significantly different for most of wines to which SO_2 was added, except for wines 7, 9 and 13 for which no significant differences were revealed between the two SO_2 treatments. The slope of oxygen decrease was at least from two to ten times higher in wine to which SO_2 was added (results not shown). The rate of oxygen decrease was significantly different for all the wines, except for wine 9, where the SO_2 added did not significantly change the final oxygen concentration or the rate of oxygen decrease.
Moreover, for all the compounds analyzed, the linear regression was performed for the data at the beginning and end time, in order to find the straight line which could explain the influence of that compound on the oxygen consumption. Among these 21 parameters analyzed, 13 of them were selected. They had the best linear model which showed the higher value of linear correlation (R^2). The compounds that showed the higher R^2 were copper, ferulic acid and total SO_2. For these compounds, the linear regression was separately performed which R^2 was 0.68. It means these three parameters can explain 68% of the variance predicted for oxygen consumption rate.
Copper is involved in oxygen consumption as effective catalyst in the Fenton reaction (Gunther et al., 1995) which causes oxygen reduction to hydrogen peroxide. The latter is degraded through a reaction catalyzed by metals (Danilewicz, 2003). The main antioxidant function of SO_2 is its reaction with hydrogen peroxide, limiting the oxidation of ethanol and other saturated hydroxy compounds, as Boulton et al. (1996) observed

either in wine or in synthetic wine solution. SO_2 is also known to reduce oxidized phenolics (Danilewicz et al, 2008). However, the antioxidant capacity of the ferulic acid is not well known in wine (Kilmartin et al., 2001; Waterhouse, 2002; Li et al., 2008) and should be further investigated.

3.14 Conclusion

The novel UPLC method developed allows the detection and quantification of GSH, catechin and caffeic in one rapid chromatographic analysis. The GSH derivatization is fast and the preparation of samples is quick and easy and no purification step is required. The validation has shown good repeatability, intermediate repeatability and linearity both in juice and in white wine, as well as a good recovery in both of the matrices for all the compounds investigated. The chromatographic method is also easy, sensitive, robust and fast, making it excellently suited for routine analysis. The reduced analytical run time allowed less solvent use, which reduced the waste generation, rendering the method more environmentally friendly and more cost efficient.
The validated analytical method allowed us to perform the experiment concerning the rate of oxygen consumption in white wine. In only one short run, the quantification of derivatized GSH, caffeic acid (and catechin) was carried out. The data obtained in the experiment focused on the caffeic acid additions in real wine or synthetic wines confirmed that antioxidant compounds, such as GSH and SO_2, could increase the oxygen consumption. However, the complexity of real wine makes direct comparisons with model wine systems very hard.
In the 13 Sauvignon Blanc analyzed, the oxidation rate was increased significantly by the addition of SO_2 in 12 of these wines. The compounds mainly affecting the oxidation rate were copper, total SO_2 and ferulic acid content; they constituted the 68% of the variation of the oxidation rate observed, a good value considering the matrix, the considered variables and the experimental conditions. A novel UPLC method has thus been developed for the simulations analyses of GSH, caffeic acid and catechin in white wines, which was used to assess of these compound's concentrations in South African white wines. The interactions between GSH, phenols and SO_2 in oxygen consumption in wine were also preliminary evaluated using this UPLC method.
Further investigations into the interactions between GSH, phenols and SO_2 are required to clearly elucidate the oxidative reactions taking place during white wine ageing.

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4 ABBREVIATIONS AND ACRONYMS

2-aminoacetophenone	1-(2-aminophenyl)ethanone
3MH	3-mercaptohexan-1-ol
3MHA	3-mercaptohexyl acetate
3MPA	3-mercaptopropanoic acid
3MPA-HQ	S-mercaptopropionyl-p-hydroquinone
4MMP	4-mercapto-4-methylpentan-2-one
4MMPOH	4-mercapto-4-methylpentan-2-ol
4MMPOH	S-(4-methyl-2-hydroxypent-4-yl)-L-cysteine
Ageing *sur lies*	traditional enological practice, used during the manufacture of wine, consisting in a variable time of contact between lees and wine
Caffeic acid	trans-3,4 dihydroxycinnamic acid
Caftaric acid	trivial name for caffeoyltartaric acid
Coutaric acid	trivial name for coumaroyltartaric acid
Cys	cysteine
Cys-HQ	S-cysteinyl-*p*-hydroquinone
DTNB	5,5'-dithiobis-2-nitrobenzoic acid (Ellman's reagent)
E	yeast extract
Fertaric acid	trivial name for feruloyltartaric acid
Ferulic acid	trans-3-methoxy-4-hydroxycinnamic acid
GPI	glycosylphosphatidilinositol
GRP	grape reaction product (2-*S* glutathionyl caftaric acid)
GRP2	2,5-diglutathionyl caftaric acid
GSH	reduced glutathione
GSH-HQ	S-glutathionyl-*p*-hydroquinone
GSSG	oxidized glutathione
H	yeast hull
HPLC	high pressure liquid chromatography
HPLC/ESI-MS	liquid chromatography coupled to electrospray ionization mass spectrometry
Hydroxycinnamtes	term used to refer all compounds containing a hydroxylated cinnamic structure, thus including: free cinnamic acids and their esters
L	yeast lysate
M	mannoprotein
mBB	monobromobimane
OTA	ocratoxin A
P3MH	S-(1-hydroxyhex-3-yl)-L-cysteine
P4MMP	S-(4-methyl-2-oxopent-4-yl)-L-cysteine
pBQ	*p*-benzoquinone

p-coumaric acid	trans-4-hydroxycinnamic acid
pHQ	*p*-hydroquinone
Pir protein	family of covalently linked cell wall proteins of *S. cerevisiae*), are directly linked to the β-1,3 glucans
PPO	polyphenols oxidase
PVPP	polyvinylpolypirrolidone
RCP	reduced protein cysteine
SDS	sodium dodecyl solfate
Sotolon	3-hydroxy-4,5-dimethyl-2(5H)furanone
TFA	trifluoroacetic acid
TNB	5-thio-2-nitobenzoic acid
UPLC	ultra performance liquid chromatography
UV	ultraviolet light
YCWF	yeast cell-wall fractions

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Appendix 1 *Copy of papers, oral comminications and posters*

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY ARTICLE

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Determination of Reduced Cysteine in Oenological Cell Wall Fractions of *Saccharomyces cerevisiae*

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Compounds containing cysteine residues, such as glutathione, can affect the redox potential of must and wine by reduction of *o*-quinones and hydrogen peroxide. The oenological yeast cell wall fractions contain cysteine residues in their protein structure, and they could affect both oxidative and odor properties of wine. An analytical approach based on the derivatization of cysteinyl residues with *p*-benzoquinone followed by reversed-phase high-performance liquid chromatography separation was developed to quantify glutathione and free and protein cysteine in 16 *Saccharomyces cerevisiae* strains and 12 commercial samples of yeast mannoproteins, hulls, and lysates. The chemical modifications induced by the Maillard reaction following the industrial preparation of such fractions were evaluated as well. Lysates showed the highest protein cysteine content and high contents of glutathione and free cysteine. Mannoproteins showed an intense Maillard reaction (furosine >60 mg/100 g protein), and most of the samples were able to bind thiol compounds with a potentially detrimental effect toward the thiol-related odors in wine.

KEYWORDS: Cysteine; glutathione; mannoprotein; yeast; hull; lysate; HPLC; quinone

INTRODUCTION

Thiol compounds of grape and yeast can strongly affect the sensorial properties of wine. Some aliphatic thiols are involved in the varietal aroma of wine even though their perception threshold is in the order of a few tens of nanomoles per liter (*1*). Barrel aging and bottle storage can decrease the concentration of such compounds below the perception level in wine because of their reaction with *o*-quinones and hydrogen peroxide arising from phenol oxidation (*2*). The reaction of quinones with the odor-related thiols, as well as other thiol compounds, produces thiol-substituted hydroquinones (*3*) or disulfide compounds lacking of aromatic properties (*4*, *5*). Glutathione (GSH) can preserve wine odor since it can compete against the odor-related thiols, for oxidation if its concentration exceeds a few micromoles per liter (*6*). Cysteine (Cys) residues can also hinder the alterative or atypical aging of white wine by preventing the formation of Maillard reaction-related compounds such as sotolone (*7*) and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (*8*). The reducing property of GSH toward the *o*-quinones produced by the tyrosinase activity in must is well-known as well (*9*).

The mercaptans responsible for the reduced odors in wine represent a further group of thiol compounds, and they can be removed by racking wine under aerating conditions or by barrel aging on yeast lees. Mercaptans are converted to thiol-substituted hydroquinones by reaction with the *o*-quinones or with the Cys residues of the yeast cell wall to give disulfides (*4*). The antioxidant properties of the reducing protein Cys (RPC) from yeast lees have not been extensively investigated in wine, but some results show the Cys residues of yeast mannoproteins (MPs) to be effective antioxidants if they are accessible to oxidizing molecules (*10*). Moreover, it is well-known that white wines and sparkling wines develop faster nonenzymatic browning, oxidation, and loss of odor-related thiols after yeast lees are removed (*6*, *11*). The release of MPs from yeast lees as well as the addition of yeast glycoproteins to wine could increase the antioxidant Cys content. Nevertheless, little is known about the Cys content of yeast cell wall fractions as well as about the antioxidant behavior of yeast lysates, hulls, and MPs in wine.

The chemical modifications of the oenological yeast fractions following their preparation procedures have not been evaluated to date. Indeed, the technologies applied to produce oenological yeast fractions can chemically modify the MP moiety of the yeast cell wall. Heat treatments and drying conditions, as those adopted to produce yeast fractions, can promote browning due to Maillard reaction (*12*). The formation of α-dicarbonyl compounds (*12*) and degradation of Cys residues (*13*) occur as well. Such reactions likely modify the RPC content of the yeast fractions, affecting their antioxidant properties. Despite the growing interest in exploiting such properties of yeast cell wall fractions in winemaking, a reliable analytical method is not currently available to quantify RPC. The adoption of classical analytical approaches such as the Ellman's method (*14*) for the determination of glycoprotein Cys, is hindered by the dark-yellow color produced by the MP solutions. On these bases, the present paper describes a reliable analytical method to quantify both the glycoprotein and the nonproteinaceous Cys content of yeast fractions. The method was applied to assess the antioxidant properties of commercial samples of yeast lysates, hulls, and MPs on the basis of their Cys content. The furosine index was

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applied for assessing the heat damage promoted by the technological processes adopted for the production of yeast fractions.

MATERIALS AND METHODS

Chemicals. Cys, 3-mercaptopropionic acid (3MPA), and *p*-benzoquinone (pBQ) were purchased from Fluka (Switzerland). GSH, starch, and trifluoroacetic acid (TFA) were from Sigma-Aldrich (St. Louis, MO). Citric acid was purchased from J. T. Baker (Phillipsburg, NJ), and the oenological lysozyme was from Intec (Verona, Italy). High-performance liquid chromatography (HPLC) grade methanol was purchased from Panreac (Barcelona, Spain), and HPLC water was obtained by Milli-Q system (Millipore Filter Corp., Bedford, MA).

Samples. The following commercial samples of yeast fractions for oenological use supplied by six different producers as dried products with unknown compositions were evaluated: four MPs, four hulls, four lysates, and two extracts. Additionally, 16 commercial dried yeast starters employed in winemaking were evaluated: 11 strains were from Lallemand Inc. (Ontario, Canada), and 5 strains were from Dal Cin (Milan, Italy).

Derivatization of Cys Residues in the Samples. Samples were added with 100 μL of 400 μM methanol solution of pBQ. After 1 min of mixing, 1 mL of 500 μM 3MPA dissolved in 0.3 M citrate buffer, pH 3.5, was added.

Determination of Cys Residues in Yeast Fractions. At least 50 mg of insoluble sample (active dried yeast, yeast hulls, and yeast lysates) was dispersed in 50 mM of citrate buffer, pH 5.0, to obtain a 2 mL suspension with sample concentrations in the range of 20−100 g/L. The dispersed sample was centrifuged at 5000*g* for 15 min at 15 °C by a thermostatted Sorvall centrifuge (Thermo, Waltham, MA). The supernatants were diluted 1−10 fold, and 2 mL was submitted to derivatization. The precipitated material was resuspended in 5 mL of 50 mM citrate buffer, pH 5.0, and submitted to the derivatization reaction. After derivatization, 1 mL of the suspension was centrifuged at 14000*g* for 5 min at 25 °C by a thermostatted benchtop centrifuge (Hettich, Tuttlingen, Germany), and the supernatant was submitted to HPLC separation. To recover the derivatized 3MPA potentially adsorbed on the insoluble yeast fraction, the precipitated material was rinsed with 2 mL of 0.1% hydrochloric ethanol as described by Ummarino et al. (*15*), carefully resuspended, and centrifuged at 14000*g* for 5 min. The supernatant was dried under vacuum, redissolved in 1 mL of water, and injected into HPLC.

MP and yeast extract samples were prepared by dissolving 100−200 mg of sample in 2 mL of 50 mM citrate buffer, pH 5.0. The solution was submitted to a derivatization reaction, and then, it was ultrafiltered using 3 kDa cutoff Microcon membranes (Millipore, Billerica, MA) at 14000*g* for 100 min at 25 °C by the thermostatted benchtop centrifuge. The permeate was submitted to HPLC separation.

The retentate was added with 1 mL of 0.1% hydrochloric ethanol and centrifuged at 14000*g* for 5 min. The supernatant was dried under vacuum, and the dried material was redissolved in 500 μL of water before the HPLC separation. Each sample was analyzed in at least duplicate.

Precision Parameters. The repeatability of the Cys residues evaluation method was assessed by submitting one sample of yeast hulls and two samples of yeast lysates to five determinations each. The response linearity of the method was assessed for RPC concentrations up to 180 μM by dispersing 27−100 g/L of a yeast hull sample containing 0.18 mmol RPC/100 g product in the 50 mM citrate buffer. The response linearity of the method for higher RPC concentrations (210−800 μM) was attained by analyzing dispersions (10−40 g/L) of a yeast hull sample containing 2.1 mmol RPC/100 g product.

Evaluation of Matrix Effects on the Cys Residues Determination. To evaluate possible interferences arising from high polysaccharide contents, one sample of yeast lysate (L2) containing GSH and protein Cys was assayed with or without addition of 30 or 80 g/L of amylose or caramelized amylose. The caramelization was obtained by exposing 2 g of amylose suspended in 2 mL of 0.1% HCl at 110 °C for 24 h. Interference of protein disulfides (cystine) was evaluated by submitting 5 g/L lysozyme solution in 50 mM citrate buffer, pH 5.0, to protein Cys evaluation.

HPLC Separation of Derivatized Thiol Compounds. The reversed-phase (RP)-HLPC of the thiol-substituted hydroquinones and *p*-hydroquinone (pHQ) was performed with a Waters Alliance 2695 (Milford, MA) equipped with a photodiode array detector Waters 2996. The separation column was a hexyl-phenyl column, 250 mm × 4.6 mm, 5 μm, 110 Å (Phenomenex, Torrence, CA). Eluting solvents were water/trifluoroacetic acid (0.05% v/v) and methanol; the concentration of the latter increased from 10 to 35% in 18 min during the elution gradient at 1.0 mL/min flow. Chromatographic data were acquired and processed by Millenium software v. 4.0 (Waters).

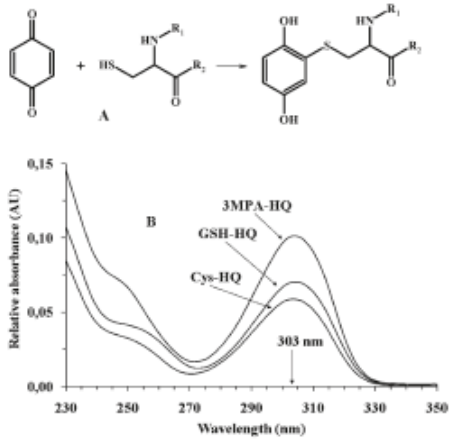

Figure 1. (A) General scheme of the derivatization reaction of Cys thiols with pBQ and (B) UV spectra obtained for derivatized Cys (Cys-HQ), GSH (GSH-HQ), and 3-mercaptopropanoic acid (3MPA-HQ).

HPLC/Electrospray Ionization−Mass Spectrometry (ESI-MS). For MS detection, the LCQ Deca XP spectrometer, controlled by the Excalibur software (Thermo Finnigan, San Jose, CA), was operated in positive ion mode. A postcolumn flow splitter was used to introduce 1:15 of the HPLC flow stream into the ESI source. The ESI interface and the ion optics settings were as follows: spray potential, 5.0 kV; nebulization gas (nitrogen) relative flow value, 10; capillary temperature, 275 °C; and cone voltage, 30 V. Full-scan mass spectra were acquired scanning the range 50−800 m/z. Mass accuracy was ensured by calibration with a mixture of caffeine, reserpine, and the tripeptide PFK (in methanol:water 1:1, 0.1% acetic acid) infused separately.

Quantification of Thiol Compounds. Cys and GSH were quantified chromatographically by the external standard method in both the soluble and the insoluble fractions. Standard solutions containing Cys and GSH concentrations up to 100 μM were prepared in 50 mM citrate buffer, pH 5.0.

The concentration of RPC in the evaluated sample fraction was calculated as follows:

$$[\mathrm{RPC}] = [\mathrm{pBQ}] - ([\mathrm{Cys}] + [\mathrm{GSH}] + [\mathrm{pHQ}] + [3\mathrm{MPA\text{-}HQ}])$$

where [pBQ] = concentration of pBQ used for the derivatization, [Cys] = concentration of Cys quantified chromatographically, [GSH] = concentration of GSH quantified chromatographically, [3MPA-HQ] = concentration of *S*-3-mercaptopropionyl-hydroquinone quantified chromatographically, and [pHQ] = concentration of the underivatized hydroquinone quantified chromatographically.

Evaluation of Protein Content and Furosine Level. The protein content and furosine level were determined as described by Resmini et al. (*16*) with the following modification: 200 mg of sample (corresponding to about 20 mg of protein) was added with 8 mL of 8 M HCl and submitted to acid hydrolysis at 110 °C for 23 h before solid-phase extraction (SPE).

Total Cys Content. The total amount of Cys (i.e., Cys + 2 × cystine) was assayed on the acid-hydrolyzed sample obtained from the furosine determination. After purification with SPE on a C18 cartridge, the sample was dried under vacuum and redissolved in 0.15 M sodium carbonate buffer, pH 8.6. The buffered solution was then submitted to Cys determination according to Krause et al. (*17*).

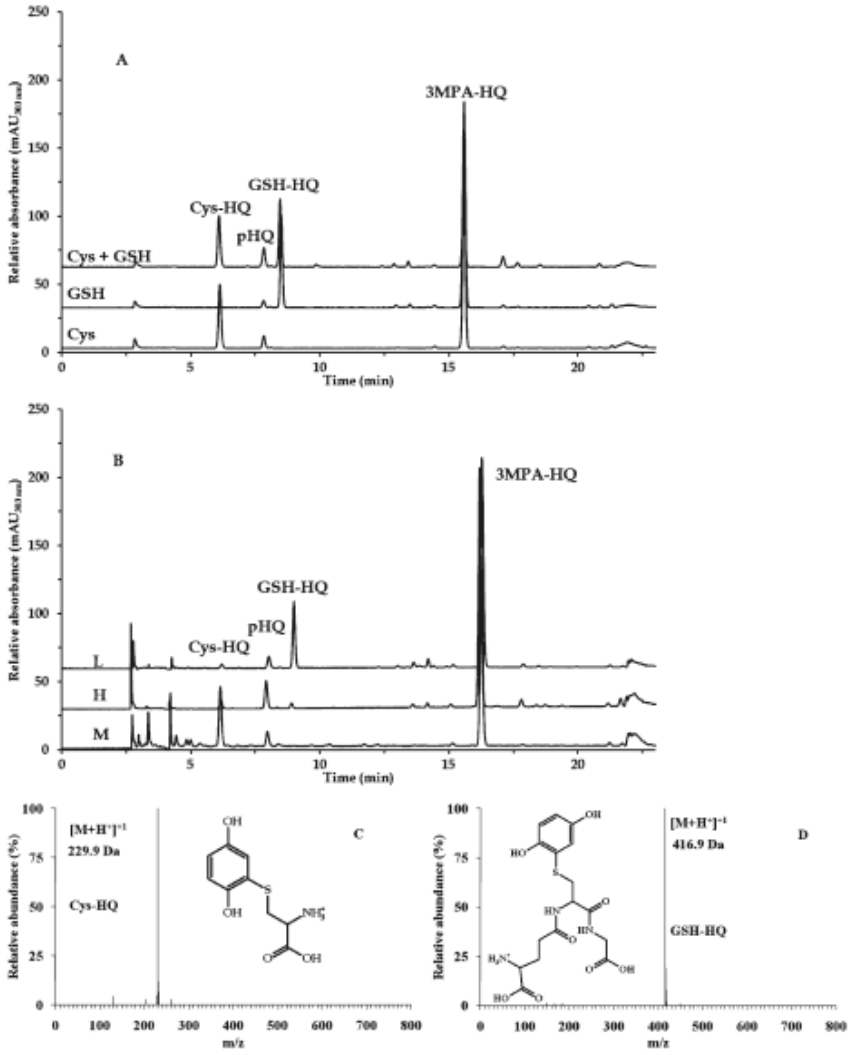

Figure 2. HPLC separation of Cys-HQ, GSH-HQ, 3MPA-HQ, and pHQ obtained from derivatization with pBQ of (A) standard water solutions of Cys, GSH, and their mixture and (B) MP sample added with Cys (M), yeast lysate (L), and yeast hull (H). HPLC-ESI/MS spectra of Cys-HQ (C) and GSH-HQ (D) are reported.

RESULTS AND DISCUSSION

The thiol property to reduce quinones to thiol-substituted hydroquinones, as it spontaneously occurs for the *o*-quinones in must and wine, was adopted to assess the Cys residues in the yeast fractions. For this purpose, the symmetric pBQ was used as a derivatizing agent to prevent the formation of multiple hydroquinone derivatives and to obtain the single mono derivative for each thiol molecule. The molecular size of pBQ is comparable to that of the *o*-quinones detectable in must and wine, and it can react with the thiol residues actually accessible in the glycoprotein structures. The formation of thiol-substituted hydroquinones (Figure 1A) is fast and stoichiometric at room temperature, and it allows Cys and GSH to be detected spectrophotometrically as *S*-cysteinyl-*p*-hydroquinone (Cys-HQ) and *S*-glutathionyl-*p*-hydroquinone (GSH-HQ), respectively (Figure 1B). The thiol derivatization procedure was followed by the addition of 3MPA to remove the exceeding amount of pBQ. The latter could be reduced to pHQ by oxidation of the thiol-substituted hydroquinones to thiol-substituted *p*-quinones, which are able to bind a further thiol molecule, so forming a dithiol-substituted hydroquinone (*18*). Moreover, pBQ can readily polymerize to produce brown compounds.

The formation of Cys-HQ and GSH-HQ in citrate buffer solution/suspension containing Cys and GSH (**Figure 2A**) or yeast fractions (**Figure 2B**) was confirmed by HPLC-ESI/MS (**Figure 2 C, D**). Such thiol-substituted hydroquinones, including *S*-mercaptopropionyl-*p*-hydroquinone (3MPA-HQ), showed a maximum absorption at 303 nm wavelength (**Figure 1B**). The HPLC pattern of **Figure 2A** also shows the formation of both pHQ and minor amounts of dithio-substituted hydroquinones following the derivatization reaction. The exceeding amount of pBQ could be effectively removed by the addition of sulfur dioxide, but the amount of the products obtained after such a reaction is affected by a number of factors (*19*). On the contrary, the addition of 3MPA allows us to verify whether the amount of pBQ exceeds the content of Cys residues and to assess the level of residual pBQ after thiol derivatization. As a result of the developed analytical approach, the amount of the RPC was calculated by the difference between the amount of added pBQ and the amounts of GSH, Cys, pHQ, and 3MPA-HQ chromatographically evaluated.

Because the insoluble cell wall fractions were submitted to derivatization with pBQ after removal of the soluble Cys residues, the amount of pBQ reacted with γ-glutamyl-Cys and cysteinyl-glycine potentially present in yeast preparations was assumed as negligible. Such dipeptides represent about 4% of GSH in yeast (*20*); therefore, their contribution was not taken into account also for the MP samples where the nonproteinaceous Cys thiols were never detected. The amounts of dithiol-substituted hydroquinones were not included in the calculation since their values were close to the limit of quantification (signal-to-noise ratio >10). The removal of the soluble material from the samples allows an effective RPC determination in wine yeast lees since sulfur dioxide interferences are minimized (data not shown).

The precision parameters for the quantification of Cys, GSH, and RPC were assessed by analyzing one sample of yeast hull and two samples of yeast lysates (**Table 1**). An average value of 5.4% can be assumed for the relative standard deviation (RSD) of Cys thiol concentrations exceeding 10 μM.

The calibration curves of Cys and GSH (**Figure 3A**) showed linear and similar analytical responses for concentrations up to

Table 1. Analytical Values (μM) and RSDs Obtained from Five Replicated Determinations Performed on One Sample of Yeast Hull (S4) and Two Samples of Yeast Lysates (L2 and L4)

	Cys			GSH			RPC		
	S4	L2	L4	S4	L2	L4	S4	L2	L4
	43.3	0.0	1.8	13.4	10.0	92	173	266	246
	43.0	0.0	2.1	15.0	9.7	101	171	299	241
	43.8	0.0	2.9	16.3	10.4	102	165	311	254
	44.2	0.0	2.9	13.8	11.0	103	164	286	269
	43.1	0.0	1.9	14.6	11.3	89	186	280	248
mean	43.0		2.3	14.6	10.5	97	172	288	252
RSD (%)	1.2		23.4	7.7	6.4	6.9	5.1	6.0	4.2

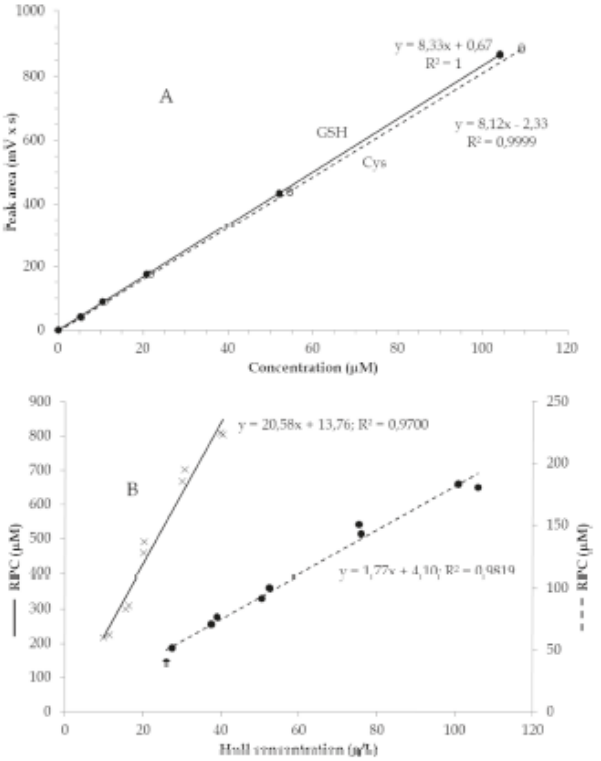

Figure 3. Analytical response of the proposed analytical method obtained for (A) standard solutions of Cys and GSH and (B) high (solid line) and low (dashed line) RPC concentrations. Data of duplicated determinations are reported.

Table 2. Effect of Amylose or Caramelized Amylose on the Quantification of the Cys Residues in a Sample of Yeast Lysate[a]

	amount (mmol/100 g sample)			
	amylose		caramelized amylose	
added amount (g/L)	GSH	RPC	GSH	RPC
0	0.22−0.21	1.59−1.44	0.23−0.23	1.38−1.35
30	0.22−0.22	1.52−1.56	0.25−0.26	1.35−1.33
80	0.21−0.22	1.57−1.60	0.27−0.28	1.38−1.35

[a] Results of duplicated determinations are reported.

100 μM. Because no standard material is commercially available for RPC quantification, the range of linear response was evaluated in yeast hull suspensions containing RPC levels up to 800 μM. Suspensions containing RPC amounts higher than 210 μM were prepared by dispersing 10−40 g/L of a yeast hull sample containing 2.1 mmol RPC/100 g product. To not affect the representativeness of the sampling, amounts higher than 50 mg were used to prepare the suspensions intended for linear response assessement. For this reason, the linear response for low (<180 μM) RPC concentrations was evaluated analyzing suspensions (27−100 g/L) of a yeast hull sample containing 0.18 mmol RPC/100 g product. Under the adopted conditions, linear responses were observed for the entire range tested (Figure 3B).

According to the signal-to-noise ratio, the detection limits for Cys and GSH by means of HPLC analysis were 0.30 and 0.26 μM, respectively; for the same compounds, the quantification limits were 1.0 and 0.85 μM, respectively. To assess whether RPC quantification was affected by matrix effects arising from polysaccharides or disulfide bonds, the quantification was performed after the addition of increasing amounts of amylose or caramelized amylose to a yeast lysate. No significant difference ($p < 0.05$) was found for RPC and GSH quantification in the presence of either amylose or caramelized amylose (Table 2). Similarly, no interference was observed when 5 g/L lysozyme, a protein containing four disulfide bonds, was added to water solutions containing GSH or Cys.

The analytical approach was first applied to the characterization of 16 commercial samples of oenological dry active yeasts to evaluate their natural thiol content. Because the yeast samples tested were aimed to both effective propagation and alcoholic fermentation, a very low intensity of Maillard reaction was expected. Indeed, the furosine values were lower than 8 mg/100 g protein (Table 3), and they agree with the values detected in other unprocessed biological material (*21*−*23*), although no data are reported in the literature for dry active yeasts. Amounts of RPC in the range 0.76−1.28 mmol/100 g were detected in yeast samples as well as GSH levels up to 0.92 mmol/100 g (Table 3). GSH is a cytoplasmatic metabolite, and it likely arises from the lysis of yeast cells during drying. None of the studied samples contained free Cys. Because the MP fractions represent about 10% of the yeast cell wall and they are mainly linked to the outer layer of the glucan backbone (*24*), oenological glycoprotein fractions with a higher content of Cys residues can be potentially obtained. The commercial MP samples showed levels of RPC very different from each other. Samples M1 and M2 had the lowest Cys levels (Table 4). Sample M2 presented the highest furosine level, thus suggesting a strong heat damage, which agreed with the dark brown color and the burnt odor characterizing the sample. The RPC level of sample M4 was close to the values found in the dry yeast samples. Overall, the RPC levels were far from those needed to obtain effective antioxidant activity if the usual amounts of MPs added to wine (200−500 mg/L) are taken into account. Neither GSH nor Cys was detected in the commercial MP samples. Surprisingly, the amount of 3MPA-HQ increased when Cys was added to samples before derivatization. Such a behavior occurred with most of the samples not containing free Cys and GSH, and it was likely due to the presence of oxidized phenol amino acids in the protein structure (*10*). The sensorial properties of wines containing odor-related thiols can be detrimentally affected by the addition of MPs capable of binding high amounts of thiols like sample M4. In this regard, 200−400 mg/L of such MP could deplete up to 250−500 μM of thiols from wine. Contrarily, such MPs could be usefully added to wine as an early treatment for removing the reduced odors since they can link

Table 3. Characterization of 16 Samples of Oenological Dry *Saccharomyces cerevisiae* Strains According to Their Content of Cys Forms, Protein Content, and Intensity of the Maillard Reaction Expressed as Furosine Level[a]

			reducing Cys mmol/100 g		
sample	protein (g/100 g)	furosine (mg/100 g protein)	GSH	RPC	overall Cys (mmol/100 g)
1	11.3	2.7	0.92	0.76	7.1
2	11.0		0.45	0.80	
3	10.5		0.71	0.86	
4	12.0	3.6	0.39	0.88	6.8
5	12.0		0.73	0.89	
6	10.3	7.6	0.63	0.90	6.9
7	12.0		0.77	0.91	
8	12.0		0.60	0.91	
9	11.2		0.83	0.91	
10	13.9	7.8	0.55	0.95	4.5
11	11.0		0.45	0.96	
12	9.2		0.54	0.97	
13	16.9	2.3	0.58	0.99	7.8
14	13.6	6.4	0.82	1.02	7.8
15	14.9		0.58	1.05	
16	11.2		0.63	1.28	
average	12.1	5.1	0.64	0.94	6.8

[a] Overall Cys refers to Cys + 2 × cystine.

Table 4. Characterization of Commercial Yeast Cell Wall Fractions and Yeast Extracts According to Their Contents of Free Cys, GSH, RPC, and Overall Cys (Cys + 2 × Cystine)[a]

			Cys mmol/100 g				
sample	protein (g/100 g)	furosine (mg/100 g protein)	unrecovered	free	GSH	RPC	overall
M1	2.86	28	<0.01	0	0	0.03	0.55
M2	10.51	254	<0.01	0	0	0	0.54
M3	9.02	62	0.04	0	0	0.03	2.3
M4	8.77	67	0.41	0	0	0.51	3.1
H1	7.48	17	0.04	0	0	0.08	3.3
H2	8.75	12	0.03	0	0	0.07	4.4
H3	8.87	6	0.04	0	0	0.14	3.3
H4	11.88	12	0	2.6	0.85	0.86	10.6
L1	9.96	3	0	0	0.45	0.73	6.9
L2	17.94	5	0	0	0.33	1.4	15.8
L3	16.84	38	0	0.32	4.6	0.81	12
L4	14.33	3	0	0.07	2.8	1.3	8.1
E1	21.68	154	0.46	0	0	1.1	37.4
E2	23.87	20	0.29	0	0	0.76	25.4

[a] The protein content and the intensity of the Maillard reaction expressed as furosine level are reported. The unrecovered Cys refers to the amount of analytically missing Cys after the addition of known amounts of Cys to samples lacking in nonproteinaceous Cys residues (M, MP; H, hull; L, lysate; and E, extract).

mercaptans, so avoiding exposure of wine to oxygen or the addition of copper sulfate. The high furosine values of MP samples indicate a strong extent of the Maillard reaction. These commercial MPs are capable of rapidly increasing the formation of atypical aging-related compounds in wine. Therefore, their addition to wine to improve colloidal and tartaric stabilities or to modify the astringency and the viscosity can also deplete wine odor and decrease wine shelf life.

Yeast hulls having a high RPC content could protect wine during barrel aging from the oxidative effect of micro-oxygenation. The analyzed commercial samples presented RPC levels close to the values detected for MPs as well as similar capability to bind free Cys. The amounts of GSH and free Cys revealed in sample H4 were likely due to GSH and Cys addition during manufacturing to increase the reducing properties of the product.

Amounts of RPC effective as antioxidant were detected in the yeast lysates L2 and L4. The former also contained high levels of GSH. The amounts of GSH detected in samples L2 and L3 accounted for 0.8−1.2% of yeast dry weight as reported in the literature (*25*). Nevertheless, their overall Cys content was about twice higher than the level found in the dry yeast samples, and likely, an enrichment with exogenous GSH occurred.

Because the cytoplasmic GSH represents about 0.5−1% of yeast dry weight, high amounts of GSH were expected to be found in yeast extracts. Moreover, in spite of the high value of total Cys (about 30 mmol/100 g sample), no GSH was detected in the two extract samples, and up to 0.50 mmol Cys/100 g sample was combined after the addition of Cys to the same samples. Such data suggest an intense oxidation of the yeast extracts likely due to the chemical/heat damage arising from the industrial production as supported by the high furosine values observed.

A wide range of oenological yeast fractions are commercially available, and yeast hulls, lysates, and MPs effective against wine oxidation and wine atypical aging can be potentially obtained. The data reported in this work show that a number of such oenological samples do not have useful reducing properties. Moreover, the technologies applied for their production are not suitable for preserving the RPC content of the yeast fractions. On these bases, both odor and antioxidant properties of wine could be potentially endangered by using most of the studied samples, which cannot be considered profitable in winemaking.

The RPC content of yeasts suggests that more useful fractions could be obtained to protect the odor-related thiols and to increase the shelf life of wine. In this regard, the adoption of specific culture media to increase GSH and RPC contents in yeasts, the selection of yeast strains with improved MP release properties, and the use of specific enzymatic procedures could greatly enhance the oenological properties of yeast fractions.

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Short Communication

Sulphur dioxide affects culturability and volatile phenol production by *Brettanomyces/Dekkera bruxellensis*

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ABSTRACT

The effect of different sulphur dioxide concentrations on culturability and viability of seven strains of *Brettanomyces bruxellensis* was tested in a synthetic wine medium (SWM) and a different response to molecular SO_2 among strains was detected. Sulphur dioxide induced a viable but non culturable (VBNC) state in all the strains. The greater percentage of VBNC cells were identified for five strains at molecular SO_2 concentrations of 0.2 mg/L and for two strains at the concentration of 0.4 mg/L. Vinyl phenols were detected in media containing VBNC or not viable *B. bruxellensis*, suggesting that its spoilage metabolism could be maintained during wine storage. Overall, this study indicates that SO_2 is a chemical stressor inducing VBNC state in *B. bruxellensis* grown in synthetic wine medium. Further studies are needed to evaluate the effects of SO_2 on the metabolism of this yeast in wine spoilage.

1. Introduction

Wine composition at the end of malolactic fermentation (high ethanol, low content of fermentable sugars and low pH, presence of sulfur dioxide) prevents most bacteria and yeasts from growing. Nevertheless, *Brettanomyces bruxellensis* (teleomorph *Dekkera bruxellensis*) can grow in these conditions and leads to the production of volatile phenols responsible for the off-flavour described as animal odours, farm, horse sweat, medicine and animal leather (Chatonnet et al., 1992). Moreover this yeast is able to produce biogenic amines, nitrogen compounds noxious to human health (Caruso et al., 2002; Vigentini et al., 2008; Agnolucci et al., 2009). For these reasons the spoilage by *B. bruxellensis* yeasts can lead to relevant economic losses for the wine industry.

During the last years *Brettanomyces* has been extensively studied in order to gain knowledge on its ecology and to establish a strategy for adequate control measures. Specific molecular methods for the identification of *Brettanomyces* yeasts, directly from wine (Tessonnière et al., 2009) and from grape varieties (Renouf and Lonvaud-Funel, 2007; Agnolucci et al., 2007), have been recently developed. Furthermore several different molecular methods (Miot-Sertier and Lonvaud-Funel, 2007; Martorell et al., 2006; Curtin et al., 2007; Agnolucci et al., 2009; Oelofse et al., 2009) have been applied to study the correlation between the genetic diversity of *Brettanomyces* isolates, collected worldwide, and their spoilage ability. These studies show that: (a) *B. bruxellensis* is the only species found in wines, (b) this yeast can be present on grapes, (c) all the strains of this species investigated so far can produce volatile phenols, (d) the level of volatile phenols production can vary among strains, but is not directly correlated to their molecular diversity.

Isolation and characterization of microorganisms from wine have been usually carried out assuming that all the cells were able to grow, multiply and give rise to colonies (the viable and culturable population, VC) in the growing media. Thus, the density of the viable but non-culturable (VBNC) microbial population cannot be evaluated. Indeed, the entry into VBNC state in response to stresses such as osmotic pressure, temperature, oxygen concentration was shown in microbial cells, particularly bacteria. The same environmental stressors could occur also through the winemaking process. Moreover, VBNC cells can recover a culturable state when favourable environmental conditions are restored (Millet and Lonvaud-Funel, 2000; Divol and Lonvaud-Funel, 2005). The existence of a VBNC-like phenomenon in *B. bruxellensis* has been suggested by Millet and Lonvaud-Funel (2000) and observed only in *B. bruxellensis* strain B3a by du Toit et al. (2005).

Sulfur dioxide (SO_2) is the preservative most commonly added to grape must and wine to control the growth of the spoilage micro-organisms during the wine-making process but its effects on *B. bruxellensis* populations are poorly understood. The aim of this study was to investigate the effect of sulfur dioxide on the viability and

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culturability of seven *B. bruxellensis* strains and their production of volatile phenols and biogenic amines.

2. Materials and methods

2.1. Yeast strains and maintenance conditions

Seven strains of *B. bruxellensis* (1 L, 20 T, BD2, BD7, BF4, 3 T and 12 T), collected from Chianti area throughout the vinification of Sangiovese grapes and genetically and physiologically characterised in a previous work (Agnolucci et al., 2009), were used. The yeasts, stored at −80 °C (Collection of Department of Crop Biology, University of Pisa) in glycerol 20% (v/v), were revitalized and maintained on YPD agar (1% yeast extract, 2% peptone, 2% glucose, 2% bacteriological agar; Oxoid, Milan, Italy).

2.2. Sulfur dioxide effect on culturability and viability

The effect of different concentrations of sulfur dioxide added to a synthetic wine medium (SWM) on the culturability and viability of *B. bruxellensis* strains has been evaluated for 24 h. The composition of SWM was the following: 6.7 g/L yeast nitrogen base (Difco), 1 g/L fructose, 5 g/L glycerol, 5 g/L tartaric acid, 0.5 g/L L-malic acid, 0.2 g/L citric acid, 4 g/L L-lactic acid, 0.12 g/L NH_4Cl, 0.02 g/L uracil, 5 mg/L oleic acid, 0.5 mL/L Tween 80 and 15 mg/L ergosterol, 0.18 g/L peptone (Vigentini et al., 2008). The medium was adjusted to pH 3.5. After autoclaving, it was supplemented with ethanol 11% (v/v) and ten milligrams per litre each of p-coumaric and ferulic acid. The final pH value was 3.67. This medium was supplemented with different amounts of potassium metabisulphite (PMB) sterile water solution to obtain final concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1 mg/L molecular SO_2 (as described in paragraph 2.4). Four milliliters of each solution were distributed in two 2-mL microcentrifuge tubes (one for the culturability and viability assessment at the beginning of the experiment and the other one for the 24 h assessments). Analyses were performed in triplicate.

For each strain, cells from YPD agar plates were inoculated and grown at 25 °C without shaking in YPD liquid medium containing ethanol at 10% (v/v). The cells were harvested after 72-h growth, washed with sterile physiological solution, checked by cell counting in a Thoma counting chamber and inoculated at an initial concentration of 10^5 cells/mL. Samples were completely used for the analyses. Culturability was evaluated at the beginning of the experiment (at the moment of the inoculum addition) and after 24 h of incubation at 25 °C without shaking. One out of two-mL of each sample was used to prepare the appropriate decimal dilutions in 0.1% peptone water solution, YPD plates were spread with 0.1 mL and incubated at 25 °C for 7 days.

Different cell viability staining techniques, using methylene blue (Uscanga et al., 2000), trypan blue (Xu et al., 1999), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Hjertstedt et al., 1998), fluorescein diacetate-ethidium bromide (Nikolova et al., 2002) and LIVE/DEAD yeast viability kit (Molecular Probes, Inc., OR, USA, Millard et al., 1997) were evaluated in preliminary studies to distinguish between viable and not viable cells, and the staining with trypan blue gave the best data.

Viability of *B. bruxellensis* was assessed at the same time points (0–24 h), by trypan blue staining. The remaining 1-mL of each sample was centrifuged at 5000 ×*g* for 5 min and washed twice with 0.1 M phosphate-buffered saline (PBS, pH 7.2). The preparations, and the appropriate dilutions thereof, were resuspended in 100 μL trypan blue (0.4 mg/mL in PBS) and incubated for 60 min statically. The number of not viable and viable cells was counted in a Thoma counting chamber by Reichert-Jung Polyvar microscope (Leica, Milan, Italy). Typically, about 250 cells were counted for each strain in each assay, and these were repeated at least twice.

2.3. Sulfur dioxide effect on volatile phenols and biogenic amines production

In order to study the influence of sulfur dioxide on volatile phenols and biogenic amines production, a 55-day experiment was carried out. In this experiment the strain *B. bruxellensis* 1 L was used because of its low sensitivity to the stressor, and because it was the most represented and widely distributed in the areas of isolation among the strains tested (Agnolucci et al., 2009).

Nine Erlenmeyer flasks (labelled from A to I) with a capacity of 250 mL containing 250 mL of SWM medium were inoculated with *B. bruxellensis* strain 1 L, adapted to the medium as described in paragraph 2.2, at an initial concentration of 10^5 cells/mL. In flasks A, B and C no PMB was added (control), flasks D to I were added with amounts of PMB sterile water solution to obtain final concentrations of 0.4 mg/L (D, E, F) and of 2.1 mg/L (G, H, I) molecular SO_2. Incubation was carried out at 25 °C without shaking.

Aliquots of 15 mL sampled from each flask after 0, 17 and 55 days from inoculation were filtered through a membrane filter with a pore size of 0.2 μm into 15 mL Falcon tubes and stored at 4 °C until analysis. Volatile phenols and biogenic amines were analyzed as reported by Agnolucci et al. (2009).

At 0, 1, 3, 10, 17 and 55 days culturability and viability were evaluated as described in paragraph 2.2.

To minimize oxygen diffusion during measurements, the determination was carried out by flushing certified pure nitrogen gas (Arcogas Srl, Italy) (flow rate of 20 L/min; purity >99.999%). Sulfur dioxide concentration was checked at the beginning and at the end of the experiment, as described in paragraph 2.4.

At the 14th day of incubation 2 mL samples from flasks D (containing 0.4 mg/L molecular SO_2) and G (containing 2.1 mg/L molecular SO_2) were centrifuged and the pellet was resuspended in peptone water in order to remove the stressor. Cells were then inoculated into liquid YPD medium, and after incubation for 5 days at 25 °C with aeration, viability and culturability were determined.

2.4. Determination of free SO_2

SWMs containing 0.2, 0.4, 0.6, 0.8, 1 and 2.1 mg/L molecular SO_2 were prepared according to Usseglio-Tomasset and Bosia (1984) for pH 3.67 and 11% (v/v) ethanol solutions at 25 °C. The free SO_2 concentration was analytically assayed by iodometric titration according to the Ripper method (Iland et al., 1993) 24 h after PMB solubilisation. The differences between added and assayed free SO_2 were negligible.

2.5. Statistical analysis

Two-way variance analysis of data was performed using SPSS v.12.0.1 software (SPSS Inc., Chicago, IL, USA), after appropriate transformations. Mean values were separated applying the Tukey's test. The difference of CFU between 0 and 24 h divided by CFU at 0 h was used to assess the effect of SO_2 on culturability. The ratio viable to total cells at 24 h minus the ratio viable to total cells at 0 h was used to assess the effect of SO_2 on viability.

3. Results and discussion

3.1. Sulfur dioxide effect on culturability and viability

A rapid antimicrobial activity of sulfur dioxide on *B. bruxellensis* has been described by Du Toit et al. (2005). Therefore, the effect of SO_2 on viability and culturability of the seven strains was evaluated by a short-term liquid medium assay (24 h). Analyses of variance performed on viability and culturability data showed statistically significant differences ($P<0.001$) for molecular SO_2 concentrations as well as for strains, also pointing out a significant interaction ($P<0.001$) between the two factors.

Table 1
Viability (viable cells) and culturability (culturable cells), expressed as mean percentage ± standar error, of *Brettanomyces bruxellensis* isolates in SWM after 24 h exposure to different molecular SO_2 concentrations.

Strains	% Viable cells						% Culturable cells					
	SO_2 mg/L						SO_2 mg/L					
	0	0.2	0.4	0.6	0.8	1	0	0.2	0.4	0.6	0.8	1
1 L	84,5 ± 2.6	45,0 ± 9.6	32,4 ± 2.5	19,2 ± 3.2	10,0 ± 6.6	11,7 ± 1.8	78.6 ± 4.5	16.1 ± 2.3	0	0	0	0
20 T	99,2 ± 2.9	59,0 ± 3.8	25,3 ± 9.6	26,8 ± 6.9	17,5 ± 0.1	10,2 ± 1.3	78.5 ± 4.3	12.0 ± 2.5	0	0	0	0
BD 2	82,5 ± 7.6	54,1 ± 0.2	6,7 ± 1.8	6,8 ± 1.5	4,6 ± 0.2	4,6 ± 1.4	50.6 ± 7.4	21.1 ± 2.6	0	0	0	0
BD7	88,4 ± 1.5	67,0 ± 5.3	16,9 ± 1.2	4,5 ± 1.4	11,3 ± 0.6	2,9 ± 1.4	64.7 ± 5.3	60.5 ± 4.55	3.1 ± 0.3	0	0	0
12 T	100,0 ± 1.9	62,0 ± 2.2	14,6 ± 2.0	9,1 ± 2.0	10,9 ± 4.8	14,7 ± 2.0	100,0 ± 29.5	45.4 ± 4.7	0	0	0	0
3 T	97,9 ± 2.1	66,7 ± 1.4	29,0 ± 4.3	12,0 ± 2.0	13,4 ± 2.3	10,0 ± 0.9	79.8 ± 11.0	36.3 ± 1.8	0	0	0	0
BF4	88,2 ± 4.2	58,0 ± 4.1	12,7 ± 1.8	11,7 ± 3.3	10,8 ± 1.6	12,6 ± 2.8	47.2 ± 9.7	32.5 ± 1.7	0	0	0	0

Viability and culturability data of *Brettanomyces bruxellensis* isolates are shown in Table 1 as percentage of the viable cells and percentage of culturable cells, respectively, after 24 h exposure to different molecular SO_2 concentrations. In particular, after exposure to 0.2 mg/L SO_2, culturability has decreased for all strains, ranging between 12% and 60.5% culturability depending on the strain. When the SO_2 concentration was increased to 0.4 mg/L SO_2, only strain BD7 gave culturable cells, and no colonies were formed on YPD agar by the other six strains. The percentage of viable cells, as measured by direct staining, also decreased on exposure to molecular SO_2. At 0.2 mg/L SO_2, the percentage of viable cells ranged between 67 and 45%. At 0.4 mg/L SO_2, the percentage of viable cells ranged between 6.7 and 32.4% depending on strain. The difference between percentage culturability and percentage viability suggests that a good proportion of the cells after exposure to SO_2 were in a VBNC state (for example, with strain 20 T, no culturable cells were detected after exposure to 0.4 mg/L SO_2, but 25.3% were still viable by direct staining). At SO_2 concentration of 0.6–1.0 mg/l no culturable cells were detected for all strains, but some 2.9–26.8% of the cells were still viable. These results indicate that the response to SO_2 is concentration dependent up to 0.4–0.6 mg/L. The greater amounts of VBNC cells were identified for five strains at SO_2 concentrations of 0.2 mg/L and for two strains at the concentration of 0.4 mg/L.

The VBNC state in *B. bruxellensis* yeasts was observed by du Toit et al (2005) for the strain B3a in wine containing 0.25–0.8 mg/L of molecular SO_2. On the contrary, Barata et al. (2008), analysing a wide range of *B. bruxellensis* strains, did not observe the existence of an active but non-culturable population after the addition of SO_2 to wine.

In the oenological practices, the levels of free SO_2 recommended for red wine ageing range from 20 to 30 mg/L (Ribéreau-Gayon et al., 2006), corresponding to 0.67–1 mg/L molecular SO_2 respectively, when conditions are pH 3.5, 13% ethanol v/v and 19 °C storage temperature (Usseglio-Tomasset and Bosia, 1984). According to our data, such values could not effectively eliminate all the *B. bruxellensis* strains. Therefore, low pH wines containing higher concentrations of molecular SO_2 will be better protected from this spoilage yeast.

Further research in wine is needed, to gain deeper insight on the occurrence and on the variability of this oenological trait among population of *Brettanomyces* spp strains.

3.2. Sulfur dioxide effect on volatile phenols and biogenic amines production

The production of volatile phenols and biogenic amines was monitored in a 55-day experiment in order to assess these enzymatic activities in VBNC cells (0.4 mg/L molecular SO_2) in comparison to VC cells (control) and not viable cells (2.1 mg/L molecular SO_2).

The results about viability and culturability of *B. bruxellensis* strain 1 L are reported in Fig. 1A and B. The highest molecular SO_2 concentration (2.1 mg/L), resulted in a dramatic loss of both culturability and viability in *B. bruxellensis* 1 L: growth on agar plates was inhibited and all the cells were not viable after one day. VNBC cells are evident by comparing curves in Fig 1A and B for the 0.4 mg/l data. Actually, when 0.4 mg/L molecular SO_2 was added to the medium, after 1 day we observed a decreased viability (from $1.60 \pm 0.26 \cdot 10^5$ to $8.46 \pm 2.05 \cdot 10^3$ cells/mL) and, at a greater extent, a decreased culturability (from $8.40 \pm 0.41 \cdot 10^4$ to $3.67 \pm 1.53 \cdot 10^1$ CFU/mL). Moreover, from the 10th day onwards, no cells were able to grow on the agar plates, whereas trypan blue staining showed that part of *B. bruxellensis* 1 L population was still viable and stable over time. These cells showed a decreased size (data not shown). This suggests that they might have entered a VBNC state. Indeed, the reduction of cell size, minimizing its energy needs, is described in the literature as a phenomenon often observed in VBNC bacterial cells (McDougald et al., 1998; Divol and Lonvaud-Funel, 2005).

Moreover, analyses carried out on non-culturable cell samples from flasks D (containing 0.4 mg/L molecular SO_2) and G (containing 2.1 mg/L molecular SO_2) after stressor removal, showed culturability of sample D cells. The culturability and viability analyses of sample D showed the presence of $4.47 \cdot 10^6$ CFU/mL and $7.91 \cdot 10^7$ viable cells/mL,

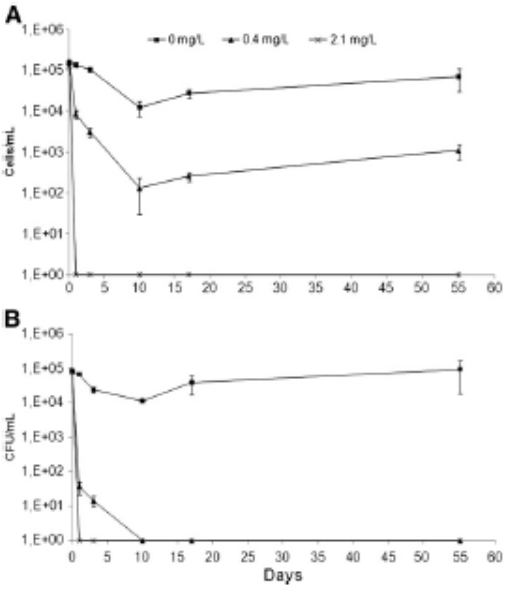

Fig. 1. Effect of different molecular SO_2 concentrations on the viability and culturability of *Brettanomyces bruxellensis* 1 L in SWM. (A) number of viable cells/ml, calculated from microscopic cell counts in a Thomas chamber after viability staining with trypan blue; (B) number of CFU/ml, calculated from CFU counts after plating on YPD agar medium. The value 10° indicates the absence of colonies in the volume of 0.1 mL of sample.

Table 2
Amounts of volatile phenols, expressed as mean ± standar error, produced by *B. bruxellensis* 1 L. exposed up to 55 days to different concentrations of molecular SO_2 in SWM.

Metabolite									
	SO_2 (mg/L) 0			0. 4			2.1		
	Days 0	17	55	0	17	55	0	17	55
4-Vinyl phenol	n.d.	0.03 ± 0.01	0.62 ± 0.07	n.d.	0.02 ± 0	0.07 ± 0	n.d.	0.02 ± 0	0.06 ± 0
4-Vinyl guaiacol	0.01 ± 0.01	0.02 ± 0.01	0.11 ± 0.08	0.07 ± 0.02	0.14 ± 0.02	0.37 ± 0.04	0.05 ± 0.01	0.14 ± 0.01	0.32 ± 0.05
4-Ethyl phenol	0.20 ± 0.04	0.10 ± 0.03	0.94 ± 0.21	0.08 ± 0.03	0.04 ± 0.02	n.d.	0.05 ± 0.02	0.03 ± 0.02	n.d.
4-Ethyl guaiacol	n.d.	0.09 ± 0.02	0.74 ± 0.08	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. — not detectable.

respectively. No development of colonies was observed for sample G where culturability and staining data showed that all the cells had been killed.

Ethyl phenols were produced throughout the 55 days of incubation when the strain 1 L was grown without SO_2 (Table 2) but neither ethyl phenol nor ethyl guaiacol was detected for VBNC and not viable cells. Nevertheless, vinyl phenols were still produced and accumulated in the SWM in the presence of SO_2. Three times higher amounts (c.a. 0.3 mg/L) of vinyl guaiacol were produced when SO_2 was added to the medium, and vinyl phenol production strongly decreased. Amounts lower than 50 μg/L of biogenic amines were detected in all the samples analyzed. Such amounts have no physiologic activity in humans. No data are reported in the literature concerning the enzymatic activity of VBNC yeast cells, although results are available on VBNC and not viable bacterial cells (Coton et al., 1998). The production of vinyl-guaiacol in VBNC and not viable cells detected in this work might be explained by considering the ethyl-phenols formation pathway. The two-step conversion of hydroxycynnamic acids to ethyl-phenols plays different roles in the cell metabolism. The activity of cynnamate decarboxylase could be aimed at counteracting the inhibitory effects of weak organic acids passively diffused across the cell membrane as it occurs in *S. cerevisiae* (Piper et al., 2001). At wine pH both coumaric and ferulic acids are mainly under undissociated form (pK = 4.5) and they have lipophylic properties allowing to cross the periplasmatic membrane and decrease cytoplasmatic pH. Yeast cells adapted to media containing weak acids can counteract the intracellular acidification either by degrading the acid (Sousa et al., 1996) or extruding protons by the membrane ATPase. In the VC cells vinyl-phenols to ethyl-phenols hydrogenation is directed at the redox metabolism but it is strongly reduced in the VBNC cells or absent in not viable cells, as shown by ATP accumulation and gene expression analyses (Lleò et al., 2000; Yaron and Matthews, 2002). Moreover, the stability of vinyl-phenol reductase is sensitive to ethanol (Godoy et al., 2008) and the occurrence of SO_2 might increase the inactivation effect. Therefore, the ethyl derivatives are not produced and the soluble lipophylic vinyl-phenols can accumulate in the media. The formation of vinyl-phenols by the not viable cells is in agreement with the synthesis of cynnamate decarboxylase during the adaptation to the acidic media and shows that the activity is not lost after the cell death. According to the stability data reported by Godoy et al. (2008) for the purified cynnamate decarboxylase the inactivation of the protein was expected. Our results suggest a different sensitivity to the inactivation factors for the intracellular decarboxylase of not viable cells.

In conclusion, the results obtained from the present work indicate that SO_2 is a chemical stressor inducing the non-culturable state in *B. bruxellensis*, grown in synthetic wine medium, depending on doses and strains. Therefore it appears that further studies are needed to evaluate the effects of SO_2 on the metabolism of this spoilage yeast in wine.

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Caratterizzazione di frazioni parietali di lievito in base alle forme cisteiniche ridotte

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La presenza di forme tioliche cisteiniche ridotte (FTCR) nei preparati enologici da parete cellulare di lievito potrebbe contribuire a prevenire lo sviluppo di fenomeni ossidativi nel vino tramite la riduzione delle forme chinoniche derivate dall'ossidazione fenolica. Un approccio analitico innovativo basato sulla reattività dei guppi tiolici verso il p-benzochinone, è stato utilizzato per caratterizzare mannoproteine, scorze, lisati, estratti e fecce di lievito di origine commerciale. Molti di tali additivi enologici hanno mostrato una spiccata attività legante verso le molecole tioliche libere ed una generale povertà in FTCR sia libere che proteiche. Questi prodotti potrebbero impoverire il patrimonio aromatico del vino e favorire lo sviluppo di fenomeni ossidativi, ma possono anche promuovere la rimozione di precoci difetti di ridotto. Alcuni campioni di scorza o lisati cellulari sono risultati contenere oltre 4 mmol/100 g di Cys e GSH liberi ridotti oltre che quantità solo poco inferiori di tioli di diversa origine la cui ascrivibilità a forme di Cys proteica ridotta da lievito richiederebbe opportuna conferma. I preparati mannoproteici sono risultati ampiamente danneggiati da un'intensa reazione di Maillard, misurata come indice di furosina, e particolarmente poveri in FTCR. La metodica proposta potrebbe aiutare a isolare frazioni di parete, e soprattutto mannoproteine, con migliori proprietà antiossidanti, nonché a valutare ulteriori aspetti dell'attività enologica delle FTCR.

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Determinazione delle forme tioliche in prodotti enologici

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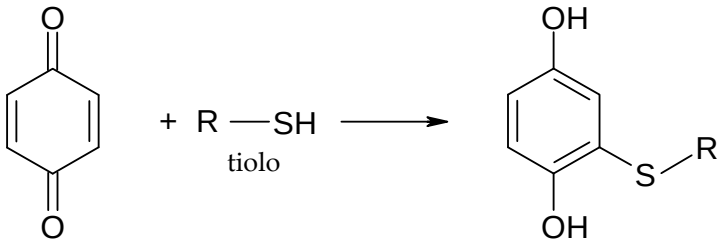
I gruppi tiolici ridotti possono svolgere in mosto e vino numerose attività tra le quali quella antiossidante, di prevenzione dall'invecchiamento atipico, di protezione delle caratteristiche sensoriali di vini bianchi e spumanti nel corso dell'invecchiamento.
Le forme più abbondanti sono quelle cisteiniche che possono essere presenti libere, legate alle proteine o come glutatione. Queste si possono ritrovare in vino e mosto in quanto già presenti nell'uva, in quantità correlate alla varietà e alla tecnologia di vinificazione, e in seguito alla crescita e lisi cellulare di *Saccharomyces cerevisiae*.
L'attività antiossidante è svolta riducendo i chinoni ad idrochinoni; in questo modo risultano sfavoriti l'imbrunimento e lo sviluppo di difetti sensoriali.
L'approccio analitico messo a punto si basa sul principio di tale reazione, da cui si ottengono i tioeteri corrispondenti alle molecole reagite. Nello specifico si esegue la derivatizzazione delle forme SH con un eccesso di benzochinone; la quota eccedente non reagita viene poi combinata con acido mercaptopropanoico.



p-benzochinone S-cisteinil-idrochinone

Fig. 1: reazione tra p-benzochinone e generico composto tiolico.

L'identificazione e la quantificazione di cisteina e glutatione avviene direttamente per HPLC e rilevazione spettrofotometrica; la quantificazione dei gruppi SH legati alle proteine è calcolata per differenza rispetto alla quota di acido mercaptopropanoico reagita e determinata cromatograficamente.
La rilevabilità riscontrata per il glutatione è di 1μM.

2nd Microsafety wine, 19-20 November, 2009 (Marina Franca (TA), Italy)

Characterization of oenologic yeast-cell wall fractions by reducing cysteine content

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Thiols can perform several activities in must and wine. The antioxidant activity of glutathione is well known: it can bind with the o-quinones so preventing browning of must and wine (Salgues et al, 1986). Glutathione can also reduce the loss of thiol aromas and it slows down the atypical aging of white wines (Dubourdieu and La Vigne-Cruège, 2004). Similar behaviour could be exerted by the reduced cysteine of yeast cell-wall and it can also bind molecules responsible of reduced odours.
The main yeast cell-wall fractions are represented by hulls, lysates and mannoproteins and they can be used in winemaking in order to improve wine properties such as prevent protein haze, improve foaming properties, affect mouth feel and protect wine from oxidations while barrel aging. The oenological properties of mannoproteins can be influenced by yeast strains, yeast growth conditions, drying process and extraction method since strong heat treatments can reduce the content of cysteinyl residues.
An analytical method based on the reaction between thiols and p-benzoquinone was developed for the evaluation of the reduced cysteine content. Different commercial samples of yeast cell-wall fractions and active dried yeasts were characterized by the content of reduced cysteine (free and protein bound) and glutathione. Moreover, the intensity of the Maillard reaction was determined in order to better understand the effect of the different industrial preparations.
Samples of yeast cell-wall fractions showed very heterogeneous contents of reduced cysteine: some of then can behave as antioxidant in must and wine, while some samples can decrease the free thiol content in wine since they can bind such molecules so depleting wine from its varietal flavours or removing mercaptans responsible for the reduced odours. The active dried yeast samples were characterized by glutathione and protein cysteine contents in the range from 0.39 to 0.92 mmol/100 g of product and from 0.76 to 1.28 mmol/100 g of product, respectively.
The production of yeast cell-wall fractions having higher reduced protein cysteine contents and lower heat damage should be pursued.

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2nd Microsafety wine, 19-20 November, 2009 (Marina Franca (TA), Italy)

Determination of reduced glutathione content in must and wine

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The antioxidant activity of glutathione in must and wine is well known: it reduces the o-quinones preventing their polimerization, browning of wine and depletion of flavour-related thiols. Glutathione can hinder the formation of sotolone (3-hydroxy-4,5-dimethyl-2(5H)furanone) in wine, a major responsible of the atypical wine aging.
Glutathione content ranges from 56 μmol/kg to 372 μmol/kg in grape (Cheynier et al, 1989), depending on grape cultivar, environmental conditions and agronomic practises. The winemaking procedures before the alcoholic fermentation influence its concentration in must, since they can affect the activity of tyrosinase, the presence of oxygen and the integrity of grape skin.
The amount of glutathione decreases to 10-40 μM (Cassol and Adams, 1995) following winemaking according to the yeast strain and the aging conditions.
Determination of glutathione in grape, must and wine can permit to estimate the antioxidant potential and the shelf-life of wine.
The analytical method described for glutathione quantification is based on the derivatization reaction with p-benzoquinone. The S-glutathionyl-p-hydroquinone is separated by HPLC and detected spectrophotometrically at 303 nm. The detection limit and the lowest quantifiable amount are 0.42 μM and 1.41 μM, respectively. The analytical approach allows the cysteine content to be determined as well.
The method was applied to investigate 8 different winemaking processes performed under different conditions (5 grape cultivars, 7 yeast strains and 2 redox conditions).
The glutathione content ranged from 1.28 μM to 3.66 μM after crushing and racking when must was exposed to air. Higher contents (18.22 μM) could be obtained pressing the grape under controlled atmosphere. Glutathione concentration rapidly increased at the end of alcoholic fermentation and the final levels (0-92.50 μM) were affected by exposure of must to oxygen, yeast strain and nitrogen availability to yeast.
The cysteine was absent in many samples or its concentration did not exceed 13.51 μM.

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32nd SASEV Congress, 18-19 November, 2010 (Somerset West, South Africa)

Effect of glutathione and sulfur dioxide on phenol oxidation in Sauvignon Blanc wine

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The antioxidant activity of glutathione and sulphur dioxide in must and wine is well known. Glutathione reduces the o-quinones preventing their polimerization, browning and depletion of flavour-related thiols. It hinders the formation of sotolone (3-hydroxy-4,5-dimethyl-2(5H)furanone), a major responsible of the atypical wine ageing.
Sulfur dioxide has both antioxidant and antiseptic activities, decreasing the formation of undesirable color and the development of secondary fermentations. It can react with oxygen, thus protecting sensitive wine compounds, and with the hydrogen peroxide, deriving from the polyphenol oxidation.
Oxidative browning occurs either during pressing and is mediated by the grape polyphenol oxidase, or during winemaking and storage in absence of the active enzymes.
This research was aimed to understand the oxidation rate and products derived from caffeic acid as phenol model in presence of different concentrations of glutathione and sulfur dioxide in Sauvignon Blanc wine. The compounds were added at equimolar concentration, in accordance to the amount typically found in white wine.
To increase the oxidation rate, the wine was stored at 37°C.
Glutathione and sulfur additions to wine resulted in the oxygen being drastically consumed in less than 30 days, while when they were not added the oxygen was detected longer than 70 days, showing clearly the oxygen consumption is strictly correlated to presence of these compounds in wine. Glutathione, sulfur dioxide and caffeic acid concentrations were also monitored during the course of the experiment.
Moreover, the oxidation rate was evaluated in 13 Sauvignon Blanc wines, with and without addition of sulfur dioxide. Besides the glutathione and sulfur amounts, the phenols quantification was carried out.

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Quantification of glutathione in must and wine by ultra-performance liquid chromatography

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Glutathione can perform several activities in must and wine. Its antioxidant activity is well known: it can bind with o-quinones thus preventing browning of must and wine by forming the Grape Reaction Product. Glutathione can also reduce a loss in certain aroma compounds and could slow down the atypical ageing of white wines. Glutathione content ranges from 5 mg/L to 80 mg/L in grape juice, depending on grape cultivar, environmental conditions and winemaking practises. The winemaking procedures before alcoholic fermentation influence its concentration in must, since they can affect the activity of tyrosinase, the presence of oxygen and the integrity of grape skin. The amount of glutathione decreases to 3 to 12 mg/L during winemaking, according to the yeast strain used and the ageing conditions.
The determination of glutathione concentration in must and wine could give an indication of the antioxidant potential and the shelf-life of wine.
The analytical method described was based on the derivatization reaction of glutathione with p-benzoquinone. The S-glutathionyl-p-hydroquinone was separated by UPLC and detected spectrophotometrically at 303 nm. The analysis was performed with a C18 column and the run time was less than 10 minutes. The minimum detection and quantification limits were 0.02 mg/L and 0.06 mg/L, respectively, with the linear range up to 100 mg/L.
Glutathione determination was carried out in 12 South African white juices and 43 white wines produced from different grape cultivars, vintages and winemaking. Glutathione concentration ranged from 1.1 mg/L to 42.3 mg/L in juice. In wine, the maximum amount detected was 27.4 mg/L and the highest levels were observed, as expected, in young wines. This novel method of glutathione determination could have a large impact on time and costs of the analyses for the wine industry and it could enable routine quantification of this important wine compound.

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Development and validation of an analytical method for the quantification of glutathione in white wine and juice by ultra-performance liquid chromatography (UPLC)

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The ever-increasing demand for higher throughput in analytical laboratories necessitates the continuous development of rapid analytical methods. Ultra-performance liquid chromatography (UPLC) has received significant attention in during the last few years as result of the superior peak capacity, resolution and efficiency, consequently offering considerably faster separation of complex samples such as wine. Higher efficiency is achieved using shorter columns packed with smaller (< 2 μm) particle sizes; however, the use of such small particles results in considerably higher back pressure. The development of instruments able to handle these increased pressures opened up the possibility of significantly faster separations in LC.
Catechin and caffeic acid are two of the most abundant phenols in wine. These two compounds can via non-enzymic oxidation, produce *o*-quinones witch are significantly correlated to browning often occurring in white wines. Glutathione is an important antioxidant in wine as it can bind with the *o*-quinones to prevent browning, by formation of the Grape Reaction Product (GRP). It can also help to prevent the loss of aroma compounds in wine commonly associated with atypical ageing.
In this study a simple, robust and rapid UPLC-PAD method was developed and validated allowing for the simultaneous quantification of derivatized glutathione, catechin and caffeic acid with analysis time of 8.5 minutes. By using a fast and simple derivatisation procedure with p-benzoquinone prior to UPLC, the overall analytical process remains very rapid. The method was found to be reproducible (RSD < 5%, n =10) and gave good recovery for all three analytes of interest (> 85%). Limits of quantification (LOQ's) are 0.05 mg $L^{-1}$for glutathione and catechin; and 0.009 for caffeic acid respectively. The method was applied for the analysis of 43 South African white wines (5 cultivars) of vintages 2004-2010 and 12 South African juices (3 cultivars) and the results are presented.

Appendix 2 *List of Tables*

Appendix 3 *List of Figures*